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**Re-use of seedling containers and *Fusarium circinatum* association with asymptomatic *Pinus patula* planting stock**

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*Fusarium circinatum* is a pathogen causing serious post-planting mortality of *Pinus patula* seedlings in southern Africa. Containerized planting stock that is asymptomatic but associated with *F. circinatum* in the nursery is thought to be the cause of this problem. The aim of this study was to determine if re-use of seedling containers could be a source of inoculum resulting in asymptomatic planting stock and increased post-planting mortality of *Pinus patula*. Two experiments were conducted in successive years comparing nursery cull of symptomatic seedlings, seedling growth, association of *F. circinatum* with asymptomatic seedlings and post-planting mortality for crops raised in re-used containers, with and without sanitation, and factory new containers. Each experiment consisted of a nursery production trial followed by out-planting into pots to assess post-planting mortality. Our results show that re-use of containers without sanitation increases the cull of symptomatic seedlings, incidence of *F. circinatum* associated with asymptomatic seedlings and post-planting mortality compared to the re-use of containers after steam sanitation or factory new containers. Growth of asymptomatic seedlings was unaffected by container treatment or association with *F. circinatum* and in the absence of wilt symptoms the root system did not exhibit typical discoloration. Watering frequency did not influence post-planting mortality in pots. The comparison of two open-pollinated seed mixes of *P. patula* that, based on seedling stem inoculation screening, represented susceptible and tolerant material did not show differences in nursery cull or post-planting mortality. This work demonstrated that natural contamination of re-used containers can be a primary source of inoculum producing asymptomatic seedlings associated with *F. circinatum* that will succumb to the pathogen after field planting. The process of seedling infection, apparent latent infection in the seedling and expression of disease after planting needs greater understanding to improve nursery hygiene measures to control this disease.

Key words; *Pinus patula*, *Fusarium circinatum*, containerized seedling nursery, post-planting mortality

## Introduction

*Fusarium circinatum* is a serious pathogen of pine that has become established in many countries around the world (Wingfield et al 2008). A wide range of disease symptoms can be associated with *F. circinatum* infection ranging from classic pitch soaked stem and branch cankers on large trees, planting stock mortality after transplanting to wilt related mortality in nursery crops (Mitchell et al 2011). In South Africa, *F. circinatum* is associated with nursery and post-plant mortality of *Pinus patula* (Viljoen and Wingfield 1994, Mitchell et al 2004, Crous 2005, Mitchell et al 2011) but has not yet been found causing classic pitch canker disease in plantations of this species despite classic pitch canker occurring in *P. radiata* plantations in South Africa (Coutinho et al 2007). Post-planting mortality of *P. patula* results from the transfer of *F. circinatum* with asymptomatic seedlings from the nursery (Crous 2005, Jones et al 2014). Effective control of the pathogen in the nursery is therefore necessary to address post-planting mortality associated with the pathogen.

Hygiene measures are an important component of the control of diseases in containerized forest nurseries (Dumroese and James 2005, Wingfield et al 2008). Re-use of seedling containers in forest nurseries is necessary to achieve cost-effective production of planting stock. It is now commonly recognised that some form of sanitation is needed for containers between cropping cycles to reduce build-up of pathogens in successive crops (Dumroese and James 2005, Peterson, 2008). The efficacy of a number of sanitation techniques have been tested with a focus on control of damping off and root rot diseases; particularly by *Fusarium* species (Sturrock & Dennis 1988, James & Woollen 1989, Peterson 1990, Dumroese et al 1993a, James & Trent 2001, Cram 2002, Dumroese et al 2002, Kohmann & Børja 2002, Van Wyk et al 2012). Various biocides and pasteurization techniques have been compared. The most effective techniques have been pasteurizing at high temperatures, typically using steam (Sturrock & Dennis 1988, Peterson 1990, Trent et al 2007), and relatively strong disinfectants such as hydrogen peroxide (Peterson 1990, Van

Wyk et al 2012) or sodium metabisulphite (Sturrock & Dennis 1988, Peterson 1990, Dumroese et al 1993a). The use of hot water dipping (Sturrock & Dennis 1988, James & Woollen 1989, Peterson 1990, Dumroese et al 2002, Kohmann & Børja 2002) and phenolic or chlorine based disinfectants (Sturrock & Dennis 1988, Peterson 1990, Cram 2002, Van Wyk et al 2012) generally reduced *Fusarium* inoculum levels but to a lesser degree than steam or hydrogen peroxide treatment.

Most studies report a reduction rather than elimination of *Fusarium* inoculum from sanitation measures (James & Woollen 1989, James & Trent 2001, Kohmann & Børja 2002). Typically the reduction in fungal inoculum has been used as the measure of sanitation achieved with comparatively few studies focused on assessment by raising seedlings in the sanitized containers (Dumroese et al 1993a, 2002, Kohmann & Børja 2002). Dumroese et al (2002) found that *Pseudotsuga menziesii* seedlings raised in polyethylene containers that were re-used through five cropping cycles without sanitation were 24% smaller with a reduction in saleable plants per container (69% vs 78% of capacity) compared to crops where containers were sanitized by hot water dip (82°C for 90 seconds) between each cycle. They also found the number of seedlings associated with *Fusarium* species was reduced by sanitation (86% vs 59%). Container sanitation measures have been developed mainly to control damping off and root rot diseases in the nursery and the efficacy in control of *F. circinatum* as a cause of post-planting mortality have not been established.

Deploying planting stock that is genetically less susceptible to *F. circinatum* is a promising strategy to combat the risk associated with this pathogen (Mitchell et al 2012a, Kanzler et al 2014). Variation in susceptibility to *F. circinatum* has been found using stem inoculation screening trials at the between species level (Hodge & Dvorak 2000, Mitchell et al 2012b), provenance and family level within species (Hodge & Dvorak 2007, Mitchell et al 2012c, Nel

et al 2014) and for hybrid combinations of *P. patula* with species more tolerant of *F. circinatum* (Roux et al 2007, Mitchell et al 2013, Kanzler et al 2014). Use of genetically tolerant planting stock and nursery hygiene measures are both important elements of an integrated pest management strategy. The relative importance of these two interventions has not been evaluated for the control of *F. circinatum* related disease in seedling production and stand regeneration.

The objective of this study was to determine if the re-use of seedling containers represents a potential inoculum source of *F. circinatum* in *P. patula* seedling crops that contributes to post-planting mortality. A further aim was to evaluate if the use of *P. patula* seed that is genetically more tolerant of *F. circinatum* could contribute to a lower disease incidence. For this purpose two seedling production experiments were conducted in the nursery over consecutive years, both followed by out-planting into pots to assess post-planting mortality.

## **Materials and methods**

### *Nursery production system*

The facility used for the reported experiments was representative of commercial containerized seedling nursery systems in South Africa. Seedling containers were hard polyethylene containers with removable individual cavity inserts (7 x 14 rows per container). Inserts with a 90 cm<sup>3</sup> volume, square in cross-section (37 mm at top, 28 mm at base) and 100 mm deep with a single 1 mm ridge on each face to prevent root spiralling were used (UniGrow 98 containers manufactured by Plasgrow, RSA). Used containers were sourced from a commercial nursery known to be contaminated with *F. circinatum* that had produced rooted cuttings of *Pinus patula* x *P. tecunumanii* as the previous crop approximately six months previous. A commercial composted pine bark growing medium was used.

