Genetic control of *Fusarium circinatum* tolerance in *Pinus patula x P. tecunumanii* hybrid families

by

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Thesis presented in partial fulfilment of the requirements for the degree of
Master of Forestry and Wood Sciences

at
Stellenbosch University
Forestry and Wood Sciences, Faculty of AgriSciences

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March 2020

Declaration

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Abstract

This study concentrated on the genetic control of *Fusarium circinatum* in *Pinus patula* × *P. tecunumannii* high elevation (HE) and *P. patula* × *P. tecunumannii* low elevation (LE) hybrid families. A greenhouse method of artificial inoculation screening was employed. The genetic material used included 37 *P. patula* × *P. tecunumanni* LE and 32 *P. patula* × *P. tecunumannii* HE hybrid families together with their parental families of two *P. patula*, four *P. tecunumannii* LE, five *P. tecunumannii* HE and four *P. taeda.*

Greenhouse screening were performed with the inoculation of three cultures simultaneously onto the abscission growth tip of seedlings. After eight weeks, plant height and lesion length were measured to calculate stem-kill percentage as an indicator of *F. circinatum* tolerance. The smaller the stem-kill percentage, the higher the *F. circinatum* tolerance. A strong positive correlation (93.6%) was observed between stem-kill percentage and lesion length, while a weak negative correlation (40.6%) was observed between stem-kill percentage and plant length. Genetic parameters such as narrow sense heritability (h^2), general combining ability (GCA) and specific combining ability (SCA) was employed to determine which parental family contributed the most to *F. circinatum* tolerance. Analyses were conducted on two categories. The first one combined both hybrid families (combined dataset for PPTH and PPTL) and the second one separated hybrid families (PPTH separated from PPTL). The assumption was PPTL might mask PPTH hybrid families. Least square means were also calculated to rank hybrid families and species in terms of *F. circinatum* tolerance.

Significant differences ($p < 0.0001$) were obtained for hybrid families and species tested for all genetic parameters tested. Variance components estimates indicated that the male variance components (σ_m^2) of *P. tecunumanii* HE and *P. tecunumanii* LE contributed more to *F. circinatum* tolerance with a gene frequency of 4.30%, while the female variance components (σ_f^2) indicated that *P. patula,* as female parent, contributed less with a 1.60% gene frequency. Obtained narrow sense heritability (h^2) also indicated that h_{male}^2 had a strong genetic control for both male parents, while the h_{female}^2 indicated a low genetic control. This was confirmed with the GCA obtained by most of the male and female parents. The parental family (TL2) from *P. tecunumanii* LE population had a negative and low GCA (-11.42), indicating more genetic effect contribution to *P. patula* and thus, a high level of *F. circinatum* tolerance. Hybrid family P5 × TL1 achieved a low and negative SCA (-20.02), indicating a high level of tolerance thus, additive and non-additive interaction between genes influenced the phenotype of hybrids.

As a novelty, this study's results were compared to the frost tolerance of the same genetic material of a previous study by Malinga (2019). Although a negative correlation in general was observed between frost and *F. circinatum* tolerance, two hybrid families indicated stronger *F. circinatum* and frost tolerance. Therefore, breeders should consider crossing *P. patula* with *P. tecunumanii* (LE and HE) families screened in this study based on the GCA and SCA values obtained. However, the combination of frost and *F. circinatum* resistant commercial hybrids might be more difficult to obtain.

Opsomming

Hierdie studie het gefokus op die genetiese beheer van *Fusarium circinatum* in *Pinus patula* × *P. tecunumannii* hibried families. 'n Kwekery metode van kunsmatige inokulasie was toegepas. Die volgende genetiese material is gebruik: 37 *P. patula* × *P. tecunumanni* LE en 32 *P. patula* × *P. tecunumannii* HE hibried families, asook die ouers wat twee *P. patula*, vier *P. tecunumannii* LE, vyf *P. tecunumannii* HE en vier *P. taeda* families ingesluit het.

Kwekery toetse was uitgevoer deur 'n mengsel van drie kulture te inokuleer op die aktiewe groeipunt van saailinge. Na agt weke is die plant lengte en letsel lengte gemeet en stam-dood persentasie bereken as 'n aanduiding van *F. circinatum* verdraagsaamheid. Hoe kleiner die stam-dood persentasie, hoe hoër is die *F. circinatum* verdraagsaamheid. 'n Sterk positiewe korrelasie (93.6%) was waargeneem tussen stam-dood persentasie en letsel lengte, maar 'n swak negatiewe korrelasie (40.6%) was waargeneem tussen stam-dood persentasie en plant lengte. Genetiese aanwysings (parameters), byvoorbeeld eng oorerflikheid (h^2), algemene kombineer vermoë (GCA) en spesifieke kombineer vermoë (SCA), was bereken om te bepaal watter ouerfamilie die meeste tot *F. circinatum* verdraagsaamheid bydra. Ontledings was uitgevoer in twee stappe: gekombineerde hibried families (kombineer die PPTH en PPTL datastelle) asook afsondelike hibried families (PPTH afsonderlik van PPTL). Dit was gedoen weens die aanname dat PPTL dalk die effek van PPTH hibried families kan oorheers. Families is verder gesorteer volgens *F. circinatum* verdraagsaamheid.

Betekenisvolle verskille ($p < 0.0001$) was waargeneem vir hibried families en spesies vir al die getoetse genetiese aanwysings. Variansie komponente het aangedui dat die manlike variansie component (σ_m^2) van *P. tecunumanii* HE en *P. tecunumanii* LE meer bygedra het tot *F. circinatum* verdraagsaamheid met 'n geen frekwensie van 4.30%. Die vroulike variansie component (σ_f^2) het aangedui dat *P. patula,* as vroulike ouer, minder bygedra het met 'n geen frekwensie van 1.60%. Waargenome eng oorerflikheid (h^2) het ook aangedui dat h^2_{male} 'n sterk genetiese beheer oor beide manlike ouers het, terwyl h^2_{female} 'n lae genetiese beheer aangedui het. Dit was bevestig met die GCA van beide manlike en vroulike ouers. Die ouerfamilie (TL2) van *P. tecunumanii* LE populasie het 'n negatiewe en lae GCA (-11.42) gehad wat gedui het op 'n sterker genetiese effek as *P. patula*, en dus 'n hoë vlak van *F. circinatum* verdraagsaamheid. Hibried familie P5 × TL1 het 'n lae en negatiewe SCA (-20.02) gehad wat gedui het op 'n hoër vlak van verdraagsaamehid, en dus het toegevoegde en nie-toegevoegde interaksie tussen gene die fenotipe van hibriede beïnvloed.

Uniekheid van die studie was die bepaling van 'n moontlike korrelasie tussen koue en *F. circinatum* verdraagsaamheid van dieslefde genetiese materiaal soos bepaal deur Malinga (2018). Alhoewel 'n negatiewe korrelasie waargeneem is, het twee hibried families 'n sterk moontlikheid getoon vir beide koue en *F. circinatum* verdraagsaamheid. Daarom moet telers oorweeg om *P. patula* en *P. tecunumanii* (LE and HE) families te toets gebasseer op die GCA en SCA waardes wat in die studie waargeneem is. Die moontlikheid om 'n hibried te kry met beide koue en *F. circinatum* verdraagsaamheid mag dalk meer kompleks wees.

Acknowledgements

The completion of this study would not have been possible without the help and sacrifices of many special people in my life who I am greatly indebted to. They constantly supported and uplifted me to new heights and this galvanised me to reach academic eminence.

Greatest words of thanks go to my husband, Setshaba Brian Mphahlele and my handsome boy, Moetapele Shammah Mphahlele, for their encouragement and endurance during many hours without a wife and a mother. The SS resilience in overcoming deeds that normally would have required my participation created very fun times in the house and they will forever be embossed on my memory.

I owe further gratitude to my parents Manase Cyril and Modjadji Julia Pilusa, my siblings Reshoketswe Mawela and Taelo Pilusa, my in-laws Salome and Sekukuti Mphahlele, my nanny Othilia Ngwamusi and my aunt, Mokgadi Senyolo, I am grateful to all of you for inspiring me to be committed to the ideals of achieving greatness.

Special mention goes to Sappi Forests for sponsoring, supporting this study and always availing information that contributed to its conclusion. This is highly appreciated as without the support from Sappi, this thesis would not have seen the light of the day.

For assistance and guidance with statistical analysis of data, I am indebted to Dr Juan Acosta and Dr Gert van Berg. Also, a special thanks to Pfarelo Tahulela for sacrificing her leisure time and contributing towards my thesis.

A special word of appreciation goes to my academic supervisor, Dr Hannél Ham. Her advice, enthusiastic support and amazing ability to enhance the quality of the thesis were pillars that I knew I could stand on no matter what.

I cannot forget my “boss” and co-supervisor, Dr André Nel. He believed in me, when academic setbacks befell my work, he will bring new hope and inject life to the project. His zealousness to overcome whatever challenges that arose.

I extend special thanks to my spiritual mentors, Pastor Richard and Thandiwe Wills for prophetic declarations and prayers throughout the cause of my study.

Thank you Jehova Shammah, I have made it! You have made a way.

This thesis is dedicated to:

- my parents, Manase Cyril and Modjadji Julia Pilusa who sacrificed their lives for my bright future, and
- my husband, Setshaba Brian and my boy Moetapele Shammah Mphahlele, they inspire me to reach greater heights.

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List of Abbreviations

ANOVA	Analysis of Variance
BLUP	Best linear unbiased predictors
CV	Coefficient variance
EL	Electrolyte leakage
F. circinatum	*Fusarium circinatum*
GCA	General Combining Ability
GLM	General linear model
h^2	Heritability
HE	High elevation
LE	Low elevation
LSMeans	Least Square Means
P. patula (female parent)	P1 to P13
P. tecunumanii HE (male parent)	PTH1 to PTH5
P. tecunumanii LE (male parent)	PTL1 to PTL10
PPTH	*Pinus patula* × *Pinus tecunumanii* HE
PPTL	*Pinus patula* × *Pinus tecunumanii* LE
Rep	Replication
SA	South Africa
SCA	Specific Combining Ability
r	Pearson Correlation

Chapter 1
Project rationale

1.1 Introduction

Forestry in South Africa (SA) is estimated to cover a total land area of 1.3 million hectares of which approximately 1.1% is used for commercial afforestation (DAFF, 2016). The forests are scattered within five Provinces, namely the northern summer rainfall areas of Mpumalanga (40.6%), KwaZulu-Natal (39.9%), Eastern Cape (11.6%), Limpopo (4.3%) and the southern winter rainfall areas of the Western Cape Province (3.6%) (Godsmark, 2017). It is estimated that 73% of all plantations are managed for commercial sawlogs, while only 26% for pulpwood (Godsmark, 2017). Commercial forestry forms an important component of the country's major contribution to its economy with an estimated Gross Domestic Product (GDP) of approximately 1%. Planted forests generally comprises of three main groups of commercial tree species which include *Pinus* (51%), *Eucalyptus* (41.8%), *Acacia* (7%) and non-forestry products (0.4%) (DAFF, 2016).

The primary pine species grown for commercial purposes are *Pinus patula, P. elliottii, P. taeda* and *P. radiata* (Mabaso, 2017). *Pinus patula* has good wood characteristics, fast growth, straight stem form and excellent pulping and saw timber properties (International *et al*., 2018; Nel *et al.,* 2017). However, pests and diseases have a significant impact on the yield, operational costs and the end-products' quality (DAFF, 2015; Morris, 2010). Although *P. patula* are affected by *Rhizina* root rot, *Fusarium circinatum* and *Diplodia sapinea* diseases, the main focus of this study will only be on *F. circinatum.*

Fusarium circinatum is an ascomycete, residing in the Hypocreales, family Nectriaceae (Fru *et al.,* 2018; Martín-García *et al.,* 2017). An estimated 60 *Pinus* species are susceptible to *F. circinatum* (Martin-*Garcia et al.,* 2019; Bezosal*, et al.,* 2017*)* and, therefore, considered as the most significant disease affecting pine trees globally (Wingfield *et al.,* 2008). It is highly infectious with some of the symptoms being: bleeding of resin; resinous cankers; wilting of needles and dieback; yellowish needles and wilting of foliage and shoots (Martin-Garcia *et al.,* 2019; Bezos *et al.,* 2017). Factors associated with its infection include moist conditions and warm temperatures (Bezos *et al.,* 2017). There are few effective options to treat or prevent *F. circinatum* infections (Iturritxa *et al.,* 2017). Methods such as biological control (Martin-Garcia *et al.,* 2019; Bezos *et al.,* 2017), nursery hygiene and fungicide application (Iturritxa *et al.,* 2017) are being tested to reduce or control infections caused by *F. circinatum*.

The ecology of *F. circinatum* in commercial plantations is unknown. However, factors such as insect vectors and environmental conditions required for the occurrence of *F. circinatum* are present (Martin-Garcia *et al.,* 2019). Therefore, *Pinus* breeding programmes globally launched initiatives to breed for *F. circinatum*

tolerance. Hybrids between *P. patula* & *P. tecunumanii* (Kanzler *et al.,* 2014) and *P. patula* & *P. oocarpa* (Mitchell *et al.,* 2013; Roux *et al.,* 2007) shows promising results. The impact of *F. circinatum* on *P. patula* pure species and *P. patula* × *P. tecunumanii* hybrids were the main focus of this study. Therefore, this study investigated the genetic control of *F. circinutum* tolerance and the inheritance thereof between pure species parents and selected *P. patula* × *P. tecunumanii* hybrids at family level.

1.2 Problem statement

Previous studies indicated that *P. patula* × *P. tecunumanii* high elevation (HE) was more tolerant to frost than *P. patula* × *P. tecunumanii* low elevation (LE) (Malinga, 2018; Mabaso, 2017; Mitchell, 2012), while *P. patula* is tolerant to frost, but highly susceptible to *F. circinutum* (Mitchell, 2012). Furthermore, *P. patula* × *P. tecunumanii* LE has high levels of *F. circinatum* tolerance with low frost tolerance, while *P. patula* × *P. tecunumanii* HE has a lower level of *F. circinatum* tolerance and higher level of cold tolerance (Malinga, 2018; Mitchell, 2012). There is a need to understand the level of genetic control of *F. circinutum* tolerance of *P. patula* × *P. tecunumanii* HE, *P. patula* × *P. tecunumanii* LE and their parents. Therefore, to determine the genetic component as part of screening methods will assist tree breeders to prioritise selections of families that are tolerant to *F. circinatum* to optimise breeding efforts.

1.3 Research objectives

Previous studies developed a laboratory based screening method to determine frost tolerance of *P. patula* × *P. tecunumanii* hybrid families (Malinga, 2018; Mabaso, 2017; van Wyk, 2011). The same genetic material used for screening techniques in Malinga (2018) and Mabaso (2017) was utilised in this study. The main objective of this study was to determine the level of genetic control of *F. circinatum* tolerance in a range of *P. patula* × *P. tecunumanii* LE and *P. patula* × *P. tecunumanii* HE hybrid families and the pure species parents. Specific objectives addressed were:

- Estimate the genetic heritability (h^2) of *F. circinatum* tolerance and variance components (V_{mom} andV_{dad}) in *P. patula* × *P. tecunumanii* LE and HE hybrid families,
- Estimate the General Combining Ability (GCA) and Specific Combining Ability (SCA) to rank hybrids and pure species,
- Determine if there is a correlation between frost tolerance (Malinga, 2018) and *F. circinatum* tolerance (this study) within the same genetic material of *P. patula* × *P. tecunumanii* LE and HE hybrid families to identify future breeding parents, and
- Identify parents for future hybrid breeding based on the genetic parameters calculated in this study indicating high levels of *F. circinatum* tolerance.

1.4 Data collection

The genetic material used in this study was from the same hybrid vegetative propagation hedges as the plant material used for previous studies on frost tolerance (Mabaso, 2017; Malinga, 2018). Seedlings of *P. patula*, *P. tecunumanii* (LE and HE) and *P. taeda* was included as controls and various families of *P. patula* × *P. tecunumanii* (LE and HE) hybrids. *Fusarium circinatum* tolerance was determined by using artificial inoculation techniques as developed by Oak *et al.* (1987). Data collection involved measurement of variables (plant length and lesion length) and expressed as a percentage of stem kill to determine *F. circinatum* tolerance. Genetic control of *F. circinatum* tolerance in *P. patula* × *P. tecunumanii* (LE and HE) was calculated with heritability (h^2) to estimate the degree of tolerance variation within families. Variance components estimates determined the gene contribution between male (σ_m^2)*P. tecunumanii* HE and *P. tecunumanii* LE, as well as female (σ_f^2) *P. patula* parents for *F. circinatum* tolerance. Genetic parameters such as general combining ability (GCA) and specific combining ability (SCA) were estimated to determine which parental family contributed the most to *F. circinatum* tolerance. Ranking of hybrid families and species for tolerance to *F. circinatum* were also calculated through Least Square Means. Thereafter, genetic correlations between *F. circinatum* and frost tolerance was calculated, and tolerant families were identified.

1.5 Significance of study

Improving the understanding of the genetic control for *F. circinatum* tolerance in Pine hybrid breeding will allow tree breeders to identify parents and specific hybrid crosses with increased tolerance levels. This will assist with the commercial deployment of tolerant interspecific hybrids. Furthermore, a better understanding of the correlation between frost and *F. circinatum* tolerance for hybrid families will also assist in producing hybrid families with both frost and *F. circinatum* tolerance and assist with improved site species matching for future hybrid deployment.

1.6 Thesis structure

This thesis comprises of six chapters. Chapter 1 stipulates the project rationale, objectives and problem statement. A comprehensive literature study (Chapter 2) highlights the general background of *F. circinatum* infestations, how it is affecting *Pinus* species, previous studies and alternative species with *F. circinatum* tolerance. Chapter 3 summarises the materials and methods employed, while results are illustrated in Chapter 4. Discussions of the results are presented in Chapter 5, while Chapter 6 consists of the conclusion and recommendations.

Chapter 2
Literature Review

2.1 Introduction

Pests and diseases are considered to have a significant impact on the quality of commercial pine production (Gordon *et al.,* 2015; Mitchell *et al.,* 2011) with poor tree growth and yield (Santana *et al.,* 2016; Mitchell *et al.,* 2011) resulting in economic losses (Bezos *et al.,* 2017; Wingfield *et al.,* 2015; Gordon *et al,* 2001). Therefore, it is important to have knowledge on the ecology and epidemiology of the host in order to manage pests and diseases properly (Wingfield *et al.,* 2008). *Fusarium circinatum* is a dynamic disease affecting trees in all stages of development: from seed to seedlings and mature trees (Pérez-Sierra *et al.,* 2007). It is also known to cause pitch canker in *Pinus* species (Mitchell *et al.,* 2011; Wingfield *et al.,* 2008). Interspecific hybridisation with more tolerant species is considered a long-term strategy to minimise the threats of *F. circinatum*. Tolerant species can be identified through the method of artificial inoculation screening whereby tolerant and non-tolerant species are screened. This study will focus on the genetic control of *F. circinatum* tolerance on *P. patula* × *P. tecunumanii* low elevation (LE) and *P. patula* × *P. tecunumanii* high elevation (HE) hybrid families to understand hybrid family variation within the tested hybrids that are tolerant to *F. circinatum.* These tolerant species or families could be useful for sustainable commercial pine production.

2.2 Fusarium circinatum and Pinus species

Fusarium circinatum is the anamorph of *Gibberella circinata*, also associated with the different mating types of heterothallic ascomycete (Nirenberg *et al.,* 1998; Viljoen *et al.,* 1997). It is usually found cross-fertile when examined under laboratory conditions (Britz *et al.,* 1999) developing sexual structures which symbolises a distinct mating population in the *Gibberella fujikuroi* complex. The genus *Fusarium* symbolises one of the most important groups of ascomycetous fungi (Kvas *et al.,* 2009). Molecular techniques can be used to distinguish between different *Fusarium* species (Steenkamp *et al.,* 1999; Nirenberg *et al.,*1998; Leslie *et al.,* 2006; Britz *et al.,* 2002). As various name changes occurred to understand the pathogen group (Morris, 2010), the fungi is currently known as *F. circinatum*. Furthermore, the pathogen is recognised as *F. circinatum* in nursery plants and as 'pitch canker' when affecting established trees in the plantation (Wingfield, 1999). Therefore, throughout this thesis the name *F. circinatum* will be used.

Pinus species grown in South Africa are all exotic and known to be pathogenetic to *F. circinatum,* causing damage to established pine plantations (Mitchell *et al.,* 2011; Wingfield, 1999; Viljoen *et al.,* 1995). It was estimated that South African forestry industry has lost approximately 42% of *P. patula* seedlings after

establishment to symptoms associated with *F. circinatum* (Crous, 2005). This relates to approximately 11 million Rand that was lost in the industry for both sawn and pulp timber. However, the cost of nursery losses has not been quantified in South Africa other than the reported estimation of 1% seedling rouging which is attributed to *F. circinatum* (Fru *et al.,* 2018; Mitchell, 2012).

2.2.1 Distribution, disease symptoms and spread

Fusarium circinatum is a very important diseases affecting various *Pinus* species (Britz *et al.,* 2005; Nirenberg *et al.,* 1998) and was first described by Hepting and Roth (1946) in southeastern United States of America affecting *P. elliottii* and *P. taeda*. The pathogen has also been reported in various other countries (Figure 2.1): Haiti (Berry and Hepting, 1959), California (McCain *et al.,* 1987), Japan (Muramoto and Dwinell, 1990), Mexico (Guerra-Santos, 1999), South Africa (Viljoen *et al.,* 1994), Spain (Dwinell *et al.,* 1998), South Korea (Lee *et al.,* 2000), Chile (Wingfield *et al.,* 2002a), Italy (Carlucci *et al.,* 2007), Portugal (Bragança *et al.,* 2009), Colombia (Steenkamp *et al.,* 2012), and Brazil (Pfenning *et. al*., 2014).

The pathogen can infect all parts of the tree (e.g. needles, stem, woody bole, roots, cones, seeds) and is more evident when trees are stressed (TPCP, 2002). Symptoms observed in nurseries are similar to those developed within the first three months after in-field planting (Crous, 2005). However, nursery symptoms differ from those observed in mature trees (Fourie *et al.,* 2014). In the nursery, symptoms associated with *F. circinatum* include pre- and post-emergence damping-off of seedlings (Gordon *et al.,* 2015; Viljoen *et al.,* 1994). The occurrence of pre-emergence damping off results in heavily colonised seed coats and affect the coleoptile of germinating seed (Viljoen *et al.,* 1994).

In the case of post-emergence damping off, symptoms are observed on the stem collar and the cotyledon node region of the seedlings (Viljoen *et al.,* 1994; Dwinell *et al.,* 1985; Barnard and Blakeslee, 1980); tip wilting and yellowing of seedlings (Gordon *et al.,* 2015; Martínez-Alvarez *et al.,* 2014; Jacobs *et al.,* 2007; Viljoen *et al.,* 1994); shoot-tip die-back; discoloration of the roots; root collar region and root rot; which eventually cause seedling mortality (Mitchell *et al.,* 2011; Wingfield *et al.,* 2008) as indicated in Figure 2.2. The symptoms of mature trees are characterised by branch die-back; stem cankers; copious pitch formation and mortality (Jacobs *et al.,* 2007); stem deformation and growth loss (Hodge and Dvorak, 2000); bleeding of resinous canker on the trunk, terminals or large branches (Figure 2.2: D, E and F) (Martín-García *et al.,* 2019; Gordon *et al.,* 2015). Infected seedlings (nursery) and mature trees (in-field) can become weak and eventually die (Hodge and Dvorak, 2007).

Figure 2.1: Global distribution of *F. circinatum* (Roux, personal communication)

Fusarium circinatum reproduces primarily by means of asexual conidia (Dwinell *et al.,* 1985), which can be distributed by insects, air, water, or soil-borne (TPCP, 2002). Fungal dispersal occurs during precipitation and turbulent air conditions. The environmental interaction factors such as soil nutrients ratio, temperature and humidity could also contribute to the spread of *F. circinatum* (Martín-García *et al.,* 2019).

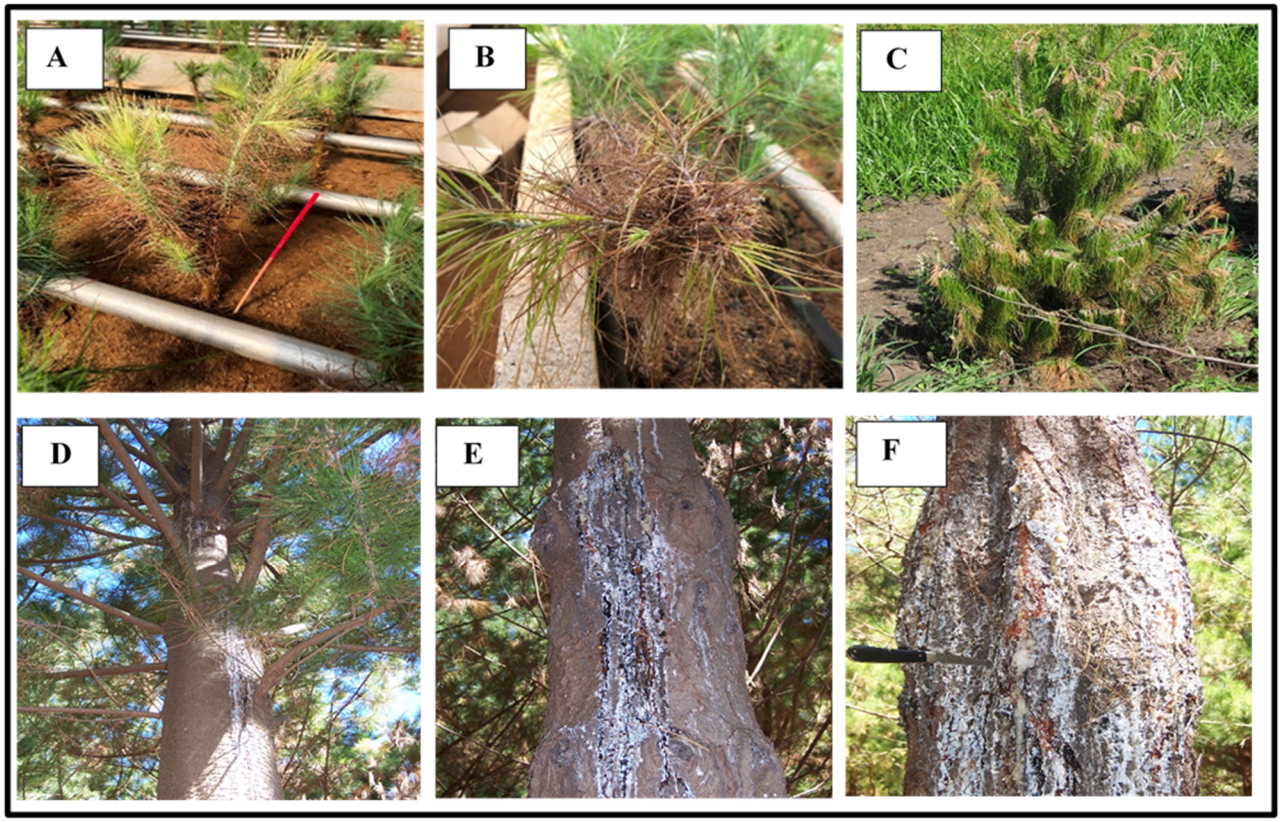

Figure 2.2: *Fusarium circinatum* symptoms observed in the nursery as tip wilting and yellowing of seedlings hedges (A); shoot-tip die-back (B). Symptoms observed in plantations include wilting of branches after establishment (C) and bleeding of resinous canker on the trunk, terminals and large branches (D, E and F) (Nel, personal communication)

2.2.2 *Fusarium circinatum* and the South African forestry industry

Fusarium circinatum was first documented in South Africa in 1990 as a root rot disease of *P. patula* seedlings in the nurseries (Viljoen *et al.,* 1994). The disease was introduced to South Africa from Mexico through importing of infested seed (Wingfield *et al.,* 2008; Couhnho *et al.,* 2001). Since then, it spread throughout South Africa (Figure 2.3) and caused an estimated nursery loss of 2.7 million (14%) of *P. patula* seedlings between 1992 and 1993 (Morris, 2010). The pathogen was noted on various *Pinus* trees: on 5 to 9-year-old *P. radiata* trees in the Tokia area (Western Cape Province) (Coutinho *et al.,* 2007); on 12 to 15-year-old *P. radiata* trees in the George area (Western Cape Province) (Steenkamp *et al.,* 2014); on 12-year old *P. greggii* trees in the Ugie area (Eastern Cape Province) (Santana *et al.,* 2016; Mitchell *et al.,* 2011); and on 10-year old *P. greggii* in the KZN Midlands area (KwaZulu-Natal Province) (Steenkamp *et al.,* 2014). Pruning wounds are suspected to be an entering point for the pathogen in matured trees (Bezos *et al.,* 2012; Gordon *et al.,* 2015). Studies indicated that approximately 30% of the trees had symptoms such as die-back of the main stems, branches and resinous pockets (Mitchell *et al.* 2012; Coutinho *et al.,* 2007).

Limited research is available on the spread of the *F. circinatum* pathogen in South African commercial *Pinus* plantations (Santana *et al.,* 2016; Steenkamp *et al.,* 2014; Britz *et al*., 2005). Therefore, screening techniques were adopted to evaluate the tolerance species and families. For example, inoculation protocols developed by Oak *et al.* (1987) and TPCP (2000) were adopted and revised to quantify *F. circinatum* tolerance on numerous pine species (Hodge and Dvorak, 2000). This technique became a popular tool for tree breeders when making parental selections (Mitchell *et al.,* 2011). A number of previous studies screened different *Pinus* species for *F. circinatum* tolerance through artificial inoculation of seedlings in the greenhouse (Mitchell *et al.,* 2014; Nel *et al.,* 2014; Hodge and Dvorak, 2000). Results indicated a significant difference in the susceptibility of *Pinus* species and hybrids to *F. circinatum.*

2.2.3 *Fusarium circinatum* in South African forestry nurseries

Since nurseries are not only used for propagation of only one species or taxonomic group, contamination of the pathogen can be through tray type, seed source, growth medium and irrigation water (Fru *et al.,* 2016; Morris, 2010; Hurley *et al.,* 2007). Nevertheless, the pathogen can be spread through movements of contaminated soil (Martín-García *et al.,* 2019; Santana *et al.,* 2016; Mitchell *et al.,* 2011). For instance, root and collar rot was one of the symptoms observed in seedlings at the Mountain To Ocean (MTO) Karatara forestry nursery and the source of the inoculum was found to be irrigation water and planting tray inserts (van Wyk, 2011).

Fusarium circinatum is usually linked to high levels of seedling death in the nursery (Santana *et al.,* 2016; Mitchell, 2012). Previous studies indicated that populations of *F. circinatum* originate in nurseries and most probably spread into plantations after establishment (Santana *et al.,* 2016). Since its discovery in South Africa, *F. circinatum* was restricted to nurseries until 2000 (Ford *et al.,* 2014) whereby, South African nurseries had experienced a decrease in propagation of *P. patula* cuttings and production of grafting material due to *F. carinatum* infestation. This infestation of *F. carinatum* also had a negative impact on activities of tree improvement and establishment of clonal seed orchards of *P. patula* species (Jones *et al.,* 2014).