Containers were placed on raised rack tables with sprinkle irrigation. Fertilizer was applied through the irrigation water in Experiment A and as slow release fertilizer (Osmocote® Exact Mini 3-4 month release) in Experiment B. The standard sanitation method used on seedling containers before re-use is steam heating in an insulated chamber and this operational equipment was used for the sanitation treatment in the experiments described below. An iButton® temperature data logger placed in the middle of the containers used in Experiment B during steam treatment recorded a maximum temperature of 74°C with 1 hour above 70°C in a 2.5 hour heating cycle.

#### *Experiment A design and conduct*

The first experiment (A) compared seedling production in factory new inserts, used inserts steam sanitized before re-use and re-used inserts without sanitizing. A commercial open pollinated seed orchard source of *P. patula* was sown into 14 containers for each treatment in November 2010. Initial germination success was 76% and one-way ANOVA on tray mean germination indicated this was unrelated to treatment. Seedlings were packed into a randomized block design 42 days after sowing (DAS). Treatment plot size was 28 seedlings with three plots per container (4 x 7 row plots with one empty row separating plots) and each container formed a randomised block. The experiment consisted of 18 containers representing a randomized complete block design with 18 replicates.

Seedling height was measured in the nursery on the inner 5 x 2 seedlings in each plot on seven occasions at 4 to 6 week intervals between 84 and 267 DAS. One randomly selected seedling was sampled from each treatment plot at 86, 121, 155, 183, 213, 240, 269 DAS (for the first five dates this was within two days of the first four height measurements). Of these, five from each treatment were used for *F. circinatum* isolation and three from each treatment

for root development measurements. Height and root collar diameter were measured on each sampled seedling.

At 283 DAS, seedlings were transplanted into polypot bags (3 L volume) filled with composted pine bark growing medium. A 3 x 2 factorial experiment was established with the three container treatments compared at two watering frequencies (weekly and fortnightly). A randomized complete block design was used with five replicates and treatment plots of 20 seedlings (4 x 5 pots). Post-planting mortality was monitored weekly for 180 days after planting (DAP).

#### *Experiment B design and conduct*

The second experiment (B) aimed to verify trends observed in Experiment A and in addition evaluated the importance of genetic variation in tolerance to *F. circinatum* as a means of reducing disease losses. A combination of container treatment and seed source was tested in a 3 x 3 factorial. The container treatment factor was the same as that used in Experiment A. Three seed sources were compared as the second factor; a *P. tecunumanii* seed mix and two *P. patula* seed mixes. Each mix was a combination of three open pollinated seed orchard families. The two *P. patula* mixes were based on *F. circinatum* susceptibility screening tests on seedlings (Nel et al 2014); a susceptible mix of families ranked 236<sup>th</sup>, 241<sup>st</sup> and 243<sup>rd</sup> poorest and a tolerant mix of families ranked 3<sup>rd</sup>, 5<sup>th</sup> and 6<sup>th</sup> out of 246 families, respectively. The *P. tecunumanii* families were chosen at random from available seed. Family identity was retained, by sowing separately, to allow an equal mix per tray treatment of each family in both the nursery and post-planting survival experiments but only seed mix results are presented in this paper.

Experiment B was conducted similarly to Experiment A. Seed was sown in October 2011 into two containers per family per container treatment. Initial germination was 69% with no statistically significant effects due to treatments. Seedlings were packed into a split-plot randomised block design 73 DAS. Treatment plot size was 42 seedlings with two plots per container (6 x 7 rows with two empty rows separating plots). Seed source was the main plot treatment (formed by two containers) with the four container treatments as the split plot. There were five randomized block replicates. Seedling height was measured for each treatment plot (on the centre row of six seedlings per plot) at 371 DAS. One randomly selected seedling was removed per plot at 94, 122, 157, 183 and 370 DAS, height and root collar diameter were recorded and *F. circinatum* isolations performed.

At 374 DAS seedlings were transplanted into polypot bags (3 L volume) filled with composted pine bark growing medium. A 3 x 2 factorial treatment combination was used with the three container treatments and the two *P. patula* seed mixes. The *P. tecunumanii* seedlings were omitted from the pot trial as very little post-planting mortality was anticipated. A randomized complete block design was used with four replicates and a 12 seedling plot size (3 x 4 bags). All treatments received the same watering. Post-planting seedling mortality was assessed weekly for 180 days.

#### Isolation of *Fusarium circinatum*

Samples of seed, composted pine bark and container inserts were examined for the presence of *F. circinatum*. Samples of seed (200 per seed lot used) were crushed to break the seed coat and plated directly onto *Fusarium* selective medium (FSM) after Leslie and Summerell (2006); half the seed was plated without sterilizing and half was surface sterilized using a 60 second sodium hypochlorite (2%) treatment, followed by soaking in 70% ethanol for 60 seconds and a final rinse with sterile distilled water. Two composite samples of the composted pine bark growing medium used in Experiment A were taken from the inside of

the nursery storage pile and one from the surface of the pile. For each bark sample, multiple pieces of bark were aseptically spread across thirty FSM plates. Random samples of 60 container inserts per treatment from Experiment A and 50 per treatment from Experiment B were taken immediately prior to sowing. The inside of each insert was swabbed using a sterile cotton bud starting at the base and moving upwards before streaking onto FSM.

Symptomatic and asymptomatic seedlings were sampled both to detect the presence of *F. circinatum* and to determine the frequency of its association with root tips. Root systems were gently washed free of growing medium before sampling. For the purposeful detection of *F. circinatum* two pieces of tissue (preferentially those showing signs of infection) from root tips, root collar, stem and growing tip, respectively, from each seedling were plated on FSM. The root system was then separated into two halves with one half surface sterilized in 2% sodium hypochlorite for one minute followed by rinsing in sterile distilled water. From each half eight root tips were randomly selected and plated onto FSM.

Following incubation of inoculated FSM plates at 25°C for 7 days under cool, white fluorescent light, colonies resembling *Fusarium* were transferred to half-strength potato dextrose agar medium and incubated for 7 days at 25°C. Isolates were examined microscopically to allow for morphology-based identification of *F. circinatum* (Nirenberg and O'Donnell 1889). The identity of presumptive *F. circinatum* isolates were then confirmed using PCR-based species-specific diagnosis as previously described (Steenkamp et al. 2012).

#### *Seedling root development measurements*



Root length and root tip counts for each of 36 random samples of asymptomatic seedlings taken from Experiment A was estimated by a line intersect method (Tennant 1975) using digital images of root systems that were carefully washed to remove growing medium, cut into parts (mostly at intersect of tap root with secondary roots) and spread onto a white surface marked with a 10 x 10 mm grid. Total root length and white root length was estimated using counts of root intersects with the grid. Counts of total root tips and white root tips were also made. White roots refer to one of the three distinct anatomical zones of pine roots described by Peterson et al. (1999). The white root tip with its live cortex is located nearest the growing tip followed by the condensed tannin having a dead cortex with tannin deposition in cells and the cork zone that is characterised by development of secondary xylem and bark separated by a cambial zone. Only the transition from white to condensed tannin can be distinguished macroscopically based on colour and reduced root diameter as the condensed zone forms.