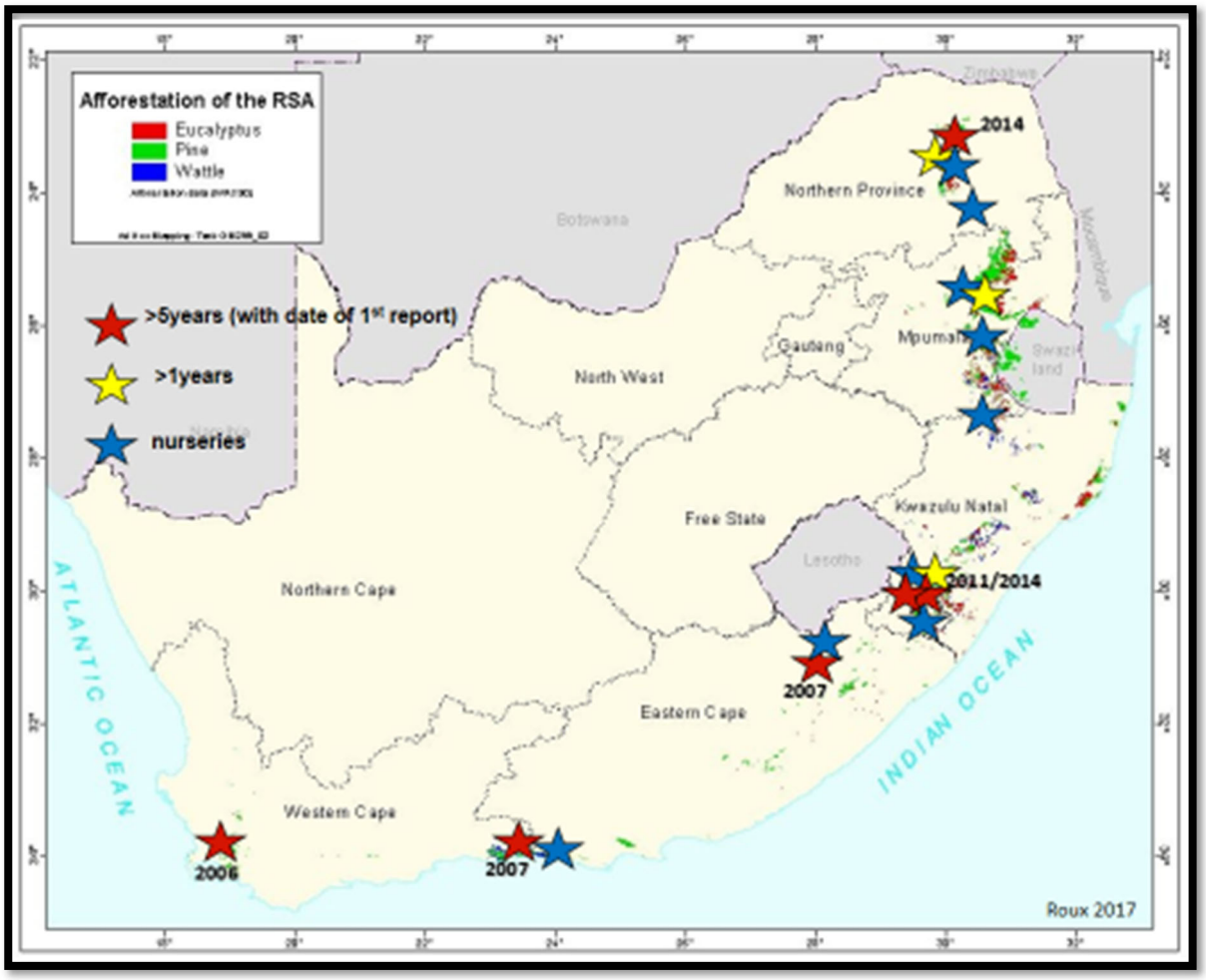

Figure 2.3: Regional distribution of *F. circinatum* in South Africa (Roux, personal communication)

The introduction of control measures, such as hot water seed treatments; quarantine; biocontrol; removing diseased plants and various hygiene methods (e.g. sterilising trays), were found to be effective in nurseries (Iturritxa *et al.,* 2017; Morris, 2010). With hot water and hydrogen peroxide treatments, *F. circinatum* contamination on *P. radiata* seeds were significantly reduced with an overall disease incidence lower than 0.8% (Morales-Rodríguez *et al.,* 2018). *Trichoderma* species commonly used as biological control agents was effectively used to control damping-off caused by *F. circinatum* (Morales-Rodríguez *et al.,* 2018). Application of fungicides such as Folicure® (active ingredient terbuconazole) reduced the spread of the *F. circinatum* (Morris, 2010). Benomyl and Thiram applications have been used to treat seed for *P. patula* (Mitchell *et. al.* 2012). However, Benomyl had no significant effect on the control of *F. circinatum* in the nursery (Jones and Kanzler, 2008). Although fungicides application may promote resistance, application of fungicides such as Benomyl are not allowed in certain countries because of negative environmental impact (Iturritxa *et al.,* 2017).

2.3 *Fusarium circinatum* in other countries

In Mexico, *F. circinatum* has been recognised in nine states (Sinaloa, Nayarit, Mexico, Nuevo Leon, Puebla, Michoacán, Jalisco, Durango and Tamaulipas) (Guerra-Santos, 1999). Based on the levels of genetic diversity, it is believed that it was the central origin of *F. circinatum* (Britz *et al.,* 2005). Typical symptoms of canker caused by *Fusarium* species were observed on various species such as *P. patula, P. greggii, P. teocote* and *P. leophylla* trees (Britz *et al.,* 2005). Literature stated that in 1946 at California, plantations, seed orchards and the natural population of *P. radiata* were severely affected by *F. circinatum* (Gordon *et al.,* 2001; Dwinell *et al.,* 1998; Dwinell *et al.,* 1985). The infections were through insect activities such as bark beetles (Sakamoto and Gordon, 2006). The disease then spread from Mendocino County north of San Francisco to San Diego (Gordon *et al.,* 2001; Dwinell *et al.,* 1998; Correll *et al.,* 1991).

The pathogen was first discovered in the North of Spain in 1997 in the isolated community of Pais Vasco, Basque Country (Dwinell *et al.,* 1998). *Fusarium circinatum* introduction was caused by clonal populations in this community (Fru *et al.,* 2016). Outbreaks of this pathogen left the forestry industry in Spain with poor crop yield, affecting the economy as costs were invested in the monitoring and controlling of *F. circinatum* (Bezos *et al.,* 2017; Pérez-Sierra *et al.,* 2007). The disease caused major damage on mainly *P. radiata* seedlings in bare-root nurseries. Recent reports indicated that the disease spread to five regions within Spain: Galicia, Asturias, Cantabria, País Vasco and Castilla León (Bezos *et al.,* 2017).

Although the outbreak of *F. circinatum* was first documented in 1989 in the United State of America, it was first discovered in 1945 on *P. virginiana* in Florida (Hepting and Roth, 1946). Unlike other countries, the disease caused economic losses only in forest managed stands and was hardly found in native pine stands (Blakeslee *et al.,* 1978). Mortality of *P. virginiana* was moderately low in Florida with stem deformation and severe growth loss. A previous study indicated that on 5-year old *P. elliottii,* 59% of the population had only one infection with 2% mortality, while 22% of the population had suffered from stem deformities and a 15% loss in volume growth increment (Hodge and Dvorak, 2000). The state of infection in Chile was similar to that of South Africa, whereby *F. circinatum* threatens mainly *P. radiata* trees in commercial plantations (Coutinho *et al.,* 2007). After its discovery in South Africa, it was believed that *P. radiata* stand was established from infected nursery seedlings in 2002 (Wingfield *et al.,* 2002b). Symptoms discovered included tip die-back and root collar diseases, as a result, long-term strategies such as tree breeding and selection was employed to minimise the threat of *F. circinatum* (Wingfield *et al.,* 2002b).

2.4 Species tolerance to *Fusarium circinatum*

Species responds differently to disease infections and strategies used by plants are either to be tolerate or resistant. Tolerance is the inability of a plant to limit growth and development of specified pest, while

resistance is the ability of a plant variety to limit the growth and development of a specified pest or damage they cause when compared to susceptible plant varieties under similar environmental conditions and pest pressure (ISF, 2017; Koch *et al.,* 2016). Tolerance benefits both the host and the parasite, yet resistance benefit the host only (Horns and Hood, 2012; Politowski and Browning, 1978). Plant varieties that are resistant show some disease symptoms or damage under continued pressure. These varieties of resistance have two levels (ISF, 2017):

- High pressure: plant varieties that restrict the growth and development of the specified pest under normal pest pressure when compared to susceptible varieties. These plant varieties may, exhibit some symptoms or damage under heavy pest pressure (ISF, 2017). For example, *P. patula* in case of *F. circinatum*.
- Intermediate: plant varieties restrict the growth and development of the specified pests but may exhibit a greater range of symptoms or damage compared to high resistance varieties. Plant varieties will still show less severe symptoms or damage than susceptible plant varieties when grown under similar environmental conditions or pest pressure (ISF, 2017). For example, *P. patula* × *P. tecunumanii* (HE) to *F. circinatum* infestation.

There is convincing evidence that interspecific hybridisation is the key to introduce *F. circinatum* tolerance into *P. patula* interspecific breeding (Mitchell *et al.,* 2013; Roux *et al.,* 2007). As a result, the hybrids of *P. patula* × *P. tecunumanii* and *P. elliottii* × *P. caribaea* have been deployed commercially in the forestry industry in South Africa (Nel *et al.,* 2017; Kanzler *et al.,* 2014). The *P. elliottii* × *P. caribaea* hybrid was first produced and evaluated in 1960 by the South African Forestry Research Institute (Hongwane *et al.,* 2017; Nel *et al.,* 2017), while *P. patula* × *P. tecunumanii* was produced in 1990 (Kanzler *et al.,* 2014; Dungey, 2001). Furthermore, several field studies indicated that species such as *P. oocarpa*, *P. jaliscana*, *P. pringlei* (Hodge and Dvorak, 2007), *P. elliottii* (Mitchell*,* 2012) and *P. caribaea* (Mitchell*,* 2012; Roux *et al.,* 2007) are very tolerant to the disease, while *P. patula, P. greggii* (Hodge and Dvorak, 2007) and *P. radiata* (Coutinho *et al.,* 2007) were highly susceptible to *F. circinatum*. *Pinus taeda* ranged amongst species that are moderate susceptible to *F. circinatum* (Mitchell*,* 2012). Hybrids between some of these species have been found to be tolerant or less susceptible to *F. circinatum* infection (Morris, 2010). Table 2.1 indicates the level of tolerance to *F. circinatum* of *Pinus* species grown in South Africa.

2.5 Description of species used in the study

Pinus patula, P. tecunumanii (LE and HE) and *P. taeda* were included as controls together with interspecific hybrids families of *P. patula* × *P. tecunumanii* (both LE and HE) as treatments. These taxonomic groups were screened to investigate the genetic control of *F. circinatum* tolerance on *P. patula* × *P. tecunumanii* LE and HE families. Only *P. patula* and *P. tecunumanii* (parents) and their interspecific hybrids were reviewed in this chapter as this study focused only on the *F. circinatum* tolerance of these taxonomic groups and species.

2.5.1 *Pinus patula*

Pinus patula originates from Mexico and has two varieties: *P. patula* Schiede ex Schlect. & Cham. var. *patula* and *P. patula* Schiede ex Schlect. & Cham. var. *longipedunculata*. The two varieties are found in different geographic regions with different morphological characteristics. The geographic regions of the two varieties overlap in northeastern Oaxaca with *P. patula* var. *patula* variety ranging from Tamaulipas to northeastern Oaxaca in Sierra Madre Oriental, and *P. patula* var. *longipedunculata* ranging from northeastern Oaxaca to Guerrero in Sierra Madre del Sur (Dvorak *et al.,* 2000a). The natural geographical regions of the two *P. patula* varieties are indicated in Figure 2.4.

Pinus patula var. *patula* grows well between the 18° and 24° N latitude, while *P. patula* var. *longipedunculata* grows between 16 and 17° N latitude. The species performs well at altitudes of 1 500 to 3 100 m.a.s.l. and mean annual precipitation of between 600 and 2 500 mm (Dvorak *et al.,* 1992; Wright, 1994). The variety of *P. patula* var. *patula* from northern Oaxaca are cold tolerant and can survive in freezing weather (Dvorak *et al.,* 1995). However, *P. patula* var. *longipedunculata* from Southern and Western Oaxaca are more susceptible to cold weather. In South Africa, the variety of *P. patula* var. *longipedunculata* do not perform well in cold weather when planted in high altitude areas (Dvorak *et al.,* 1995). This species grows best on cool and moist sites in the summer rainfall region (Kanzler *et al*., 2012) and it performs well in Mpumalanga, Limpopo, KwaZulu-Natal and the Eastern Cape Provinces (DAFF, 2014). The mean annual temperatures average 16.5 ºC with an annual precipitation of between 780 and 880 mm (Mitchell, 2012).

Pinus patula is known to have superior growth, good stem form and desired wood properties (Mabaso, 2017; Dvorak *et al.,* 2000a). It hybridises easily with other species such as *P. tecunumanii* LE, *P. tecunumanii* HE and *P. oocarpa* (Nel *et al.,* 2017; Roux *et al.,* 2007). Nevertheless, it exhibits moderate resistance to frost and drought. However, *P. patula* is severely susceptible to *F. circinatum* (Dvorak *et al.,* 2000a). Therefore, there is a strong movement to replace *P. patula* commercial plantations with interspecific *Pinus* hybrids between *P. patula* and *P. tecunumanii* (Kanzler *et al.,* 2014).

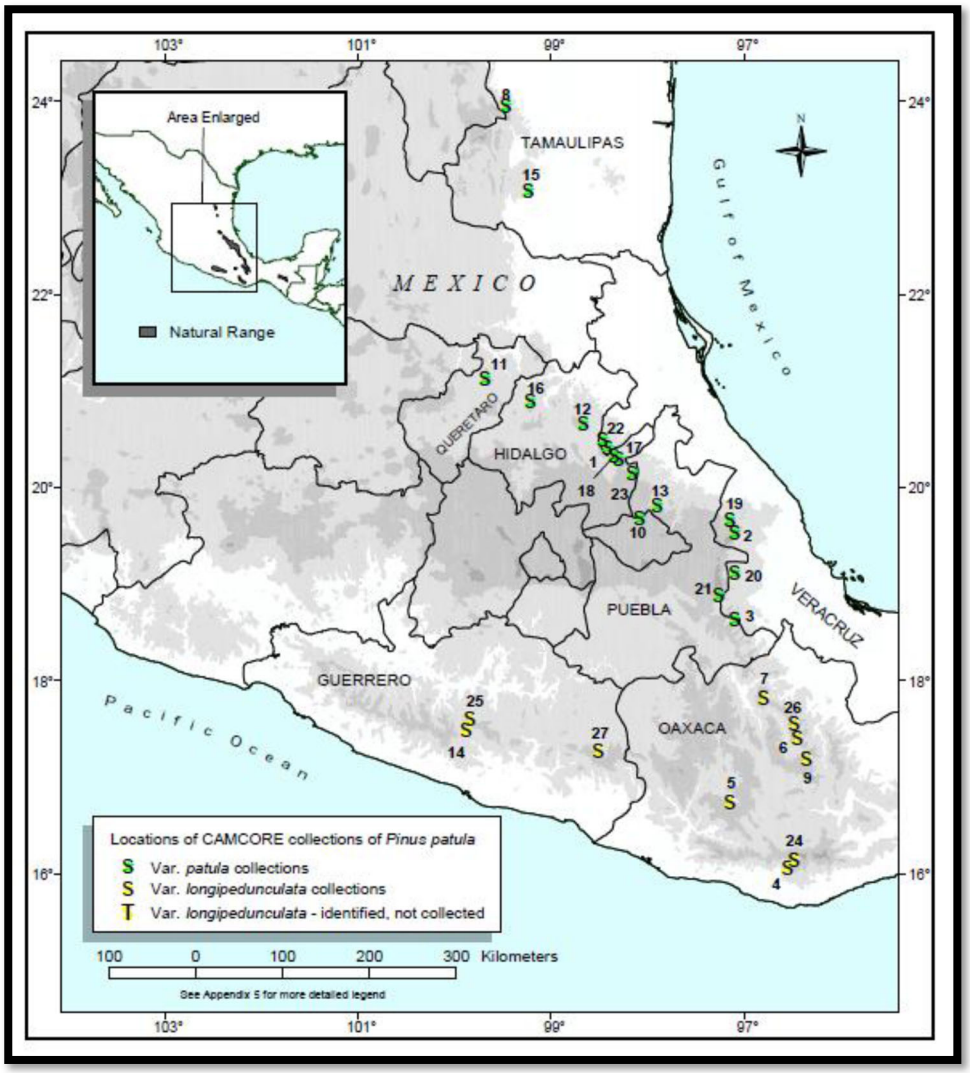

Figure 2.4. Natural range of the two varieties of *Pinus patula* in Mexico (yellow indicating variety *longipenduculata* and green indicating variety *P. patula*) (Dvorak *et al.,* 2000a)

Table 2.1: *Pinus* species grown in South Africa and the level of *Fusarium circinatum* tolerance (Mabaso, 2017; Morris, 2010; Dvorak *et al.,* 2000a)

			***Fusarium circinatum* tolerance**		
Species Name	**Common name**	**Geographical range**	**Highly susceptible**	**Moderate tolerant**	**Relatively resistance**
P. patula	Mexican yellow pine	Mexico, north eastern Oaxaca, Siera Madre	X		
P. radiata	Monetary pine	Central Coast of California and Mexico.	X		
P. greggii	Gregg's pine	Eastern Mexico	X		
P. elliottii (improved)	Slash pine	George Town, Central Florida. North central Georgia and Alabama		X	
P. caribeae var bahamensis	Caribbean pine	Central America and Mexico (Honduras, Belize, Nicaragua)		X	
P. caribeae var hondurensis	Caribbean pine	Central America and Mexico (Honduras, Belize, Nicaragua)		X	
P. tecunumanii HE	Schwerdtfeger's pine	Guatemala, Chiapas and Mexico		X	
P. pseudostrobus	Pino Blanco pine	Mexico: Chiapas, Guerrero, Hidalgo, Puebla, Tlaxcala, W-C Veracruz, México, Oaxaca, and Veracruz.		X	
P. taeda	Loblolly pine	Southern United State (Georgia and Northern Nicaragua)		X	
P. maximinoi	Thin-leaf pine	Mexico, Guatemala and northern Nicaragua.			X
P. tecunumanii LE	Schwerdtfeger's pine	Belize (northern Guatamela), Honduras, Nicaragua			X
P. oocarpa	Mexican yellow pine	Mexico, South Sonora and North Nicaragua			X
P. pringlei	Spanish pine	Eastern Mexico and Central America			X

2.5.2 *Pinus tecunumanii*

Pinus tecunumanii is a medium to very large tree that originates from the highlands of central Chiapas, Mexico to central Nicaragua (Dvorak *et al.,* 2000b). The species is found in two distinct groups, according to morphology and adaptability differences namely *P. tecunumanii* HE and LE (Figure 2.5). *Pinus tecunumanii* HE occurs from approximately 1 500 to 2 900 m.a.s.l. in Honduras, Guatemala and Mexico, while *P. tecunumanii* LE occurs from approximately 450 to 1 500 m.a.s.l. in Nicaragua, Honduras and Belize. The *P. tecunumanii* LE populations, in general, have better seed yield (Dvorak *et al.,* 2000b).

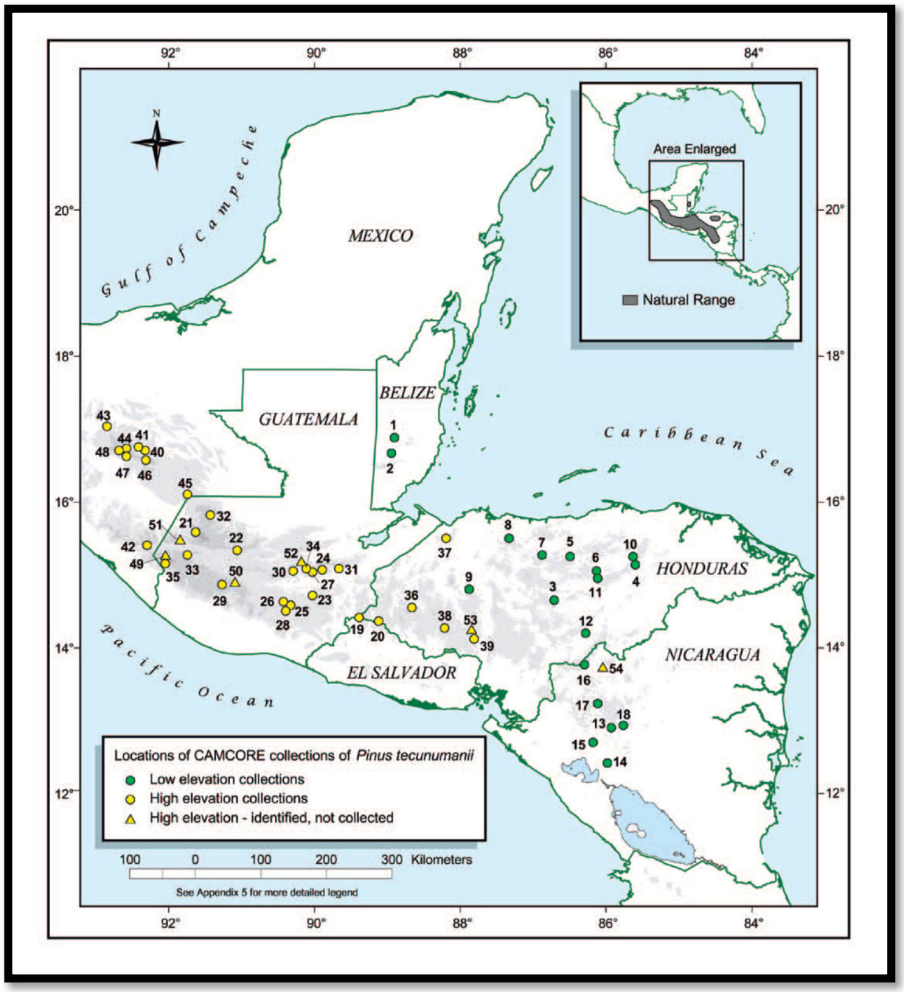

Figure 2.5: Natural range of *Pinus tecunumanii* with the HE populations in yellow and LE populations in green (Dvorak *et al.,* 2000b)

Pinus tecunumanii (HE) provenances differ on the level of tolerance to frost and *F. circinatum*, as some provenances can tolerate light frost with intermediate *F. circinatum* tolerance. However, the LE populations are sensitive to frost, but can tolerate *F. circinatum* (Mitchell, 2012). This species has numerous advantages compared to *P. patula,* such as rapid growth in the nursery, drought tolerance in case of the HE populations (South Africa), higher wood density (South Africa and Colombia), generally more uniform wood, better

tolerance to *F. circinatum*, and hybridises easier with other pine species (Kanzler *et al.,* 2014; Dvorak *et al.,* 2000b; Malan, 1994).

2.5.3 *Pinus patula* × *P. tecunumanii* hybrid

Commercial deployment of interspecific hybrids has been adopted by many forestry companies such as Australia, Korea, USA and South Africa (Nel *et al.,* 2017). In 1990, the first initiative to cross *P. patula* × *P. tecunumanii* occurred after tree breeders were convinced that this hybrid could be utilised to replace pure *P. patula* (Kanzler *et al.,* 2014). Field trials were established in Mpumalanga, KwaZulu-Natal, Eastern Cape and Western Cape provinces of South Africa (Hongwane *et al*., 2017), relying on the adaptability of *P. patula* as the female parent to these sites (Kanzler *et al.,* 2014). The hybrid between *P. patula* and *P. tecunumanii* HE grows more vigorously on a wider range of sites including temperate sites (Mitchell, 2012). However, it has moderate tolerance towards *F. circinatum* (Hodge and Dvorak, 2000), while *P. patula* × *P. tecunumanii* LE has good *F. circinatum* tolerance.

Research and development conducted in South Africa for the past 20 years indicated that these two hybrids can potentially outcompete *P. patula* with superior growth (Kanzler *et al.,* 2012). Findings also indicated that this hybrid has a large breeding base in provenance trials and breeding banks. Other advantages include: early availability of pollen; close genetic distance with *P. patula*; good wood properties (pulp and sawn timber) and *F. circinatum* tolerance. In field trials advantages such as rapid site capture, reduce prolonged weed competition and relatively good drought tolerance were observed (Kanzler *et al.,* 2014). Having all these advantages, it is imperative to screen the individual full-sib crosses for *F. circinatum* tolerance at the family level. This could benefit tree breeders in case of future deployment of new hybrid family crosses. Interspecific hybrids offer opportunity of producing superior offspring with good traits from both parents. Good traits can contribute towards pest and disease tolerance, drought, cold resistance depending on specific environmental conditions.

2.6 Genetic studies on *Fusarium circinatum* tolerance

World-wide, forest tree species are under increasing threat from pest and diseases. Breeding programs can offer a better solution to these threats (Meseka *et al.,* 2018) by breeding disease or pest tolerant germplasm. In many cases, genetic resistance offers the key to restoration of forest trees and may even prevent the loss of some tree species. Understanding the level of disease frequency, durability and stability of tolerance and its limitations will help to contain the damage caused by the pests and diseases (Gao *et al.,* 2013). In order to quantify the level of tolerance available in a specific species or family, the heritability for resistance needs to be quantified and the combining ability determined for tolerant individuals (Meseka *et al.,* 2018).

The type and level of combining ability is useful since it explains the contribution of each specific parent to the performance of their progeny (Nel, 2013). This approach also allows for the separation of the combining ability among progenies into a general combining ability (GCA) and a specific combining ability (SCA) (Gao *et al.,* 2013). GCA is the average performance of a trait in hybrid combinations and is a measure of additive gene action (Gao *et al.,* 2013; Dwivedi *et al.,* 2012). The estimate of GCA of a parent is an important indicator of its potential for generating superior populations (Arashida *et al.,* 2017). SCA is the deviation from expected average performance of traits (Gao *et al.,* 2013; Dwivedi *et al.,* 2012). Its effects represent dominance and epistasis gene actions which can be used as a guideline to determine the usefulness of a particular cross combination in the exploitation of hybrids (Gao *et al.,* 2013).

The differences between progeny in GCA are mainly due to the additive and additive × additive gene interactions, whereas in the SCA hybrid combination are attributable to non-additive, often dominant epistatic interactions for interspecific hybrids (Meseka *et al.,* 2018). The GCA and SCA can also be referred to as General Hybridisation Ability (GHA) and Specific Hybridisation Ability (SHA). Information on the combining ability of parents and the genetic diversity of tolerant species is useful when initiating a new breeding programme. Promising genotypes are selected on the basis of their performance in various hybrid combinations (Gao *et al.,* 2013). In this study, the most interest is on parents that show a negative and low GCA and SCA values as this indicates *F. circinatum* tolerance. It shows that the average of the parent is inferior or superior to overall average. Furthermore, it represents a strong evidence of favourable gene flow of the parent for the progeny at a high frequency and informs on the predominantly additive genetic concentration (Arashida *et al.,* 2017).

2.6.1 Previous genetic studies on *Fusarium circinatum* tolerance of *Pinus* species

Genetic selection for genotypes that are more tolerant to *F. circinatum* would reduce further development of the disease (Vivas *et al.,* 2012). This can be achieved through the development of non-tolerant genotypes since there is a potential to select for disease tolerant families in a large pool of various species. Thus, the forestry industry can benefit from development, testing, crossing and selection of best hybrid combinations. Therefore, variation within species families can be determined through estimation of genetic components and heritability. Previous studies to determine the tolerance of *F. circinatum* are summarised in Table 2.2.

Table 2.2: Estimates of individual and family heritability for disease tolerance of different species as determined by previous studies

Species	Individual heritability (h^2)	Family heritability (h^2)	Country of study	Reference
P. patula	-	0.76 - 0.87	USA and RSA	Nel *et al.,* 2014
P. tecunumanii HE	0.08	-	RSA	Mitchell *et al.,* 2013
P. tecunumanii LE	0.07	-		
P. patula	0.06	-		
P. patula *P. elliottii*	0.25 0.52	- -	RSA	Mitchell *et al.,* 2014
P. pinaster	-	0.43 - 0.58 to 0.51–0.8	Spain	Elvira-Recuenco *et al.,* 2014
P. pinaster	0.2 – 0.5		North West Spain	Vivas *et al.,* 2012

2.6.2 Genetic parameters of *Fusarium circinatum* tolerance within open-pollinated families of *Pinus patula* tested at screening facilities in South Africa and the United State of America

The genetic parameters for *F. circinatum* tolerance on five open-pollinated *P. patula* families was investigated by Nel *et al.* (2014). The study was conducted at two different screening laboratory facilities: (1) Forest Service Resistance Screening Center (RSC) in Bent Creek, North Carolina, USA, and (2) at the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa. From the study, findings indicated that there was significant genetic variation in tolerance to *F. circinatum* among a large number of open-pollinated *P. patula* families. Heritability (h^2) estimates for dieback and stem-kill percentage were moderately high, ranging from 0.22 to 0.31, indicating genetic family variation between tested families. Genetic correlations (r) between experiments in the same laboratory were very high, ranging from 0.78 to 1.00, indicating a clear tolerant in both laboratories. This was confirmed by a group of families that had a negative predicted GCA values (-6.5), indicating less stem-kill percentage. Considered all the data, it seemed clear that families identified as tolerant in one laboratory will also be identified as tolerant in another laboratory (Nel *et al.,* 2014).

2.6.3 The tolerance of *Pinus patula* × *Pinus tecunumanii* and other pine hybrids to *Fusarium circinatum* in greenhouse trials

In a study by Mitchell *et al.* (2013), three greenhouse experiments were carried out to examine the tolerance of *P. patula* × *P. tecunumanii* HE and *P. patula* × *P. tecunumanii* LE hybrid families to *F. circinatum*. The study also indicated a wide array of tolerant hybrid families that could also be used as possible replacements for *P. patula*.

The results of the study indicated that family differences in *P. patula* × *P. tecunumanii* were due to specific interaction between the *P. patula* and *P. tecunumanii* parents. The variance components of *P. patula* × *P. tecunumanii* HE has accounted for 9.6% of the phenotypic variance, while that of *P. patula* accounted for 6.4%. This was lower than *P. patula* × *P. tecunumanii* HE as *P. tecunumanii* HE had 2.1% and *P. tecunumanii* LE 1.8% of the phenotypic variance. This indicated that *P. patula* × *P. tecunumanii* HE was less tolerant to *F. circinatum* than *P. patula* × *P. tecunumanii* LE with a 4.2% phenotypic variance (Mitchell *et al.,* 2013). This explains that family variation in tolerance to *F. circinatum* is typically because of the combination of specific parents that are non-tolerant to *F. circinatum*. Therefore, there is still a need for further testing *P. tecunumanii* HE individual families before deployment. *Pinus tecunumanii* LE demonstrated a very small genetic variation while *P. patula* had a high genetic variation. Therefore, there is no need for further testing *P. tecunumanii* LE individual families before deployment since most individual families demonstrated tolerance to *F. circinatum* (Mitchell *et al.,* 2013).

2.6.4 Comparison of the tolerance of *Pinus patula* seedlings and established trees to infection by *Fusarium circinatum*

A comparison between seedlings and established trees of *P. patula* to the tolerance of infection by *F. circinatum* was made by Mitchell *et al.* (2014). The study aimed to determine whether the screening of families as seedlings in a greenhouse will provide information equal to that of mature trees in plantations. Variation in the tolerance of *P. patula* to *F. circinatum* was assessed to identify tolerant families. These was attained by inoculating a total of 141 *P. patula* families in an event of two consecutive greenhouse trials and 96 *P. patula* families from nine-years-old trees. Obtained results were then compared to the tolerance of those families tested as nine-years-old trees and again with those seedling families raised from seeds collected from the mature trees. Treatments included was *P. elliottii, P. radiata, P. patula* × *P. tecunumanii* LE, *P. patula* × *P. oocarpa*, *P. patula* × *P. greggii* var. *greggii*, *P. patula* × *P. caribaea*, *P. elliottii* × *P. caribaea*, *P. tecunumanii* LE × *P. caribaea* and *P. tecunumanii* LE × *P. oocarpa* (Mitchell *et al.,* 2014).

Analysis confirmed that breeding for tolerance to *F. circinatum* is feasible. Screening large numbers of open-pollinated families in greenhouse trials provided opportunity to identify more tolerant clones. Furthermore, identification of tolerant clones based on the performance of their open-pollinated progeny as seedlings can

lead to healthier plants if seeds are harvested from orchards of such trees (Mitchell *et al.,* 2014). It was estimated that approximately 5% of *P. patula* trees found to be as tolerant as *P. elliottii.* A good individual h^2 was also obtained in two greenhouse studies and ranged between 0.25 and 0.52. This indicated that breeding for *F. ciricinatum* is possible, therefore, selection for tolerance to *F. circinatum* within such population will allow for further improvement of tolerance levels.

2.6.5 Adaptive potential of *Pinus pinaster* populations to the emerging Pitch canker pathogen, *Fusarium circinatum*

The study reported by Elvira-Recuenco *et al.* (2014) was carried out in Spain to predict whether *F. circinatum* will have an impact on *P. pinaster,* examined the genetic mechanisms and the host resistance of *P. pinaster* species . A total number of 670 ramets of three-year-old cuttings from clonal provenance progeny trials were used. Artificial inoculation was carried out under maintained controlled environmental conditions. Interestingly, a high genetic variation was found with and estimates of between 0.43 and 0.58 and between 0.51 and 0.8, depending on the resistance traits measured (lesion length, lesion length rate, time to wilting, and survival). High values of h^2 and a high capacity of breeding response are good indication of tolerance species to the *F. circinatum* pathogen. This phenotyping resistance results was seen on both clonal provenances and progeny trial (Elvira-Recuenco *et al.,* 2014).