### *Statistical analysis*

Where data were obtained in a structured orthogonal form from the designed nursery and pot planting experiments ANOVA was used to determine significant treatment effects. Significant differences between treatment means were identified using the HSD Tukey test. Other comparisons between groups used statistical tests as described. Data were analysed using Statistica version 6.0.

## **Results**

*Fusarium circinatum* associated with seed, composted pine bark and seedling containers

*Fusarium circinatum* was not detected in any of the seed or composted pine bark samples examined in this study. However, seedling container cavities were found to be contaminated. The frequency of *F. circinatum* contamination of container cavity inserts found in both experiments was consistent with the subsequent cull of symptomatic seedlings in the nursery experiments. No *F. circinatum* was detected in factory new inserts. The re-used sanitized containers had some *F. circinatum* (3.3% of inserts in Experiment A, none in Experiment B) and without sanitation a higher frequency of contaminated insets were found (6.6% in Experiment A, 18.3% in Experiment B). A good correlation was found between container insert contamination and cull of seedlings in the nursery across the two experiments (Figure 1).

#### *Cull of symptomatic seedlings in the nursery*

The culling of symptomatic seedlings, that were showing signs of wilt, began between 60 and 120 DAS in both experiments and 80% of culled seedlings were removed by 220 DAS (Figure 2). This timing is as typically observed in commercial *P. patula* seedling production where *F. circinatum* is present the nursery.

In Experiment A the total cull of symptomatic seedlings differed between container treatments ( $p < 0.01$ ) with a significantly higher cull occurring in containers that were re-used without sanitation compared to low culling in factory new and re-used sanitized containers that were not significantly different (Figure 3). In Experiment B the cull of symptomatic seedlings was influenced by both container treatment ( $p < 0.0001$ ) and seed source ( $p < 0.01$ ) with a significant interaction between these factors ( $p < 0.02$ ). As in Experiment A, re-use of containers without sanitizing resulted in significantly higher cull of *P. patula* seedlings than with factory new or sanitized containers (Figure 4). Although in each container treatment the susceptible *P. patula* seed source required more culling than the tolerant *P. patula* seed source, overall the difference was not statistically significant (Table 1). Both *P. patula* seed

sources required considerably more culling than the *P. tecunumanii* seed source which received low culling irrespective of container treatment producing the significant two-way treatment interaction.

#### *Fusarium circinatum* associated with symptomatic seedlings

During the course of Experiment A, culled symptomatic seedlings were also sampled for *F. circinatum*. Of 28 seedlings sampled, *F. circinatum* was isolated from 46%. This level is comparable to that reported by Jones et al (2014) on *P. patula* seedlings culled at similar rates in the nursery and from *P. patula* seedlings that experienced post-planting mortality (42%) (Crous 2005). The frequency of *F. circinatum* isolation from purposeful root collar and root tip samples was much higher in symptomatic seedlings and the incidence of false negatives was greatly reduced (Table 2).

#### *Fusarium circinatum* associated with asymptomatic seedlings

The association of *F. circinatum* with symptomatic seedlings was determined by isolation from all sampled tissues (purposeful samples and random root tips). No significant correlation was found between frequencies of *F. circinatum* associated asymptomatic seedlings and seedling age. Sample dates were therefore treated as replicates in testing for treatment effects in both experiments. Although not statistically significant, in both experiments the proportion of asymptomatic seedlings with associated *F. circinatum* was highest in containers that were not sanitized and lowest in new containers. In Experiment A, seedlings in new containers had 17% seedlings associated with *F. circinatum*, re-used sanitized containers 23% and re-used without cleaning 34% (Experiment B results in Figure 5). In Experiment B seed source was associated with statistically significant differences (Table 1) in proportions of *F. circinatum* associated asymptomatic seedlings ( $p < 0.05$ ); being higher in *P. tecunumanii* seedlings than *P. patula* (Figure 5).

Overall the frequency of *F. circinatum* isolation from various tissue samples was low. Across both experiments *F. circinatum* was detected in only 0.9% of surface disinfected root tips and 2.7% of non-surface-disinfected root tips that were randomly selected from 573 seedlings (4 584 root tips of each category). This incidence was statistically significantly different ( $p < 0.05$ ) between container treatments for non-sterilized root tips in Experiment B (Table 3). When frequency of isolation is considered for only those seedlings where *F. circinatum* was isolated from at least one of the tissue samples taken, there were no statistically significant treatment effects (Table 4). This implies the likelihood of asymptomatic seedling association with *F. circinatum* is increased by some treatments but that degree of infection on these seedlings is similar across container treatment.

False negative seedlings, where *F. circinatum* was isolated from random root tip samples but not from purposeful tissue samples, were common. Approximately a third of *F. circinatum* associated seedlings in Experiment A and half those in Experiment B were false negatives (Table 2). A chi-squared test applied to determine if the frequency expected from random non-sterilized root tip samples differed from that measured taking purposeful root tips that appeared discoloured or necrotic indicated the two methods did not differ in ability to detect *F. circinatum* ( $p=0.21$ ). Chi-squared tests also indicated the frequency of false negatives did not differ between container treatments or seed source using overall trial mean false negatives as the expected outcome ( $p=0.89$  in Experiment A and  $p=0.98$  in Experiment B for tray treatments,  $p=0.29$  for seed source).

### *Seedling growth in the nursery*

At none of the height measurements did ANOVA indicate a statistically significant difference in mean height between the three container treatments in Experiment A. Two weeks prior to planting in pots (267 DAS) trial mean height was 124 mm (SE  $\pm 15$  mm) and the mean for

each container treatment was within 1 mm of the overall mean. Similarly, in Experiment B container treatment had no significant effect on seedling mean height prior to planting in pots (371 DAS). Mean height of *P. tecunumanii* (275 ±72 mm) was significantly higher ( $p < 0.01$ ) than *P. patula* (200 ±32 mm for susceptible and 208 ±40 mm for tolerant seed sources) but there was no significant interaction with container treatment.

The 315 asymptomatic seedlings randomly sampled for *F. circinatum* isolations in Experiment A provided an opportunity to compare size of seedlings with and without a known association of *F. circinatum*. After correcting for seedling age effects with standardized normal deviates a t-test indicated no statistically significant difference in either height or root collar diameter for seedlings with or without associated *F. circinatum* (Table 5). A similar comparison using the seedlings sampled from Experiment B was not possible due to sample size per treatment limitations on the number of *F. circinatum* associated seedlings.

The effect of container treatment and seedling age on root length and root tip count data from Experiment A was investigated using ANOVA with each seedling used as a replicate. None of the parameters were significantly different between the three container treatments. Total root length and count of root tips were statistically significantly related to, and increased with seedling age (Table 6). In contrast, the total length and count of white root tips was not statistically significantly different between seedling ages. White root tips formed a major part of root systems at 86 DAS (30% of total root length and 76% of root tips) but were proportionally less on subsequent sample dates (Table 6).

#### *Post-planting seedling performance in pot trials*

The purpose of the pot trials was to measure treatment effects on seedling post-plant mortality. In both Experiment A and B, container sanitation treatments produced statistically highly significant differences in survival (Table 7). Containers re-used without sanitation

produced significantly higher mortality than new or re-used sanitized containers (Fig 7). In Experiment A, by 180DAP, the longer watering interval began to experience a statistically significant higher mortality across all treatments ( $p < 0.01$ ), yet watering frequency did not interact significantly with container sanitation treatment. Seedlings raised in re-used containers without sanitation did not require water stress to induce or accentuate post-planting mortality. In Experiment B, there was no significant difference in post-planting mortality between the two *P. patula* seedling mix treatments.

Mean seedling height at 180 DAP was not influenced by container sanitation treatments in Experiments A or B (Table 7). The lower watering frequency in Experiment A resulted in statistically significant ( $p < 0.001$ ) shorter seedlings ( $225 \pm 23$  mm watered every week,  $267 \pm 15$  mm watered every second week). There was no significant difference in height between the two *P. patula* seed mix treatments.

The successful isolation of *F. circinatum* from dead seedlings removed from the pot trials occurred in 18% of plants which were still sufficiently fresh. Seedlings were only removed from the trials when recovery was not expected. *Fusarium circinatum* is not readily detectable in dead plant tissue typically requiring tissue from seedlings showing only incipient disease symptoms for isolation.

## **Discussion**

This study has shown that re-use of seedling containers without sanitation results in a substantially higher frequency of *F. circinatum* associated seedling mortality in the nursery. The higher incidence of *F. circinatum* contamination in re-used containers without sanitation was accompanied by an increased nursery cull of symptomatic seedlings and post-planting mortality. Our results clearly demonstrated that steam treatment, following a current

operational protocol, significantly reduced the incidence of *F. circinatum* to levels comparable to those observed using factory new seedling containers. These findings support container sanitation as an important measure for controlling *F. circinatum* infection of pine seedlings in containerized nurseries.

We found a higher incidence of *F. circinatum* associated with asymptomatic seedlings raised in re-used containers without sanitation. Our analyses also revealed that container sanitation had a statistically significant effect on the survival of asymptomatic seedlings that were planted out into pots. This link between *F. circinatum* contamination of seedlings in the nursery and post-planting mortality was also shown by Jones et al (2014) who demonstrated that inoculation of the seedling crop in the nursery results in post-planting mortality of seedlings due to disease. The association of *F. circinatum* with asymptomatic seedlings of *P. patula* confirms similar observations on *P. radiata* (Storer et al 1998, Swett and Gordon 2011) and *P. patula* (Mitchell et al 2011, Jones et al 2014). Our study indicates post-planting mortality of *P. patula* is linked to a natural *F. circinatum* inoculum source in nursery seedling production.

Disease symptoms observed in our experiments were distinct from other nursery root rot diseases, caused by other *Fusarium* species. Several *Fusarium* species are pathogens of conifers in nurseries and disease symptoms are well described (Dumroese and James 2005, Peterson 2008). The most common symptoms are pre- and post-emergence damping off, typically resulting from contaminated seed, and seedling root rot which in containerized nurseries often manifests near the end of the production cycle. Root rot is associated with blackened necrotic cortex and fewer actively growing root tips with a moribund root system resulting in reduced shoot growth and seedling wilt. However, colonization of conifer seedlings in the nursery by pathogenic *Fusarium* species other than *F. circinatum* has been

shown not to result in persistent post-planting infection or mortality (Smith et al 1965, Dumroese et al 1993b, Axelrood et al 1998).

In this study there was no seedling growth difference among container treatments or between asymptomatic seedlings with and without associated *F. circinatum*. Total root length, number of white root tips and height of symptomatic seedlings were unaffected by container treatment. Discolouration of the root systems of seedlings generally occurred at the same time as symptomatic shoot wilt. Swett and Gordon (2011) report symptomless infection of *P. radiata* seedling roots by *F. circinatum* that persisted for more than a year. These findings have practical importance. Our study and that of Jones et al (2014) clearly link nursery infection with *F. circinatum* to subsequent post-planting mortality. Nurserymen typically measure disease control as reduced seedling losses in the nursery; a strategy appropriate for other root rot and damping off diseases. However, the asymptomatic nature of *F. circinatum* association with seedlings in the nursery makes such measures less useful for managing this pathogen. We suggest that, for the development of effective control measures against *F. circinatum* in the nursery, a seedling quality assessment that relates to post-planting survival is required.

*Fusarium circinatum* has been shown to produce damping off following inoculation of seed and germinating seedlings (Viljoen and Wingfield 1994, Aegerter and Gordon 2006). The initial germination success of the seedlings raised for our experiments did not indicate more damping-off losses associated with container re-use without sanitation. Seed and composted bark growing medium did not yield *F. circinatum* and measured deposition of airborne spores of *F. circinatum* in a South African nursery (Fourie et al 2014) suggests this source of inoculum is considerably lower than artificial rates used to demonstrate pathogenicity in damping off. In our experiments we conclude the surface of re-used seedling containers was a major primary source of *F. circinatum* inoculum.



Many pathogens gain root access through the white zone of actively growing roots (Okubara and Paulitz 2005). This has been observed for *Fusarium* species on a range of hosts (Alabouvette et al 2009) including woody plants (Marks 1965, Farquhar and Peterson 1989, Rodríguez-Gálvez and Mendgen 1995, Salerno et al 2000). At 42 DAS, when seedlings were packed into the trial design, roots had not yet grown into contact with the seedling container cavity sides (based on six seedlings sampled data not shown here) but by 86 days nine sampled seedlings had all established roots in contact with walls of container cavities. In our experiments the location of *F. circinatum* inoculum on the seedling containers, timing of root development and first disease symptoms support this mechanism of root infection.

Post-planting water supply did not influence the progress of mortality in relation to container treatments (Experiment A) despite differences in growth rates indicating water supply was influencing plant growth. This is consistent with other observations that low water supply is not a prerequisite and does not increase mortality due to *F. circinatum* infection. The application of water at time of planting is a common practice in South Africa to reduce post-planting mortality (Rolando and Crous 2007) but has not reduced losses associated with *F. circinatum* in *P. patula* out-plantings (Crous 2005, Rolando and Little 2010, Jones et al 2014). A severe reduction in watering in the nursery did not induce greater symptomatic seedling culling of *F. circinatum* inoculated seedlings or post-planting mortality (Jones et al 2014). Shoot water potential differences in seven-month-old *P. patula* seedlings planted in pots, regulated by watering, did not influence symptom development following stem inoculation with *F. circinatum* (Nadel et al 2013). Application of fungicides at planting has been shown to only delay *P. patula* mortality associated with *F. circinatum* (Mitchell et al 2004, Crous 2005). Changing planting practice to reduce the impact of *F. circinatum* seems not to be an effective management option and indicates the need to reduce incidence of *F. circinatum* association with *P. patula* during nursery production of planting stock.