2.6.6 Screening of *Pinus pinaster* for resistance to *Fusarium circinatum*, the causal agent of pitch canker disease

The objective of this study was to determine the tolerance of *P. pinaster* to *F. circinatum* and again to find out whether selection and breeding mechanisms can be used to improve the species (Vivas *et al.,* 2012). A total of 39 *P. pinaster* clones and seedlings where evaluated for resistance. Only one isolate was used (MAT-1, code Fc7-1) because the virulence of *F. circinatum* in Spain is homogeneous and, therefore, different *F. circinatum* strains do not reveal significantly different rankings of susceptibility among the same host genotypes. The results of the study indicated that genetic variation in response to *F. circinatum* does exist. Plants h^2 of time-to-death (0.2) has proven to be moderate while of mortality (0.5) was higher. High mortality indicated a good strong genetic variation and quantified that it is possible to do selection for resistance species (Vivas *et al.,* 2012). This result was in the same range as the h^2 reported for *P. radiata* by Aegerter and Gordon (2006) and Matheson *et al.* (2006).

2.6.7 High genetic diversity of *Fusarium circinatum* associated with the first outbreak of pitch canker on *Pinus patula* in South Africa

Samples of this study was collected from three *P. patula* compartments situated in Limpopo province. Trees sampled were between three to six years and sampled again at between 12 and 19 -years old. The study was carried out to confirm the presence of *F. circinatum* on symptomatic trees (Fru *et al.,* 2018). The method used

was morphology and DNA-based diagnostic procedures. This was used to evaluate the overall management risks associated with this disease outbreak by considering the population biology of the pathogen in this region. The overall genetic diversity of 17 populations, which represent 30 alleles was estimated. The genetic diversity (G*) of *F. circinatum* was found to be high at 12.70. The high level of genetic diversity of *F. circinatum* and the presence of sexual recombination in the population of *P. patula* could pose significant management challenges. The results of the study suggested that the population was largely asexual, regardless of the presence of both mating type (Mat-1 and Mat-2) individuals in the Limpopo population (Fru *et al.,* 2018).

2.7 Breeding for *Fusarium circinatum* tolerance

Breeding to improve disease tolerance has been prioritised in the South African forestry industry after the discovery and spread of *F. circinatum* in 1990 (Viljoen *et al.,* 1994). Breeders have screened pine species for tolerance to *F. circinatum* through a technique of artificial inoculation, which provided good results in terms of identifying breeding material with increased level of *F. circinatum* tolerance (Nel *et al.,* 2014). The most effective method to manage this disease was through planting tolerant species (Mitchell, 2012).

The interspecific hybrid between *P. patula* and *P. tecunumanii* seems to be a suitable replacement to *P. patula* (Roux *et al.,* 2007). This was proven by field trials conducted in Swaziland and in the KwaZulu-Natal Midlands of South Africa. Results from these studies indicated that *P. patula* × *P. tecunumanii* outperforms *P. patula* with an average of 23% in volume at the age of five years, the hybrid also performed well at 64% survival rate when compared to its controls of *P. patula* and *P. tecunumanii* (LE and HE) (Hongwane et al., 2017). Previous studies also indicated that the hybrids *P. patula* × *P. tecunumanii* LE and *P. patula* × *P. oocarpa* survived better than pure *P. patula* on the warmer sites of South Africa, whereas the hybrid between *P. patula* × *P. tecunumanii* HE performs better at the colder sites (Hongwane *et al.,* 2017; Mitchell *et al.,* 2011).

Chapter 3
Materials and methods

3.1 Introduction

During the last decade, commercialisation of the *Pinus patula* × *P. tecunumanii* low elevation (LE) and *P. patula* × *P. tecunumanii* high elevation (HE) hybrids, as a replacement for *P. patula* pure species, proved to be an effective strategy (Fru *et al.,* 2018; Kanzler *et al.,* 2014; Mitchell *et al.,* 2013). As part of understanding the benefits of this hybrid, it is important to determine which hybrid families can tolerate *Fusarium circinatum* infection. A standard screening method to determine the tolerance of *F. circinatum* was conducted on 69 hybrid families and 15 pure species families. Quantitative approach was used to collect and analyse data in this study. Data was generated through rooted cuttings whereby lesion length and plant length were measured, and stem-kill percentage was proportionally extracted from the latter. The concept of genetic parameter estimates general combining ability (GCA), special combining ability (SCA) and genetic variance components were used to provide information to interpret the genetic influence of traits tested.

This study comprised of two experiments aimed to determine the level of *F. circinatum* tolerance of two interspecific hybrids. The first experiment was conducted at the Stellenbosch University (Stellenbosch) in August 2017 and the second at the Sappi Shaw Research Centre (Howick) during May 2018. Hybrids were produced through controlled pollination and multiplied through vegetative propagation; here after referred to as rooted cuttings. The controls (*P. taeda*, *P. tecunumanii* HE, *P. tecunumanii* LE and *P. patula*) were produced as seedlings through sowing and seed germination. Artificial inoculation following screening protocols as developed by Oak *et al.* (1987), for both rooted cuttings and seedlings, were carried out under greenhouse conditions to determine the level of *F. circinatum* tolerance. Interspecific hybrids used in this study were *P. patula* × *P. tecunumanii* HE and *P. patula* × *P. tecunumanii* LE. Pure species (controls) were: *P. tecunumanii* HE, *P. tecunumanii* LE, *P. patula*, and *P. taeda.* Both experiments contained the same number of combinations consisting of interspecific hybrid families and controls, except *P. taeda* that was not included as control in the second experiment due to limited seed. In this study, acronyms were introduced for hybrid to limit confusion and repetition. Pure species are referred to by Latin names, but when reference are made to specific families, a numeric letter will follow the acronym of species and hybrids (Table 3.1).

Table 3.1: Acronyms of interspecific hybrid families and pure *Pinus* species (controls) screened during this study

Pure species or hybrid	Acronym
P. tecunumanii HE (male parent)	PTH1 to PTH5
P. tecunumanii LE (male parent)	PTL1 to PTL10
P. patula (female parent)	P1 to P13
P. patula × *P. tecunumanii* HE	PPTH
P. patula × *P. tecunumanii* LE	PPTL

Family number are indicated as a number after said species or hybrid

3.2 Plant material

Pinus seeds were harvested from controlled pollinations and sown to be propagated as hybrid family vegetative hedges at Sappi Escarpment nursery near Barberton, Mpumalanga (25° 37′ 50.77″S, 30 ° 48′ 24.92″E). Each hybrid family consisted of at least 100 seedling hedges from which rooted cuttings were produced. The controlled pollination mating design for these hybrids were executed between 2009 and 2014 at the Sappi Shaw Research Centre (Howick, Pietermaritzburg). Cuttings from these hedges were grown in Unigro® 98-cavity plastic containers (90 ml per cavity) filled with a composted pine bark medium inside a greenhouse. Rooted cuttings were placed under 40% shade net (rooting camp) for two months. Thereafter cuttings were moved to a growing camp and kept for six months to harden off. In the rooting camp, cuttings were watered for five minutes at 30 minutes intervals, while rooted cuttings were watered once a day for 30 minutes. Seedlings were fertilised with VITAMAX 3.1.5 (38) and calcium nitrate applied when necessary.

For both experiments, a total of 12 *P. patula* open-pollinated families were used as female parents, while 10 *P. tecunumanii* LE and five *P. tecunumanii* HE families represented the male parents (Table 3.2). Different families of different species were used to determine the genetic control of *F. circinatum* tolerance. These families were: Pat 2, Pat 7, PTH1, PTH2, PTH3, PTH4, and PTH5 and PTL1, PTL2, PTL3, PTL4, PTL5, PTL6, PTL7, PTL8, PTL9 and PTL10. Seed of these families were sown in the Ngodwana nursery outside Nelspruit (25 ° 34′ 57.65″S, 30 ° 38′ 36.19″E) whereas seeds of *P. taeda* were sown at Sappi Shaw Research Centre (SRC) in Howick outside Pietermaritzburg (29 ° 28′ 35. 59″S, 30 ° 10′ 46.14″E).

The seed were kept moist in a germination chamber for seven days at a constant temperature of 25 ˚C and humidity between 90 and 100 %. After germination, seed were placed in a plastic greenhouse structure for hardening off. Watering occurred once a day for 30 minutes in the morning and fertilisation was the same as for rooted cuttings. The seed sown at SRC (*P. taeda*) were treated the same as seed sown at Ngodwana.

3.3 *Fusarium circinatum* inoculation experiment

Spore suspensions of three *F. circinatum* cultures (FCC3577, 3578 and 3579) were supplied by the Disease Clinic at the Department of Plant Pathology (Stellenbosch University). These are the same isolates used in previous inoculation studies conducted at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (Nel *et al.,* 2014; Mitchell *et al.,* 2013; van Wyk, 2011). Approximately 10 days before the inoculation of *F. circinatum*, the cultures were plated and confirmed with genetic fingerprinting. The three cultures were used in equal concentrations in preparing the inoculum. Two millimetres of a 15% glycerol solution were pipetted onto the plates and the spores were removed gently with a glass hockey stick. The inoculum solution was then washed off into a sterile 100 ml Schott bottle and filtered into another 100 ml Schott bottle using a sterilised cheese cloth ideally to remove any mycelium present. The suspension was then placed on ice until the spore count was performed.

A haemocytometer was used for the spore count and was sterilised with 70% ethanol before and between spore counts. A total of 9 µl of the spore suspension was applied to the haemocytometer and counted at a 10 X magnification (Leica Analytic Light Microscope DM300). Ten readings were taken with three repetitions each. The spore concentration was adjusted to 50 000 spores per millimetre and the spore volume was made up to 150 ml with 15% glycerol. The average germination percentage on PDA (general growth media for fungi and bacteria) was respectively 100% and 98% in Water Agar (WA). This was very important as it quantified the production of enough microconidia. A total of 10 ml of the final spore suspension was transferred into a centrifuge tube and kept on ice until inoculation started.

During inoculation, only healthy seedlings were inoculated, while unhealthy plants were discarded and marked as missing. Seedlings were wounded by removing the apical bud with sharp secateurs. After each cut, the secateurs were disinfected with 1:9 JIK (sodium hypochlorite) solution to prevent cross contamination between plants. Inoculum (10 µl) consisting of approximately 50˚000 spores were inoculated onto the wound before resin development (Figure 3.1). During inoculation, the inoculum was kept in a cooler box with ice cubes to maintain the viability of inoculum. Disposable hand gloves and regularly changing of pipette tips ensured good hygiene practices were maintained. The inoculated plants were monitored and watered daily after the inoculations were completed to keep the plants moist for further assessments.

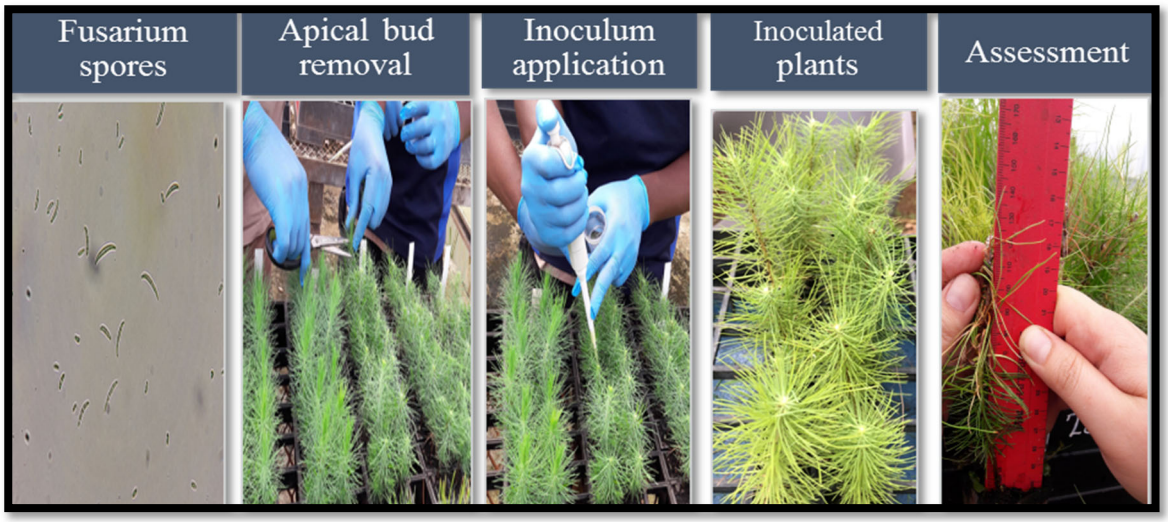

Figure 3.1: Inoculation procedure employed during *F. circinatum* screening of *Pinus* seedlings

To test whether plants were *F. circinatum* free before inoculation of experiment 1, 16 random seedlings (combination of healthy and unhealthy) were sampled and tested for *F. circinatum*. The plants were surface sterilised in a 70% ethanol solution and plated out onto PDA (a general growth medium for the isolation of fungi and bacteria) plates. All *F. circinatum* cultures were sub-cultured and isolations were made from the crown and roots of all plants. From the submitted plants, only 19% tested positive for *F. circinatum*, thus the pathogen was only present in three plants. The three positive plants could have been *P. patula* and not hybrid plants due to contamination during controlled pollination as the species is known to be susceptible to *F. circinatum*.

Lesion length of inoculated plants were assessed at eight weeks (for experiment 1) and at 20 weeks (for experiment 2) after inoculation. Experiment 2 was assessed after 20 weeks (Table 3.3) as the inoculations were done during autumn with colder temperatures, which slowed down the growth of lesions. The total length of plants and the lesion length of each seedling (mm) were measured using a ruler. The plant length was measured from the root collar to the wounded tip, whereas the lesion length was measured from the inoculation point to where the tissue displayed no lesion expression (Figure 3.1). The percentage stem-kill was expressed as the proportion of lesion length to the length of the seedling.

3.4 Experimental design

Experiment 1 consisted of 37 PPTL and 32 PPTH hybrid families and pure species as controls included two *P. patula*, four *P. tecunumanii* LE, five *P. tecunumanii* HE and four *P. taeda* families. The four *P. taeda* families were grouped together as one single species. The experiment consisted of 69 treatments and 15 controls, replicated four times. The experiment layout was an alpha lattice design with nine blocks within replication, this was designed using the CycDesignN package (Whitaker *et al.,* 1997). Plots were laid out in

two rows of 2 x 7 plants in Unigro 98 trays with a total number of 340 plots in the trial. The total number of plants per treatment was 56, subject to availability of plants.

After hardening off and packing out, plants were transported to Stellenbosch for *F. circinatum* screening. Plants were kept at approximately 27 °C in a plastic growth tunnel for four weeks to acclimatise and were irrigated twice daily for five minutes each. Experiment 2 consisted of 50 PPTL and 41 PPTH hybrid family treatments and the controls consisted of two *P. patula*, two *P. tecunumanii* LE and one *P. tecunumanii* HE families. In total, the experiment contained 91 treatments and five controls. The experimental layout was the same design as experiment 1, alpha lattice design with four replications. Due to a severe drought and water restrictions in Stellenbosch, experiment 2 was carried out at SRC. Table 3.3 summarises the treatments of both experiments.