The two *P. patula* seed mixes compared in experiment B were constituted to represent the range in susceptibility to *F. circinatum* measured by stem lesion development following inoculation. However, this difference did not transfer into significant differences in nursery cull or post-planting mortality. Aegerter and Gordon (2006) also found no correlation between families of *P. radiata* in their susceptibility to damping off following inoculation of seed and stem lesion development following seedling stem inoculation with *F. circinatum*. The response of *P. patula* to artificial stem infection with *F. circinatum* in seedlings and mature plantation trees has been demonstrated (Mitchell et al 2014). Successful improvement in tolerance to *F. circinatum* through selection may require different screening techniques to simulate nursery and field infections.

An unexpected finding of this study was the apparent increased incidence of *F. circinatum* association with asymptomatic *P. tecunumanii* seedlings compared to what were observed for the two *P. patula* seedling groups. This is despite the fact that significantly fewer *P. tecunumanii* than *P. patula* seedlings were culled during the course of the second experiment. Why *F. circinatum* was isolated more often from asymptomatic plants of the highly tolerant *P. tecunumanii* compared to susceptible *P. patula* (Hodge and Dvorak 2000) is unclear. This effect warrants further investigation.

## **Conclusions**

Results of this study demonstrate that contamination of nursery production inputs, in particular re-used seedling containers, with *F. circinatum* can result in asymptomatic planting stock of *P. patula* which will experience markedly increased post-planting mortality. Post-planting mortality can be high even when nursery cull of diseased plants is low emphasising the need for good quality control of nursery hygiene practices. Existing steam treatment of containers before re-use is effective in controlling this source of primary infection.

Management intervention to reduce post-planting mortality of *P. patula* should focus on reducing incidence of asymptomatic planting stock association with *F. circinatum* when despatched from the nursery.

The mechanism of infection, location of latent infection in the seedling and reason for ultimate disease expression in asymptomatic seedlings associated with *F. circinatum* is not understood. Evidence from this study suggests that growing root tips are a means of entry. The onset of wilt in symptomatic seedlings in the nursery was not found to be preceded by a progressive necrotic decline in the root system or by reduced seedling growth rates. This suggests a girdling canker at or near the root collar as a common means of disease expression.

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Table 1: Analysis of variance of the cull of symptomatic seedlings, final height and association of asymptomatic seedlings with *F. circinatum* in experiment B nursery results.

	df	Cull <sup>a</sup> (%)		<i>F. circinatum</i> associated asymptomatic seedlings <sup>b</sup> (%)		Seedling mean height <sup>c</sup> (mm)	
		F	p	F	p	F	p
Seed mix	2	9.5	<0.01	3.22	<0.05	27.2	<0.0001
Tray treatment	2	25.4	<0.0001	1.51	ns	0.5	ns
Treatment interaction	4	5.0	<0.01	0.10	ns	0.9	ns
Trial grand mean		1.13		0.216		231	
Main plot coeff. Variation		65.6%		80.2%		4.6%	
Subplot coeff. Variation		78.2%		46.3%		3.0%	

<sup>a</sup> Cull data is at 180 DAS and is square root transformed

<sup>b</sup> *F. circinatum* associated seedling data are means of four sample dates and were arcsin transformed

<sup>c</sup> Mean height is prior to planting in pot trial (371 DAS)



Table 2: Detection of *F. circinatum* associated with random sample of asymptomatic seedlings and culled symptomatic seedlings from different excised tissue samples.

<b>Tissue sample</b>	<b>Asymptomatic seedlings associated with <i>F. circinatum</i> (%)</b>		<b>Symptomatic seedlings associated with <i>F. circinatum</i> (%)</b>
	<b>Exp. A</b>	<b>Exp. B</b>	<b>Exp. A</b>
Purposeful shoot stem/tip	0.0	0.9	0.0
Purposeful root collar	1.0	2.6	28.6
Purposeful root tip	10.4	4.8	35.7
Random root tip	13.6	13.3	25.0
All tissue samples combined	17.1	17.1	46.4
False negative seedlings <sup>a</sup>	36%	51%	4%
Number of seedlings sampled	105	240	28

<sup>a</sup> False negatives were defined as seedlings from which *F. circinatum* was isolated from random root tip samples but not purposeful samples

Table 3: Frequency (%) of root tips associated with *F. circinatum* in asymptomatic seedlings. Mean of all random seedling samples taken at 86, 121, 155, 183, 213, 240, 269 DAS from Experiment A and 94, 122, 157, 183 DAS from Experiment B. Isolations were made from samples of surface sterilized and non-surface sterilized root tips.

Tray treatment	Experiment A		Experiment B	
	Surface sterilized root tips	Non-surface sterilized root tips	Surface sterilized root tips	Non-surface sterilized root tips
Factory new	<b>0.62<sup>a</sup></b> ±0.30	<b>0.83<sup>a</sup></b> ±0.46	<b>0.83<sup>a</sup></b> ±0.35	<b>0.00<sup>a</sup></b> ±1.73
Sanitized re-used	<b>0.83<sup>a</sup></b> ±0.10	<b>1.04<sup>a</sup></b> ±0.46	<b>0.87<sup>a</sup></b> ±0.75	<b>4.43<sup>b</sup></b> ±1.97
Re-used without cleaning	<b>0.31<sup>a</sup></b> ±0.29	<b>2.92<sup>a</sup></b> ±0.80	<b>2.55<sup>a</sup></b> ±0.96	<b>7.86<sup>c</sup></b> ±2.20
Mean	<b>0.59</b>	<b>1.60</b>	<b>1.43</b>	<b>4.31</b>
F test probability	p=0.22	p=0.06	p=0.53	p=0.04

Tray treatment means within each experiment that are significantly different at 5% level are indicated by superscript letters.

F test from ANOVA on square root transformed data.

Means shown in bold with standard error below.

Table 4: Frequency (%) of root tips associated with *F. circinatum* in asymptomatic seedlings from which *F. circinatum* had been isolated in at least one tissue sample.

	Root tip samples (%)	
	Surface sterilized	Not sterilized
Experiment A		
Factory new	5.0	6.7
Re-used sanitized	5.6	6.9
Re-used without cleaning	1.5	14.0
Trial mean	3.7	9.9
Experiment B - tray treatments		
Factory new	10.0	0.0
Re-used sanitized	6.3	32.8
Re-used without cleaning	11.5	29.8
Trial mean	8.3	26.3
Experiment B - seed source treatments		
<i>P. tecunumanii</i>	8.9	27.4
<i>P. patula</i> - tolerant	3.8	18.8
<i>P. patula</i> - susceptible	12.5	23.4
No statistical test was able to detect significant differences between treatments.		

Table 5: Height and root collar diameter of randomly sampled asymptomatic seedlings in Experiment A comparing seedlings found associated with *F. circinatum* with those where *F. circinatum* was not isolated.

DAS		Height (mm)		Root collar diameter (mm)		Count	
		Negative seedlings	Positive seedlings	Negative seedlings	Positive seedlings	Negative seedlings	Positive seedlings
86	Mean	59	60	1.22	1.21	18	27
	SE	±12	±11	±0.27	±0.18		
121	Mean	91	93	1.76	1.71	34	11
	SE	±16	±12	±0.19	±0.16		
155	Mean	106	111	2.08	2.13	38	7
	SE	±17	±15	±0.19	±0.30		
183	Mean	110	-	2.18	-	45	0
	SE	±13	-	±0.23	-		
213	Mean	114	103	2.22	2.20	37	8
	SE	±17	±9	±0.32	±0.31		
239	Mean	116	93	2.27	2.05	43	2
	SE	±15	±11	±0.26	±0.21		
269	Mean	113	115	2.34	2.33	42	3
	SE	±20	±0	±0.27	±0.15		
Standardized normal deviate <sup>a</sup>	Mean	0.013	-0.046	0.015	-0.055	212	58
	SE	1.019	0.886	1.011	0.919		
	t test		0.43		0.50		
	p		0.69		0.63		

<sup>a</sup> Standardized normal deviates were calculated to correct for seedling age and excluded seedlings from 183 DAS sampling date.

Table 6: Seedling height, root collar diameter, root length and root tip counts for 36 asymptomatic seedlings randomly sampled in Experiment A.

Variable		Period since sowing				F test significance
		86 days	121 days	155 days	183 days	
Height (mm)	Mean	60	91	107	109	<0.0001%
	SE	±1.7	±2.2	±2.4	±2.0	
Root collar diameter (mm)	Mean	1.2	1.8	2.1	2.2	<0.0001%
	SE	±0.03	±0.03	±0.03	±0.03	
Biomass Index <sup>a</sup>	Mean	93	286	476	529	<0.0001%
	SE	±6	±13	±22	±21	
Total root length (m)	Mean	1.18	4.31	5.42	7.25	<0.0001%
	SE	±0.17	±0.35	±0.33	±0.65	
White root length (m)	Mean	0.33	0.28	0.41	0.32	ns
	SE	±0.03	±0.04	±0.05	±0.05	
Total root tip count	Mean	42	102	142	184	<0.0001%
	SE	±5	±8	±18	±17	
White root tip count	Mean	31	33	41	46	ns
	SE	±3	±4	±5	±6	
White root tips (% total)	Mean	76	32	30	26	<0.0001%
	SE	±4	±2	±2	±2	
White root length (% total)	Mean	29.7	6.3	7.6	4.2	<0.0001%
	SE	±2.5	±0.5	±0.8	±0.4	

<sup>a</sup> Biomass index calculated as product of height and square of root collar diameter.

Table 7: Analysis of variance results for post-planting mortality in pots at 60, 120 and 180 DAP and mean height at 180DAP for Experiments A (a) and B (b).

a) Experiment A

	df	Mortality <sup>a</sup> (%)						Height (m)	
		60 DAP		120 DAP		180 DAP		180DAP	
		F	p	F	p	F	p	F	p
Tray treatment	2	15.8	<0.0001	19.1	<0.0001	9.06	<0.001	1.09	ns
Water frequency	1	0.04	ns	0.65	ns	3.15	<0.01	48.91	<0.001
Treatment interaction	2	0.04	ns	0.19	ns	1.10	ns	1.05	ns
Trial mean		3.2%		5.7%		11.5%		0.25 m	
Main plot coeff. variation		107.7%		90.4%		84.6%		9.8%	
Subplot coeff. variation		107.7%		85.2%		53.6%		7.0%	

<sup>a</sup> Mortality data was square-root transformed for analysis but non-transformed trial means presented.

b) Experiment B

	df	Mortality <sup>a</sup> (%)						Height (m)	
		60 DAP		120 DAP		180 DAP		180DAP	
		F	p	F	p	F	p	F	p
Tray treatment	2	3.24	ns	28.60	<0.0001	19.18	<0.001	0.61	ns
Seed source	1	0.73	ns	0.23	ns	0.01	ns	0.10	ns
Treatment interaction	2	0.73	ns	0.32	ns	0.77	ns	0.59	ns
Trial mean		2.8%		6.6%		13.3%		0.33 m	
Coefficient of variation		99.1%		86.5%		75.5%		10.4%	

<sup>a</sup> Mortality data was square-root transformed for analysis but non-transformed trial means presented.

Figure 1: The relationship between measured tray insert contamination and cull of *P. patula* seedlings in the nursery for the various tray sanitation treatments evaluated. Experiment B data are for the mean of both *P. patula* seed mixes.

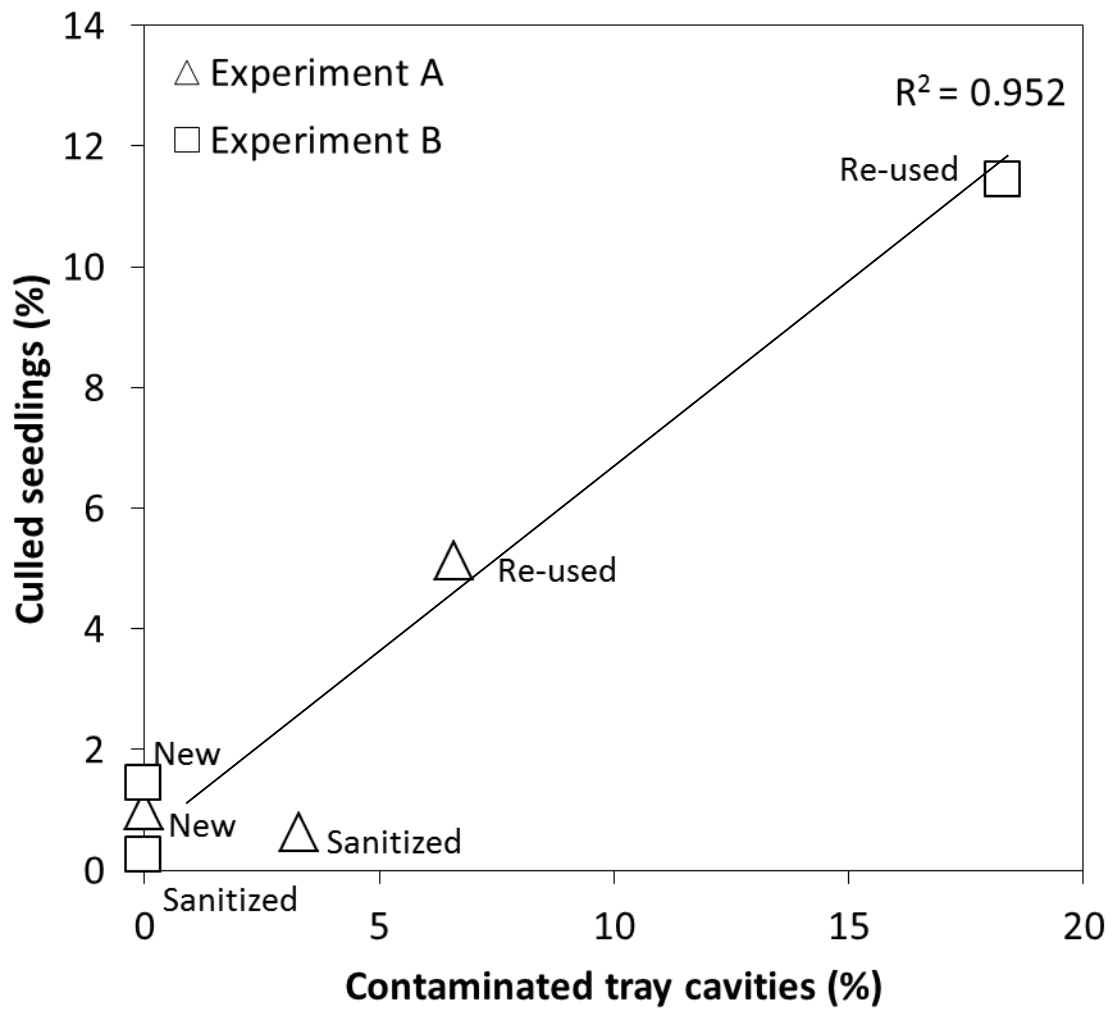


Figure 2: Cull of symptomatic seedlings raised in re-used containers without cleaning in Experiment A and B.