Table 3.2: The factorial mating design indicating interspecific hybrids between *P. patula*, *P. tecunumanii* LE and *P. tecunumanii* HE families screened during this study

		Male parents														
		***P. tecunumanii* LE**										***P. tecunumanii* HE**				
Female parents	***P. patula***	**PTL1**	**PTL2**	**PTL3**	**PTL4**	**PTL5**	**PTL6**	**PTL7**	**PTL8**	**PTL9**	**PTL10**	**PTH1**	**PTH2**	**PTH3**	**PTH4**	**PTH5**
	P1	×				×					×		×	×	×	×
	P2	×	×		×				×			×	×	×	×	×
	P3	×		×	×				×	×		×	×	×	×	
	P4	×			×				×	×		×	×	×	×	
	P5	×		×					×	×		×	×	×		
	P6	×							×		×					
	P7	×	×	×	×			×	×	×		×	×	×	×	
	P8						×					×	×		×	
	P9	×			×				×	×		×	×	×	×	
	P10	×			×		×			×			×	×		
	P11	×			×		×		×	×		×	×	×	×	
	P12	×			×	×			×			×	×	×		

Table 3.3: List of treatments in the two experiments testing the genetic control of *Fusarium circinatum* tolerance in *P. patula* × *P. tecunumanii* low and high elevation hybrid families

Trial details	**PPTL and PPTH**	**PPTL and PPTH**
	Trial 1	**Trial 2**
Propagation date	October 2016	May-17
Date inoculated	August 2017	May-18
Date assessed	October 2017	October 2018
Treatments tested	PPTL (37)	PPTL (50)
	PPTH (32)	PPTH (43)
Total plot size	384	384
Replication	4	4
Total treatments including controls	84	96
Controls	*P. taeda*	N/A
	P. tecunumanii (LE)	*P. tecunumanii* (LE)
	P. tecunumanii (HE)	*P. tecunumanii* (HE)
	P. patula	*P. patula*
Tunnel structure	Permanent structure with polycarbonate roof and brick walls	Temporary structure covered with plastic on top and fully opened on both sides
Strains	3	3
Average size of plants length	122 mm	219 mm
Period plants kept in tunnel before assessment	8 weeks	20 weeks
Average temperature	27 °C	12 °C
Watering regime	Overhead sprinklers twice a day	Overhead sprinklers twice a day before inoculation and once a day after inoculation (winter)

3.5 Statistical analyses

Microsoft Excel (version 2013) computer package was used to encode the inoculated data, while statistical analysis was done with R Commander CRAN- package Rcmdr 2016 (version 3.3.0), R Studio lme4 package (Bates *et al.,* 2015) in R (version 3.5.1, R Core Team, 2017).

Each experiment was analysed separately. R Commander CRAN- package was used to assess the fitness of dataset with one sample t-test and validated with the Shapiro-Wilk test (Shapiro and Francia, 1972) while basic diagnostic plots were used to test for normality. This was done before the results could be assumed reliable

(Clewer and Scarisbrick, 2001). Data transformation through Microsoft Excel using Arcsine transformation was carried out to standardise the data as most of the proportions were between 0 and 0.3. Basic statistics included the summary statistics (minimum, maximum, mean, median, range, skewness, standard deviation and variance). Kruskal Wallis test based on non-parametric method of ranking, was used to determine whether there was a statistically significant differences between continuous dependent variables (Kruskal and Wallis, 1952).

Results were analysed through the linear model with R Commander. Four analysis of variance (ANOVA) at 5% (0.05) confidence significant level was performed. The first analysis was performed on combined hybrid families (PPTH and PPTL), while the second analysis was performed on separated hybrid families (PPTH and PPTL). Hybrid families were combined to determine if there would be a significant difference of the mean between tested populations of PPTH and PPTL. These hybrid families were separated because the hypothesis was there could be a possibility that PPTH jeopardise the population of PPTL since it is know from literature that *P. tecunumanii* LE is more tolerant to *F. circinatum* that *P. tecunumanii* HE (Kanzler *et al.,* 2014; Mitchell *et al.,* 2013; Hodge and Dvorak, 2000). The assumption was that the mean could be skewed to PPTH since the trial mean was a combination of very tolerant and non-tolerant populations. The linear model included replication as fixed effects (families and treatments) were used as random effects.

The statistical linear model used was as follows:

$$stem\ kill\ percentage = rep + treatment \quad \ldots\ldots (1)$$

Where: stem kill percentage = proportion of lesion length and plant length, rep = replication effect, and treatment = effect of hybrids, treatment and family. Analysis were done separately for each treatment.

Treatments were grouped together to assess the mean differences at the hybrids level. Pearson correlation (r) was generated to measure the relationship between plant length, lesion length and stem-kill percentage (Clewer and Scarisbrick, 2001). LSmeans was performed through a general linear model (GLM) using R studio. The focus was on ranking hybrid families and parents tolerance to *F. circinatum.*

The statistical linear model used was as follows:

$$stem\ kill\ percentage_{mn} = \mu + rep_m + treatment_n + e_{mn} \quad \ldots\ldots (2)$$

where: μ = the overall mean, rep_m = the replication effect, $treatment_n$ = effect of hybrids, family and species and e_{mn} = within plot error.

Narrow sense heritability (h^2) and genetic parameters were calculated to determine the contributing factor of parents when hybridised (Falconer and Mackay, 1996). As hybrids were full sibs, h^2 from here onwards refers to narrow sense h^2.

Individual h^2 was estimated by:

$$h^2{}_x = 4\sigma^2{}_x/\sigma^2\text{Total} \quad (3)$$

Where: $h^2{}_x = 4\sigma^2{}_x$ is the narrow- sense heritability for both male and female parents.

Except the overall mean, all effects and replication were considered randomly and independently distributed. Assumption could then be that the epistatic effects were negligible and that the inbreeding coefficient of the parents were zero (Retief and Stanger, 2009). Variance component (V) analysis was performed using the lme4 package (Bates *et al.,* 2015) in R Studio to estimate genetic parameters for the individual families' tolerance to *F. circinatum*. As parents were from two different treatments, additive and dominance variances (σ_x^2) were estimated as follows:

$$\sigma^2{}_a = 4\,(cov\ HS) = 4\sigma^2{}_f + 4\sigma^2{}_m \quad (4)$$

$$\sigma^2{}_d = 4\,(cov\ FS - 2\ cov\ HS) = 4\sigma^2{}_{fm} \quad (5)$$

where: $\sigma^2{}_a$ = additive variance, $\sigma^2{}_d$ = dominance variance, = $\sigma^2{}_f$ = the female additive variance, $\sigma^2{}_m$ = the male additive variance, $\sigma^2{}_f + \sigma^2{}_m$ = the additive variance combining the female and the male variance.

For hybrids, the combining ability were assessed to express the contribution of each specific parent to the performance of their progeny (Nel, 2013). There are two concepts of explaining combining ability (GCA and SCA). GCA is considered as a simple and dominant measure of genetic parents. It is defined as the average performance of individual from a particular parent, compared to the population mean, when the parent is mated to a representative number of other individuals from the mating design. SCA is defined as the performance of the progeny produced from that specific combination of parents (Verryn, 2019) . Therefore, it refers to a specific cross and not to an individual parent. From a statistical point of view, the GCA is a main effect and the SCA is an interaction effect (Fasahat *et al.,* 2016). These two concepts (GCA and SCA) are also explained and defined in more detail in Chapter 2 section 2.6 (genetic studies on *Fusarium circinatum* tolerance).

The variance associated for female parents (P1 to P12) was taken as the variance of GCA for *P. patula* (σ^2 GCA-*pat*), while the variance of the male parents (PTH1 to PTH5 and PTL1 to PTL10) was taken as the variance of GCA for *P. tecunumanii* (σ^2 GCA-*tec*). The variance associated with the interspecific hybrids (PPTH1 to PPTH5 and PPTL1 to PPTL10) interaction was taken as the variance of SCA (σ^2 SCA-*pat* × *tec*).

Total $vPhen$ was calculated as the sum of all the variance components, and the percentage of variance accounted for each component was calculated. To obtain best linear unbiased predictors (BLUP) of random genetic effects (GCA and SCA) the lme4 package in R studio (R Core Team, 2017) was used as follows:

$$vPhen_{ijkl} = vFam_i + vMale_j + vFemale_k + vErr_{ijkl} \quad \text{(6)}$$

Where: $vFam_i$ = specific combining ability effect of male and female, $vMale_j$ = general combining ability effect of male parent, $Female_{k,}$ = general combining ability of female, $vErr_{ijkl}$ = random within plot error.

A Pearson's product moment correlation (r) analysis using R Commander CRAN- package, determined the relationship hybrid families had between *F. circinatum* and frost tolerance. Further correlation was performed to determine whether there was significant repeatability in tolerance of *F. circinatum* between tested hybrid families. The frost tolerance study was performed by Malinga (2018) to investigate frost tolerance of the same PPTH and PPTL hybrid families used in this study. Electrolyte leakage (EL) and the whole plant freeze testing (WPFT) methods were employed to generate numerical data from unrooted shoots and rooted cuttings at different temperatures (-3, -6 and -9 ˚C), whereas *F. circinatum* tolerant study was conducted through artificial *F. circinatum* inoculation screening of eight-month plants at the nursery. Both studies used the same genetic material from the same factorial design.

Chapter 4

Results

4.1 Introduction

Artificial screening of *Fusarium circinatum* inoculation was performed on 37 PPTL and 32 PPTH interspecific hybrid families and 15 parental families (two *P. patula*, five *P. tecunumanii* HE, four *P. tecunumanii* LE and four *P. taeda*) to determine *F. circinatum* tolerance. This involved the inoculation of field-ready plants with a pipette application of inoculum to the cut tip of plants. Assessment of variables such as plant length, lesion length and the calculation of stem-kill percentage was carried out. Statistical analyses were performed on the stem-kill percentage data as a proxy to determine hybrid family tolerance to *F. circinatum.* Data analysis included analysis of variance (ANOVA); Least Square Means (LSMeans) ranking; heritability (h^2); genetic variance components (V); General Combining Ability (GCA) and Specific Combining Ability (SCA) to determine which tolerant families breeders can incorporate into future breeding strategies. Acronyms included in this discussion were explained and tabulated in Chapter 3 (section 3.1).

4.2 Normality of data

Data were analysed through one sample T-test ($p < 0.0001$), a non-parametric test and Shapiro Wilk test ($p < 0.0001$) to determine whether the means across the dataset for both experiment 1 and 2 were normally distributed. It was found that both datasets were not normally distributed as indicated by the Normal QQ plot (Figure 4.1 A). The distribution of stem-kill percentage was skewed to the right, indicating most of the stem-kill percentage values were very small and almost all values fell under 0.3% or 30%. In this case the Arcsine data transformation was employed to stabilise variances and normalise proportional datasets that tend to be skewed when the distribution is not normal (see Chapter 3 section 3.5).

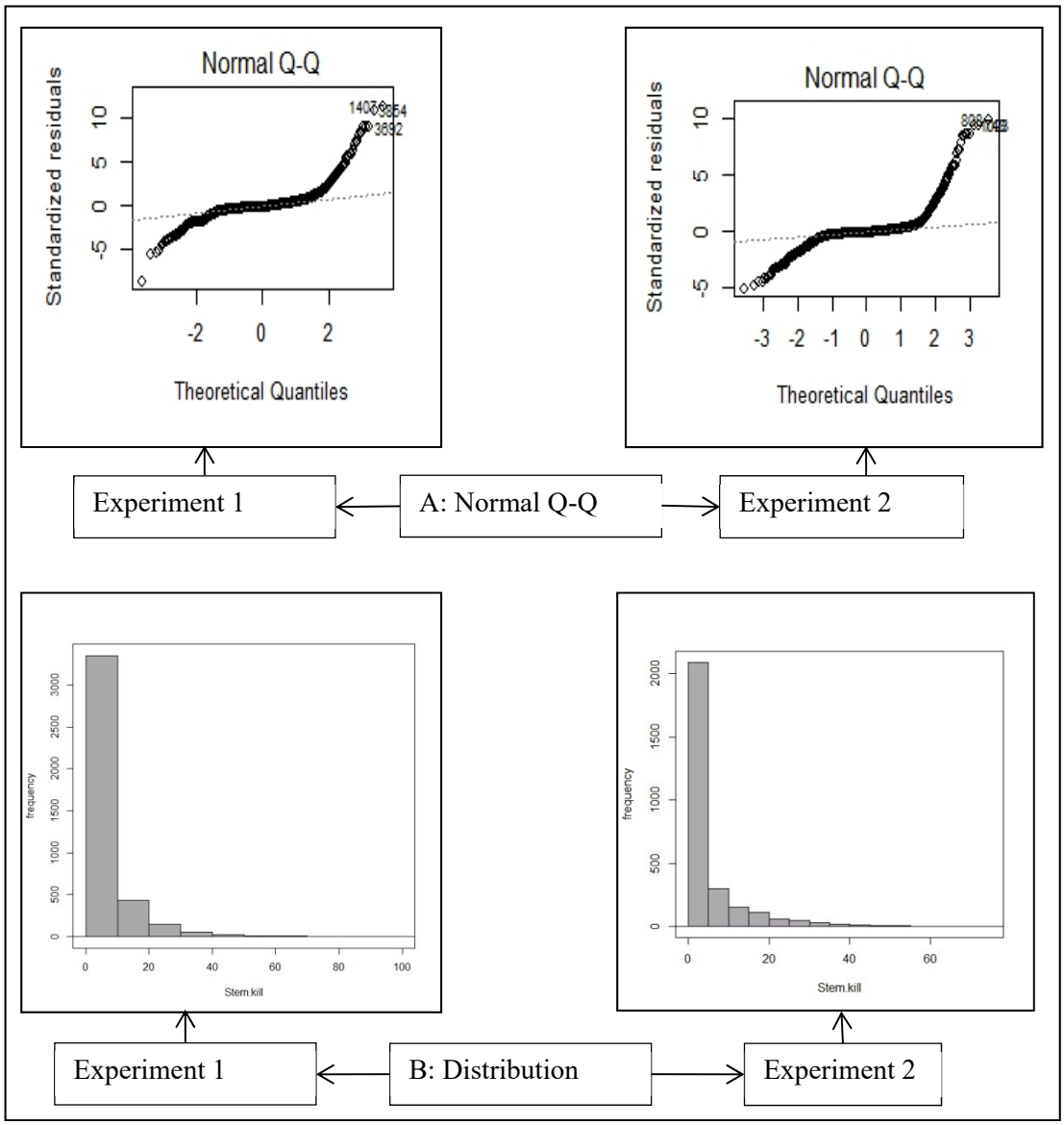

Figure 4.1: Normal Q-Q (A) and Distribution (B) graphs of experiment 1 and 2 for all combined treatments based on stem-kill percentage

4.3 Mean values for variables measured

Assessment of MEANS and UNIVARIATE procedures summarised variables measured such as plant length, lesion length and stem-kill percentage to determine the mean variation between variables and proportionally draw up the injury percentage after inoculation (Appendix A). The smaller the stem-kill percentage, the higher the *F. circinatum* tolerance. There were significant differences for stem-kill percentage between hybrids and controls ($p < 0.0001$; Table 4.1). Stem-kill percentage of experiment 1 (transformed dataset) indicated that PPTL (1.80%) had the highest, while *P. patula* had the lowest tolerance level (3.56%). For the untransformed data, *P. tecunumanii* LE (3.55%) had the lowest, while *P. patula* (24.38%) had the highest stem-kill percentage. Pearson product-moment correlation test (r) for stem-kill percentage and lesion length displayed a strong

positive relationship (r = 0.936), while a weak negative relationship (r = -0.406) was observed between stem-kill percentage and plant length.

Table 4.1: Transformed and untransformed data for experiment 1 by hybrid grouping and species comparing mean values for variables measured (PPTH, PPTL, *P. tecunumanii* HE, *P. tecunumanii* LE, *P. patula* and *P. taeda*)

Variables measured						
Experiment 1 untransformed data				**Experiment 1 transformed data**		
Treatment	**Plant length (mm)**	**Lesion Length (mm)**	**Stem-kill percentage**	**Plant length (mm)**	**Lesion Length (mm)**	**Stem-kill percentage**
PPTH	129.41	7.86	6.47	5.54	2.33	2.11
PPTL	132.45	5.37	4.69	5.53	2.01	1.80
P. patula (control)	54.15	13.44	24.38	4.47	2.95	3.56
P. tecunumani HE (control)	73.72	6.95	9.88	4.97	2.25	2.56
P. tecunumani LE (control)	97.36	3.29	3.55	5.25	1.81	1.86
*P. taeda(*control)	94.99	7.60	7.69	5.29	2.35	2.34

Results observed in experiment 2 indicated that *P. tecunumanii* LE had the highest level of tolerance with a mean stem-kill percentage of 1.45%, while *P. patula* (2.20%) had the lowest tolerance (Table 4.2). A strong positive relationship has been observed between stem-kill percentage and lesion length (r = 0.937), while a weak negative relationship (r = -0.233) was observed between stem-kill percentage and plant length.