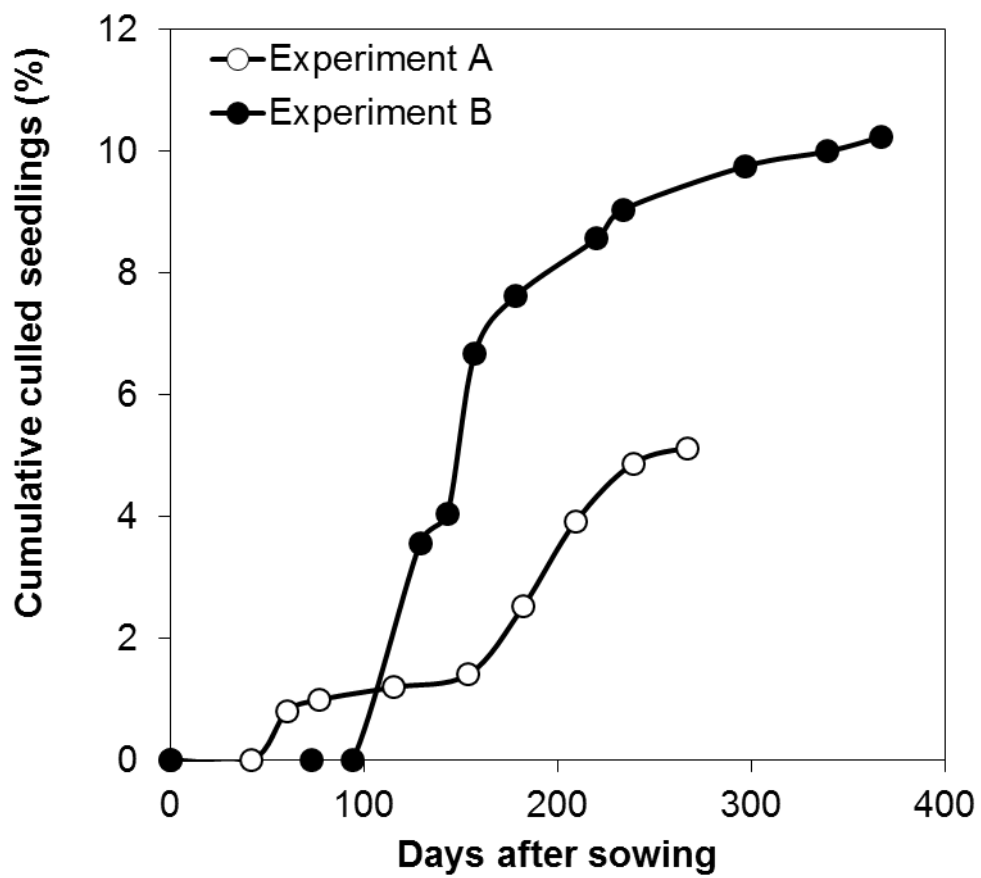




Figure 3: Total seedlings culled in nursery for Experiment A. Values with the same letter were not significantly different at the 5% level applying Tukey's HSD to square root transformed data. Transformed trial mean cull was 0.77% with SE  $\pm$  0.22%. Data presented in figure is not transformed.

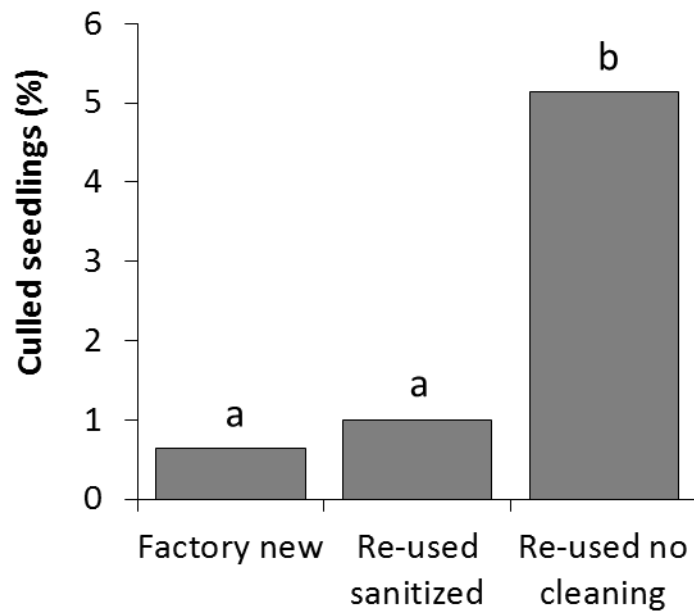


Figure 4: Total symptomatic seedlings culled in nursery for Experiment B. Values with the same letter were not significantly different at the 5% level applying Tukey's HSD to square root transformed data. Transformed trial mean cull was 1.08% with SE  $\pm$  0.28% for main plots and  $\pm$  0.34% for main plots. Data presented in figure is not transformed.

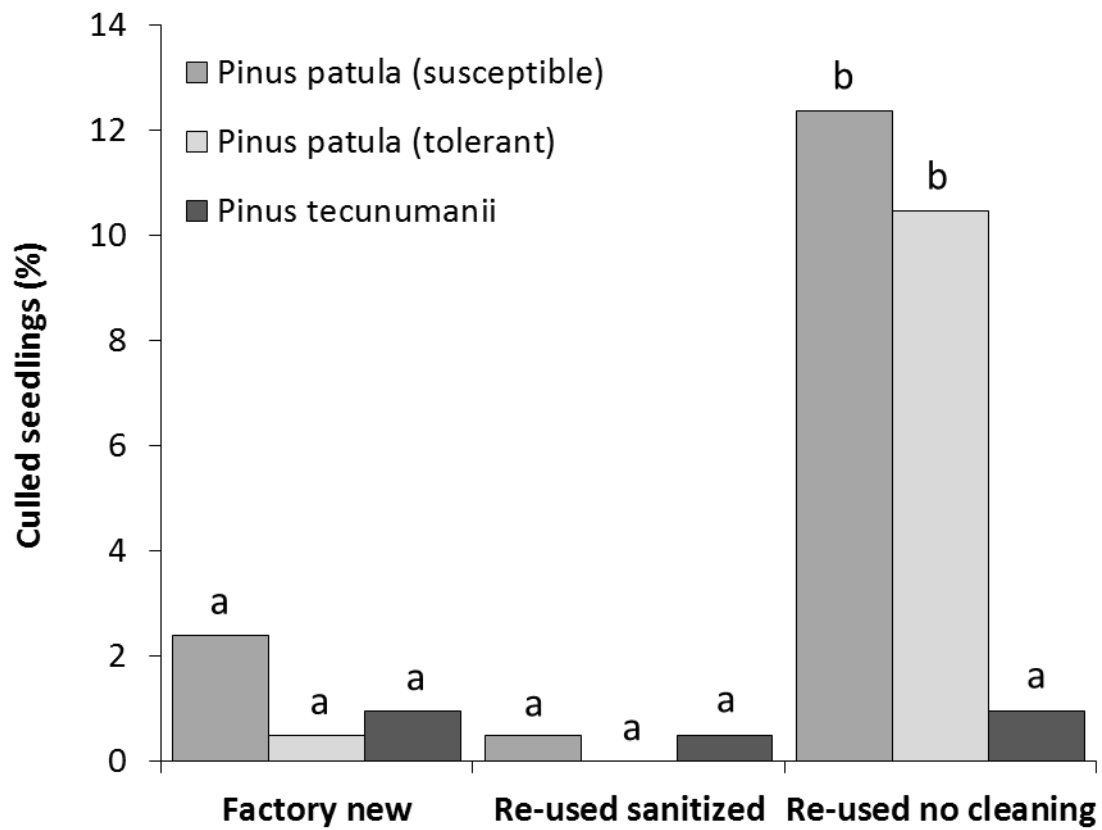


Figure 5: Proportion of asymptomatic seedlings associated with *F. circinatum* in Experiment B for the main treatment effects tray treatment and seed mix. Mean of seedlings sampled 86, 121, 155 and 183 DAS using results from all random root tips and purposeful tissue samples plated. The association of *F. circinatum* with symptomatic seedlings was determined by isolation from all sampled tissues (purposeful samples and random root tips). Values with the same letter were not significantly different at the 5% level applying Tukey's HSD to arcsin transformed data. Transformed trial mean cull was 0.300% with SE  $\pm$  0.105% for main plots and  $\pm$  0.065% for main plots. Data presented in figure is not transformed.

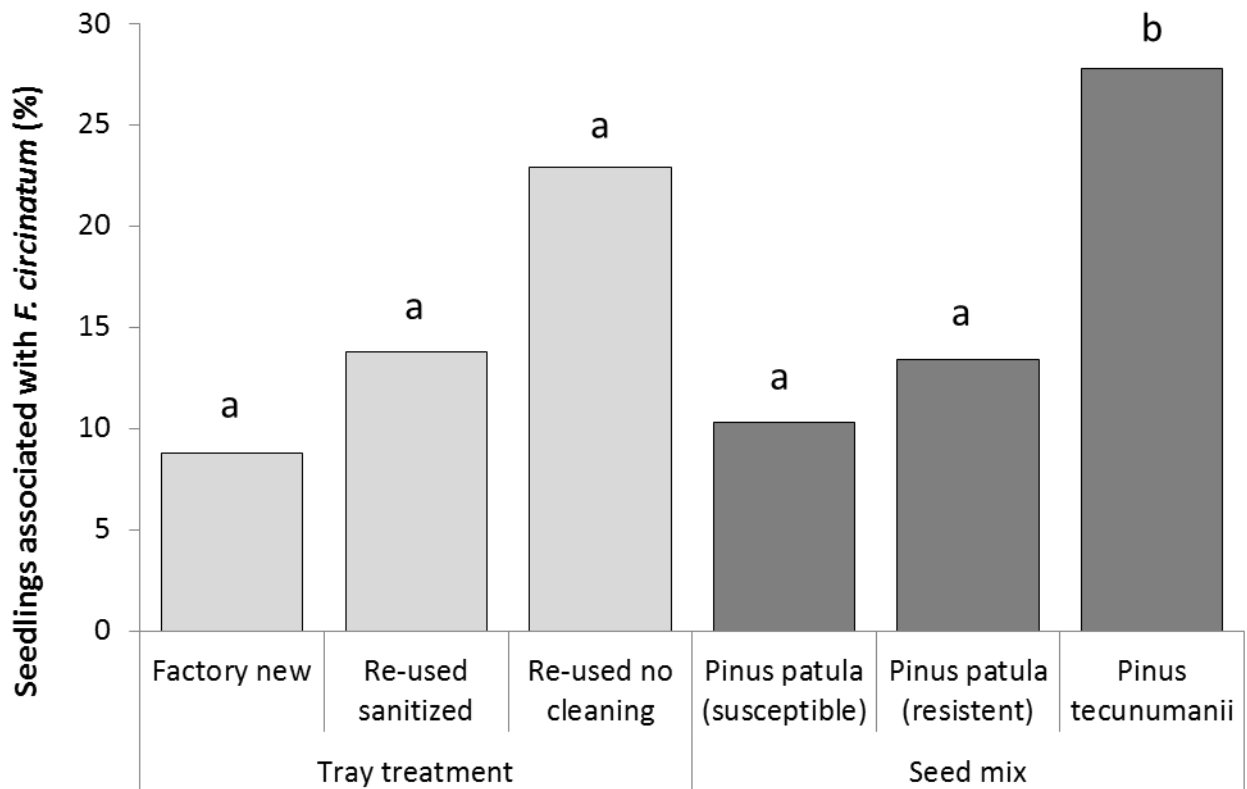
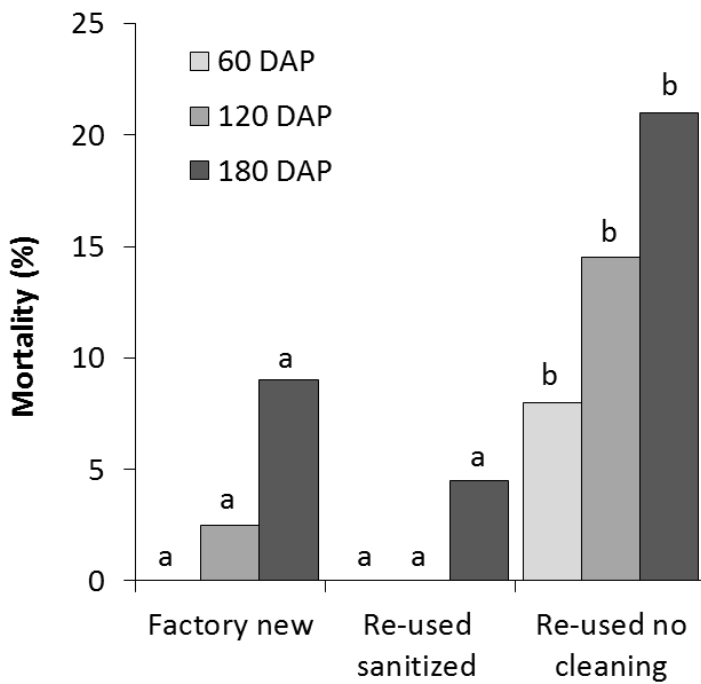


Figure 6: Post-planting mortality in a) Experiment A and b) Experiment B at 60, 120 and 180 DAP. For each experiment and at each assessment date treatments with the same letter were not significantly different at the 5% level applying Tukey's HSD test to square-root transformed data. Data presented in figure is not transformed.

a) Experiment A



b) Experiment B