Table 4.2: Comparison of transformed and untransformed data mean values for variables measured by hybrid grouping (PPTH & PPTL) and by species grouping (*P. tecunumanii* HE, *P. tecunumanii* LE, *P. patula* and *P. taeda*) of experiment 2

Variables measured						
	Experiment 2 untransformed data			Experiment 2 transformed data		
Treatment	Plant length (mm)	Lesion Length (mm)	Stem-kill percentage	Plant length (mm)	Lesion Length (mm)	Stem-kill percentage
PPTH	206.95	12.29	6.60	5.94	2.41	1.86
PPTL	226.11	8.58	7.16	5.99	2.16	1.57
P. patula (control)	172.48	20.13	21.12	5.65	2.56	2.20
P. tecunumanii HE (control)	269.22	13.09	4.01	6.22	2.73	1.85
P. tecunumanii LE (control)	347.43	8.13	3.43	6.48	2.42	1.45

4.4 Numeric variables summary for stem-kill percentage

Stem-kill percentage was the proportion of the stem infected by the *F. circinatum* inoculum after artificial screening and is a function of the lesion-length and total length of each plant. Therefore, stem-kill percentage was used for all further genetic analyses. The basic numeric statistics for stem-kill percentage for experiment 1 and 2 are illustrated in Tables 4.3 and 4.4 (Appendix B). Assessments of minimum and maximum were used to compare the distribution of data. In untransformed data of experiment 1, the maximum value ranged from 23.8% to 66.7% for both hybrid families and parental families, while the minimum of transformed data ranged from 0.30% to 0.88%. Standard deviation varied between 2.41% and 16.14% (untransformed data) and 0.49% to 1.04% (transformed data), indicating the presence of outliers. Result of the coefficient of variance (CV) varied between 0.66% and 1.48% (untransformed data) and 0.26% to 0.47% (transformed data), indicating the existence of variation in relation to the mean.

All treatments and controls, except *P. patula*, were positively skewed (to the right), ranging between 0.1% and 0.98% (Table 4.3). A negative skewness of -0.61% was observed for *P. patula*. The *P. tecunumanii* LE data deviation from the mean was 0.49%, while *P. taeda* deviation was 1.04%. The negative Kurtosis of PPTH, *P. tecunumanii* HE, *P. patula* and *P. taeda* indicated a flat distribution, while PPTL and *P. tecunumanii* LE was not normally distributed, but had a one-sided tail.

In experiment 2, the minimum value ranged from 0.05% to 0.63% (transformed data) and 0.06% to 0.68% (untransformed data) for all hybrid families and parental families, while the maximum of transformed data ranged between 3.35% and 5.0% (Table 4.3). Standard deviation was between 4.0% and 21.12% for untransformed and between 0.75% and 1.49% for transformed data, indicating the presence of outliers. Result of the coefficient of variance (CV) ranged between 0.96% and 1.62% for untransformed and between 0.42% and 0.67% for transformed data, indicating the variation of stem-kill percentage in relation to the mean.

The tested hybrid families and parental families were positively skewed to the right, ranging between 0.27% and 1.19% (Table 4.3). The negative Kurtosis on PPTH, and *P. patula* indicated a flat distribution, while PPTL, *P. tecunumanii* HE and *P. tecunumanii* LE was not normally distributed, thus, a one sided tail. From Tables 4.1 and 4.2 it is evident that experiment 1 responded better to inoculation than experiment 2. The lesion length of experiment 1 developed better compared to experiment 2. Due to the poor lesion length development of experiment 2, results in this Chapter were only based on experiment 1.

4.5 Analysis of Variance

Generalised Linear Model (GLM) for combined hybrid families of 37 PPTL, 32 PPTH and for separated PPTL and PPTH hybrid families was fitted to determine *F. circinatum* tolerance (see Chapter 3 section 3.5). Results from analysis of variance indicated significant differences ($p < 0.0001$) for both combined and separated PPTH and PPTL hybrid families (Table 4.5; Appendices C and D). The R-squared (R^2) of combined hybrid families and PPTL were 15% ($p < 0.0001$), while 8% for combined species ($p < 0.0001$) and 7% for PPTH ($p < 0.0001$). R^2 values ranged between zero and one, with zero indicating that the proposed model does not improve prediction over the mean model, and one indicating a perfect prediction. However, there are situations in which R^2 is less important especially when the interest is in the relationship between variables, not in prediction. In this study, R^2 is less important because the study focused on the relationship between variables. Nevertheless, high R^2 does not necessarily mean that the data is good because residuals could be randomly distributed around the zero line despite low R^2 values.

Table 4.3: Summary of the numeric variables of tested hybrid families (PPTH and PPTL) and parental controls (*P. tecunumanii* HE, *P. tecunumanii* LE, *P. patula* and *P. taeda*) based on stem-kill percentage for experiment 1

Variables measured												
Experiment 1 untransformed data							**Experiment 1 transformed data**					
Description (%)	**PPTL**	**PPTH**	***P. tecunumanii* LE**	***P. tecunumanii* HE**	***P. patula***	***P. taeda***	**PPTL**	**PPTH**	***P. tecunumanii* LE**	***P. tecunumanii* HE**	***P. patula***	***P. taeda***
Mean (μ)	4.69	6.47	3.55	9.88	24.37	7.69	1.81	2.11	1.86	2.57	3.57	2.34
Maximum (Max)	66.7	66.7	23.8	62.0	55.6	37.5	5.23	5.28	3.86	4.82	4.71	4.32
Minimum (Min)	0.0	0.0	0.0	0.0	2.4	0.0	0.39	0.39	0.81	0.88	1.61	0.30
Median (M)	2.4	3.3	3.2	5.0	21.5	3.8	1.61	1.91	1.88	2.31	3.76	2.31
Range (R)	93.8	69.0	23.8	62.0	53.2	37.5	4.84	4.89	3.05	3.94	3.1	4.02
Standard Error (SE)	0.16	0.20	0.16	0.67	2.52	0.61	0.02	0.03	0.03	0.05	0.14	0.07
Standard Deviation (StDev)	6.94	7.67	2.41	10.83	16.14	8.57	0.86	0.97	0.49	0.87	0.91	1.04
Coefficient of Variation (CV)	1.48	1.18	0.68	1.1	0.66	1.11	0.47	0.46	0.26	0.34	0.26	0.44
Variance (V)	48.16	58.83	5.81	117.29	260.50	73.44	0.74	0.94	0.24	0.76	0.83	1.08
Skewness (Skew)	4.60	2.61	3.76	2.16	0.18	1.43	0.98	0.42	0.58	0.61	-0.61	0.10
Kurtosis (KURT)	32.39	10.27	24.14	5.00	-1.28	1.38	0.57	-0.78	0.91	-0.51	-0.97	-1.19

Table 4.4: Summary of the numeric variables of tested hybrid families (PPTH and PPTL) and parental controls (*P. tecunumanii* HE, *P. tecunumanii* LE, *P. patula* and *P. taeda*) based on stem-kill percentage for experiment 2

Variables measured										
Experiment 2 untransformed data						**Experiment 2 transformed data**				
Description (%)	**PPTL**	**PPTH**	***P. tecunumanii* LE**	***P. tecunumanii* HE**	***P. patula***	**PPTL**	**PPTH**	***P. tecunumanii* LE**	***P. tecunumanii* HE**	***P. patula***
Mean (μ)	4.42	6.60	2.91	4.18	13.55	1.57	1.87	1.45	1.84	2.21
Maximum (Max)	59.38	66.67	14.29	18.18	74.38	4.78	4.89	3.35	3.59	5
Minimum (Min)	0.06	0.26	0.62	0.40	0.68	0.05	0.26	0.59	0.39	0.63
Median (M)	1.58	2.08	1.63	3.00	2.65	1.24	1.48	1.26	1.82	1.70
Range (R)	59.32	66.41	13.67	17.78	73.7	4.73	4.63	2.76	3.2	4.37
Standard Error (SE)	0.19	0.27	0.54	0.84	4.4	0.03	0.03	0.12	0.16	0.31
Standard Deviation (StDev)	7.16	9.74	3.43	4.00	21.12	1.00	1.44	0.75	0.77	1.49
Coefficient of Variation (CV)	1.62	1.48	1.78	0.96	1.56	0.64	0.61	0.52	0.42	0.67
Variance (V)	51.27	94.87	11.76	16.00	446.05	1.00	2.07	0.56	0.59	2.22
Skewness (Skew)	3.27	2.52	2.25	2.18	1.73	1.01	0.69	1.19	0.27	0.67
Kurtosis (KURT)	12.70	7.18	4.03	4.85	1.85	0.18	-0.69	0.74	0.03	-0.97

Table 4.5: Summary of the ANOVA results for combined and separated hybrid families of PPTH and PPTL based on stem-kill percentage

Family	DF	R^2	SS	MS	Error	Family differences	
						F-value	P-value
Combined Families (SK. ~ Rep +Family)	81	0.15	496.85	6.13	0.85	9.08	<0.0001
Combined Families (SK. ~ Rep + Species)	5	0.08	235.08	47.02	0.89	61.93	<0.0001
PPTH	32	0.07	102.12	3.19	0.94	3.62	<0.0001
PPTL	36	0.15	197.87	5.49	0.80	8.53	<0.0001

DF: Degrees of Freedom, SS: Sum of Squares, MS: Mean Square

4.6 Heritability and genetic parameters

4.6.1 Heritability

In tree breeding, heritability (h^2) is used to estimate the level of genetic variation for a phenotypic trait based on genetic variation between individuals in the population. A h^2 value of zero indicates no genetic contribution, whereas a value of one indicates complete genetic control in the tested traits. Narrow sense h^2 estimate for stem-kill percentage, lesion length and plant length are presented in Table 4.6. Three methods were applied: (1) both hybrid families (PPTH and PPTL) combined, (2) separate hybrid family (PPTH and PPTL) populations; and (3) at parental level (*P. tecunumanii* HE & *P. tecunumanii* LE and *P. patula*). These methods were applied to find out if h^2 will differ between the hybrid families for combined, separated and the reaction of parental species.

Results indicated low to moderate genetic control with h^2_{family} between 0.21 and 0.75 (Table 4.6). Significant differences were noted with the separated analyses, as PPTL had a high and strong genetic control with h^2_{family} ranging between 0.37 to 1.05, opposed to PPTH with a low h^2_{family} ranging between 0.07 and 0.36. Further analyses conducted at parental level indicated that *P. tecunumanii* LE and HE (male) had a high and strong genetic control with h^2_{male} ranging between 0 and 0.18 and *P. patula* (female) with a low h^2_{female} ranging between 0.04 and 0.1. In general, h^2 results indicated that there was a genetic family variation between tested families. Hybrid PPTL and the male parents (*P. tecunumanii* LE and HE) had a higher h^2 value and, thus, a stronger h^2 than PPTH and *P. patula*. Therefore, from the factorial mating design (see Chapter 3, Table 3.2), PPTL and *P. tecunumanii* (LE and HE) can be improved more easily by selecting and breeding for *F.*

circinatum tolerance than PPTH and *P. patula*. The low h^2_{family} of PPTH and low h^2_{female} of *P. patula* indicated that the genetic effects of these particular families were not diverse. Therefore, not much breeding improvement might be possible.

Table 4.6: Genetic parameter estimates for stem-kill percentage, lesion length and plant length from *P. patula* (female) and *P. tecunumanii* HE and *P. tecunumanii* LE (male) parents screened to explain heritability (h^2) and variance components *(V)* of PPTH & PPTL hybrid families tolerance to *F. circinatum*

Variables			**Variance components *(V)***					**Heritability *(h^2)***	
	Family	h^2_{family}	V_{fam}	V_{male}	V_{female}	V_{Err}	V_{phen}	h^2_{male}	h^2_{female}
Stem-kill percentage	Combined families	0.28	157.29	92.71	22.7	1946.8	2219.5	0.17	0.04
	PPTH	0.07	38.3	40.26	50.68	1996.83	2126.07	0.08	0.1
	PPTL	0.54	305.83	0	31.44	1932.55	2269.82	0	0.06
Lesion length (mm)	Combined families	0.21	87.38	74.58	39.29	1451.89	1653.14	0.18	0.1
	PPTH	0.14	56.78	45.17	40.49	1480.19	1622.63	0.11	0.1
	PPTL	0.37	144.88	4.33	17.56	1398.86	1565.63	0.01	0.04
Plant length (mm)	Combined families	0.78	6.27	0	0.39	25.68	32.34	0	0.05
	PPTH	0.36	2.34	0.57	0.14	23.18	26.23	0.09	0.02
	PPTL	1.05	9.59	0	0.29	26.58	36.46	0	0.03

4.6.2 Genetic Variance Components *(V)*

Variance component estimates and genetic parameters were calculated to assess the contribution of parents to *F. circinatum* tolerance for the hybrids (Table 4.6). The estimates of the male variance components (σ^2_m) ranged between 0.21 and 93.19, whereas the female variance components (σ^2_f) ranged between 0.45 and 39.1. Subsequently, the additive genetic σ^2_m of parents (*P. tecunumanii* HE and *P. tecunumanii* LE) were high with a contribution of 4.30% gene frequency. Nevertheless, the σ^2_f parents (*P. patula*) contributed less with a 1.60% gene frequency. Having additive genetic effects of male parents, indicated that genetic properties of the male parents contributed more to *F. circinatum* tolerance than the female parent.

4.7 General Combining Abaility (GCA) and Specific Combining Ability (SCA)

4.7.1 GCA and SCA for combined hybrid families of PPTH and PPTL

The assessment of combining ability indicated the performance contributed by specific parents to its progeny. In this study, GCA calculated the mean improvement of a parent's progeny over the mean of the experimental population, while SCA calculated the deviation from the expected value of a single cross. GCA values indicated that there were not big differences between *P. patula* families (Table 4.7) as GCA ranged between -4.51 and +4.82. However, family P1 had a lower GCA (-4.51), followed by P5 (-3.97), P9 (-2.72), P12 (-0.78), P10 (-0.66) and P11 (-0.33). This indicated the most tolerant female parents. For the male parents, *P. tecunumanii* LE had a GCA ranging between -11.42 and +8.05. However, the most tolerant family was TL2 with the lowest GCA (-11.42), followed by TL2 (-9.14), TL3 (-8.04), TL9 (-4.12), TL10 (-3.88), TL5 (-2.53), TL6 (-2.19) and TL8 (-0.10). *Pinus tecunumanii* HE (male parent) GCA ranged between -0.92 and +17.14 with TH5 (-0.92) being the most tolerant family, indicating a high level of *F. circinatum* tolerance. In generally, *P. tecunumanii* LE had the lowest GCA compared to *P. patula* and *P. tecunumanii* HE. Consequently, most *P. tecunumanii* LE parental families achieved a low GCA, indicating that there was a high level of *F. circinatum* tolerance.

SCA values indicated that 39 (P5 × TL1, P2 × TL2, P6 × TL1, P9 × TL9, P12 × TL4 etc.) hybrid families attained negative values ranging between -20.02 and -0.08 (Table 4.7). The hybrid family P5 × TL1 achieved a low negative SCA (-20.02), indicating a high level of tolerance. About 31 hybrid families were least tolerant to *F. circinatum* with SCA values indicating positive effects ranging between +0.28 and +34.86. Hybrid family P2 × TL1 had a high positive SCA value (+34.86), indicating a low level of tolerance to *F. circinatum*. Overall, the 39 specific crosses may offer great potential when selected for tolerance breeding.

Deviations of experimental mean for male parent *P. tecunumanii* LE was very low ranging between 1.30% and 2.02%. This was lower than that of *P. tecunumanii* HE, which ranged between 1.88% to 2.36%, while *P. patula* ranged between 1.63% and 2.33%. The lower deviation of experimental mean quantified the lower level of *F. circinatum* tolerance of *P. tecunumanii* LE.

Table 4.7: Estimated General Combining Ability (GCA) for *P. patula*, *P. tecunumanii* parents and Specific Combining Ability (SCA) of PPTL and PPTH hybrid families based on stem-kill percentage (Experimental mean = 2.09)

P. patula	**PPTL**										**PPTH**					
	TL1	**TL2**	**TL3**	**TL4**	**TL5**	**TL6**	**TL7**	**TL8**	**TL9**	**TL10**	**TH1**	**TH2**	**TH3**	**TH4**	**TH5**	**GCA**
P1	-5.62				0.66					-4.92		-3.21	-5.11	-7.77	-5.29	-4.51
P2	34.86	-19.37		4.63				-10.60			4.63	-3.61	4.06	8.54	3.73	3.88
P3	7.67		-5.42					-1.19			8.33	16.70	-1.54	-6.09		2.66
P4	6.79			-3.11				6.40			-1.64	-2.62	9.63	-14.73		0.10
P5	-20.02		-8.64						-5.11		-1.75	-1.29	9.33			-3.97
P6	-18.17							23.18		-1.67						0.48
P7	-4.01		0.43				3.04		15.02		6.21	-8.05	-5.45	-0.08		1.03
P8						4.60					7.85	-1.10		22.05		4.82
P9	-10.62			9.15					-17.39							-2.72
P10	-6.38			0.28		1.55										-0.66
P11				17.73		-9.86		-11.27	0.48		-0.22			6.68		-0.33
P12				-15.02	-4.95			-6.69			5.68	5.76	9.78			-0.78
GCA	-9.14	-11.42	-8.04	8.05	-2.53	-2.19	1.79	-0.10	-4.12	-3.88	17.14	1.52	8.77	5.08	-0.92	

The yellow shaded cells indicate the SCA values to a specific cross (e.g. = P1×TL1 -5.62) and the green cells indicates the GCA values of the parents (e.g.-9.14)

4.7.2 GCA and SCA for separated PPTH hybrid families

There was a highly significant difference between the GCA values of all five *P. tecunumanii* HE parents (Table 4.8). Results indicated that three *P. tecunumanii* HE male parents TH2 (-5.90), TH4 (-1.34) and TH5 (-4.54) had a lower negative GCA, signifying a lower level of tolerance to *F. circinatum.* A high positive GCA were only achieved by two families, TH1 (+9.55) and TH3 (+2.22), indicating a high level of tolerance to *F. circinatum.* TH2 (-5.90) was the only parent attaining a low and negative GCA, indicating a lower level of tolerance as male parent. However, TH1 (+9.55) had a high and positive value, indicating a high level and the least tolerant male parent. Furthermore, TH2 and TH4 had a positive GCA based on the combined dataset. However, the separated dataset results indicated negative values.

Five *P. patula* female parents P1 (-9.10), P4 (-3.20), P7 (-2.53), P5 (-1.81) and P11 (-1.18) indicated a lower negative GCA, while only four parents P8 (+8.15), P3 (+3.22), P2 (+3.28) and P12 (+3.17) had a high and positive GCA (Table 4.8). P1 (-9.10) had the lowest value, indicating the most tolerant female parent, while P8 (+8.15) was the least tolerant female parent indicating least tolerant female parent.

From the SCA estimates, results indicated that 19 hybrid families (P4 x TH4, P3 x TH4, P7 x TH2, P1 x TH4, P8 x TH2, P11 x TH3, P1 x TH5, P2 x TH2, P7 x TH3, P3 x TH3, P5 x TH1, P5 x TH2, P1 x TH3, P11 x TH1 etc.) had a low and negative SCA ranging between -0.01 and -8.11, while P4 x TH4 (-8.11) had the lowest negative value (Table 4.8). These cross combinations of negative values indicated a lower level of tolerance and can, therefore, be described as additive × additive gene interaction, since both parents obtained a low and negative SCA. About 14 hybrid families had a positive SCA ranging between +0.41 and +9.35 with P8 × TH4 (+9.35) having the highest positive value. The 14 positive cross combination based on a high SCA, indicated a high level of tolerance. Therefore, these hybrids can be regarded as poor combinations and described as dominance × dominance type of gene interactions.

Table 4.8: Estimated General Combining Ability (GCA) of *P. patula*, *P. tecunumanii* parents and Specific Combining Ability (SCA) of PPTH hybrid based on stem-kill percentage (Experimental mean = 2.11)

P. patula	**PPTH**					
	TH1	**TH2**	**TH3**	**TH4**	**TH5**	**GCA**
P1		-0.06	-2.16	-3.49	-2.95	-9.10
P2	1.43	-2.77	1.04	3.90	-0.48	3.28
P3	3.44	7.24	-2.39	-5.22		3.22
P4	-0.31	-0.35	5.73	-8.11		-3.20
P5	-2.38	-2.30	2.96			-1.81
P7	3.62	-3.53	-2.48	-0.01		-2.53
P8	1.66	-3.26		9.35		8.15
P11	-0.65		-3.03	2.56		-1.18
P12	0.41	0.59	2.01			3.17
GCA	9.55	-5.90	2.22	-1.34	-4.54	

The yellow shaded cells indicate the SCA values to a specific cross (e.g. P1×TH2= -0.06) and the green shaded cells indicate the GCA of parents (e.g. TH1 =9.55)

4.8 GCA and SCA for separated PPTL hybrid families

GCA values of the 10 *P. tecunumanii* LE male families ranged between -0.46 and +6.0 (Table 4.9). However, only six male parents TL1 (-3.12), TL2 (-2.39), TL3 (-2.06), TL9 (-0.88), TL10 (-0.87) and TL5 (-0.46) obtained a low and negative GCA, indicating a low level of tolerance. The four remaining male parents TL4 (+6.01), TL8 (+2.21), TL7 (+1.01) and TL6 (+0.55) obtained a high and positive GCA. The combined datasets indicated two more parental families, namely TL6 (-2.19) and TL9 (-4.12), had a low and negative GCA. TL1 (-3.12) obtained a very low GCA, indicating a high level of tolerance. However, the highest positive GCA was obtained by TL4 (+6.01), indicating a high level of tolerance.

Three female parents P1 (-3.47), P5 (-8.58) and P9 (-3.68) achieved a low GCA, indicating a low level of tolerance while nine female parents P2 (+5.34), P3 (+1.51), P4 (+3.23), P6 (+1.41), P7 (+4.04), P8 (+2.00), P10 (+2.27), P11 (+1.4) and P12 (3.43) had a high GCA, indicating a high level of tolerance (Table 4.8). P5 (-8.58) had the lowest and negative GCA, while P2 (+5.34) had the highest GCA.

However, SCA indicated 19 hybrids (P5 × TL1, P2 × TL2, P6 × TL1, P9 × TL9, P5 × TL3 etc.) had negative values ranging between -0.74 and -28.61, while 18 cross combinations had a high and positive SCA ranging between +0.10 and +46.13 (Table 4.8). The most tolerant cross was P5 × TL1 with a low negative GCA

(-28.61) indicating a low level of tolerance, while P2 × TL1 had the highest and most positive GCA (+46.15), indicating a high level of tolerance.

Table 4.9: Estimated General Combining Ability (GCA) of *P. patula*, *P. tecunumanii* parents and Specific Combining Ability (SCA) of PPTL hybrid based on stem-kill percentage (Experimental mean =1.80)

P. patula	**PPTL**										
	TL1	**TL2**	**TL3**	**TL4**	**TL5**	**TL6**	**TL7**	**TL8**	**TL9**	**TL10**	**GCA**
P1	-11.98				-0.74					-8.59	-3.47
P2	46.13	-23.27		16.25				-6.35			5.34
P3	10.58		-4.90					3.61			1.51
P4	7.20			2.46				10.17			3.23
P5	-28.61		-15.23						-8.77		-8.58
P6	-22.39							30.92		0.11	1.41
P7	-5.79		0.10				9.83		20.62		4.04
P8						12.29					2
P9	-16.38			15.71					-21.91		-3.68
P10	-9.15			7.08		3.46					2.27
P11				28.35		-10.41		-10.90	1.54		1.4
P12				-11.36	-3.74			-5.92			3.43
GCA	-3.12	-2.39	-2.06	6.01	-0.46	0.55	1.01	2.21	-0.88	-0.87	

The orange shaded cells indicate the SCA values to a specific cross (e.g. P1×TL1= -11.98) and the green shaded cells indicate the GCA of parents (e.g. P12 = 3.43)

4.9 The top 20 SCA hybrid families

The ranking of the top 20 hybrid families (Table 4.10) was drawn from the SCA combining abilities of Table 4.7. Negative SCA estimates ranged between -5.42 to -20.02, indicating a low level of tolerance. P5 × TL1 was ranked as the top performer for both combined and separated analyses, indicating a high tolerance to *F. circinatum*. These cross combinations are important in a breeding program since breeders can select from these families to breed hybrids with possible *F. circinatum* tolerance.

Table 4.10: SCA values from combined families of PPTL and PPTH indicates ranked top 20 most tolerant families

Rank	Most tolerant	
	Hybrid	**SCA effects**
1	P5 × TL1	-20.02
2	P2 × TL2	-19.37
3	P6 × TL1	-18.17
4	P9 × TL9	-17.39
5	P12 × TL4	-15.02
6	P4 × TH4	-14.73
7	P11 × TL8	-11.27
8	P9 × TL1	-10.62
9	P2 × TL8	-10.60
10	P11 × TL6	-9.86
11	P5 × TL3	-8.64
12	P7 × TH2	-8.05
13	P1 × TH4	-7.77
14	P12 × TL8	-6.69
15	P10 × TL1	-6.38
16	P3 × TH4	-6.09
17	P11 × TH3	-5.83
18	P1 × TL1	-5.62
19	P7 × TH3	-5.45
20	P3 × TL3	-5.42

4.10 Least square means ranking (LSMeans)

The LSMeans for hybrid families (PPTH and PPTL), and parent species (*P. patula*, *P. tecunumanii* LE, *P. tecunumanii* HE and *P. taeda)* were ranked from least to most tolerant families based on stem-kill percentage (Figures 4.2 and 4.3; Appendix E). There were significance differences ($p < 0.0001$) between hybrids and parental families in terms of level of tolerance to *F. circinatum*. Female parent P2 was the lowest ranking family with the highest mean (3.75%), indicating the least tolerant family to *F. circinatum*. The top-ranking hybrid obtaining a lower mean value was P2 × TL2 (1.29%), indicating a high level of tolerance to *F. circinatum* (Figure 4.2 and Table 4.11). Due to the high number of families, Figure 4.2 gets distorted when printed on A4 paper size. The hybrid P2 × TL2 (1.29%) was ranked second. In terms of hybrids alone, most

PPTL hybrid families was more tolerant to *F. circinatum* with values ranging between 1.06% and 2.78%, while PPTH hybrid families was the least tolerant ranging between 1.58% and 3.09%. Hybrid family of *P. patula* × *P. tecunumanii* LE (1.98%) obtained the lowest mean values followed by *P. tecunumanii* as male parent (1.89%). In general, *P. tecunumanii* LE was the most tolerant parent population, while *P. patula* was the least tolerant female parent with a mean of 3.07%.

Table 4.11: LSMeans stem-kill percentage per family number indicating significant differences and ranked for high to low

Family	**Significant interval**	**Groups**
P2	3.778889	a
P7	3.509063	a
TH1	2.996458	ab
P2xTL1	2.760909	abc
P8xTH4	2.658636	abcd
TH4	2.562264	abcde
P8xTH1	2.536250	abcdef
P11xTL4	2.496170	abcdefg
P6xTL8	2.493023	abcdefg
P3xTH1	2.490870	abcdefg
TH3	2.476852	bcdefg
TH5	2.470566	bcdefg
P7xTH1	2.444773	bcdefgh
P2xTH1	2.431471	bcdefghi
P3xTH2	2.424737	bcdefghi
P12xTH1	2.403871	bcdefghij
TH2	2.391607	bcdefghij
P.taeda	2.344350	cdefghij
P4xTH3	2.326304	cdefghij
P12xTH3	2.298654	cdefghij
P2xTH3	2.297273	cdefghij
P2xTH4	2.278235	cdefghij
P2xTL4	2.263171	cdefghijk
P5xTH3	2.251957	cdefghijk
P9xTL4	2.240732	cdefghijk
P11xTH1	2.234375	cdefghijk
P4xTH1	2.218958	cdefghijk

Family	Significant interval	Groups
P7xTL9	2.212692	cdefghijk
P11xTH4	2.158913	cdefghijkl
P5xTH1	2.124419	cdefghijklm
P3xTH3	2.121538	cdefghijklmn
TL7	2.098269	cdefghijklmn
P8xTL6	2.069800	cdefghijklmn
P10xTL4	2.067500	cdefghijklmn
P12xTH2	2.061273	cdefghijklmn
P2xTH5	2.055192	cdefghijklmn
P4xTL8	2.054348	cdefghijklmn
P8xTH2	2.031364	cdefghijklmno
P7xTL7	2.021915	cdefghijklmno
P7xTH4	2.020741	cdefghijklmno
P4xTL4	1.995000	cdefghijklmnop
P3xTL1	1.989200	cdefghijklmnop
P7xTH3	1.970769	cdefghijklmnop
P3xTL8	1.929362	defghijklmnop
P3xTH4	1.922979	defghijklmnop
P2xTH2	1.918636	defghijklmnop
P10xTL6	1.905179	efghijklmnop
P4xTL1	1.897660	efghijklmnop
TL1	1.895091	efghijklmnop
P4xTH2	1.886410	efghijklmnop
P11xTH3	1.874737	efghijklmnop
P1xTH3	1.871064	efghijklmnop
P11xTL9	1.849787	efghijklmnop
P5xTH2	1.837021	fghijklmnop
P6xTL10	1.808085	ghijklmnop
P7xTH2	1.802105	ghijklmnop
TL4	1.799623	ghijklmnop
P1xTH2	1.795769	ghijklmnop
P1xTL5	1.789444	ghijklmnop
P7xTL3	1.770638	ghijklmnop
P1xTH4	1.744815	hijklmnop
P12xTL5	1.737593	hijklmnop
P2xTL8	1.732075	hijklmnop

Family	Significant interval	Groups
P12xTL8	1.723333	hijklmnop
P12xTL4	1.683929	ijklmnop
P1xTH5	1.682500	ijklmnop
P3xTL3	1.678889	ijklmnop
TL9	1.665893	ijklmnop
P4xTH4	1.650638	ijklmnop
P7xTL1	1.636571	ijklmnop
P1xTL10	1.634054	ijklmnop
P11xTL8	1.628163	ijklmnop
P11xTL6	1.618000	jklmnop
P5xTL9	1.616471	jklmnop
P10xTL1	1.568043	jklmnop
P1xTL1	1.496136	klmnop
P5xTL3	1.462353	lmnop
P9xTL1	1.432182	mnop
P9xTL9	1.354400	nop
P6xTL1	1.300612	op
P2xTL2	1.299388	op
P5xTL1	1.185854	p

4.11 Correlation between *Fusarium circinatum* and frost tolerance in PPTL and PPTH

A previous study by Malinga (2018) indicated possible PPTL and PPTH families with frost tolerance (Appendix F). As this study used the same rooted cutting material to determine *F. circinatum* tolerance, a Pearson correlation (r) was run to determine whether any correlation between frost and *F. circinatum* tolerance does exist. The genetic material tested were PPTH, PPTL and four parental families (*P. patula*, *P. tecunumanii* LE, *P. tecunumanii* HE and *P. taeda*). Malinga (2018) indicated that best results for frost screening were obtained at -6 °C. Therefore, the r is only based on the -6 °C data and values obtained at -3 °C and -9 °C were omitted from this exercise.

A lower (negative) mean value for *F. circinatum* indicated that a specific family or parent is more tolerant or less tolerant than other families or parents with a higher (positive) mean value. In frost study, the low mean values also indicated that a specific family or parent is more tolerant or less tolerant than other families or parents with a higher (positive) mean value. Therefore, r were calculated to determine the relationship between *F. circinatum* (experiment 1) and frost (Malinga, 2018). A negative correlation of -0.39 with a high significant difference ($p < 0.05$; Figure 4.4) was obtained. The negative correlation confirmed that it is unlikely to breed

the same hybrid families for both *F. circinatum* and frost, because there might be different genes involved. For example, P3 × TH1, P3 × TH2, P3 × TH3, P4 × TH3, P4 × TL8 and P7 × TH3 was not tolerant to both frost and *F. circinatum.* Therefore, breeders cannot use these cross combinations to cater for both *F. circinatum* and frost. Cross combinations of P2 × TL8, P4 × TH2, P3 × TH4 and P2 × TH5 was not tolerant to frost, however, they were tolerant to *F. circinatum.* Subsequently, these cross combinations can only be deployed for *F. circinatum.* Four cross combinations found to be intermediate between *F. circinatum* and frost, which included: P2 × TH2, P3 × TL1, P7 × TL7 and P7 × TL9. However, P2 × TL2 and P3 × TL3 were found to be tolerant to both frost and *F. circinatum,* thus indicating a positive correlation.

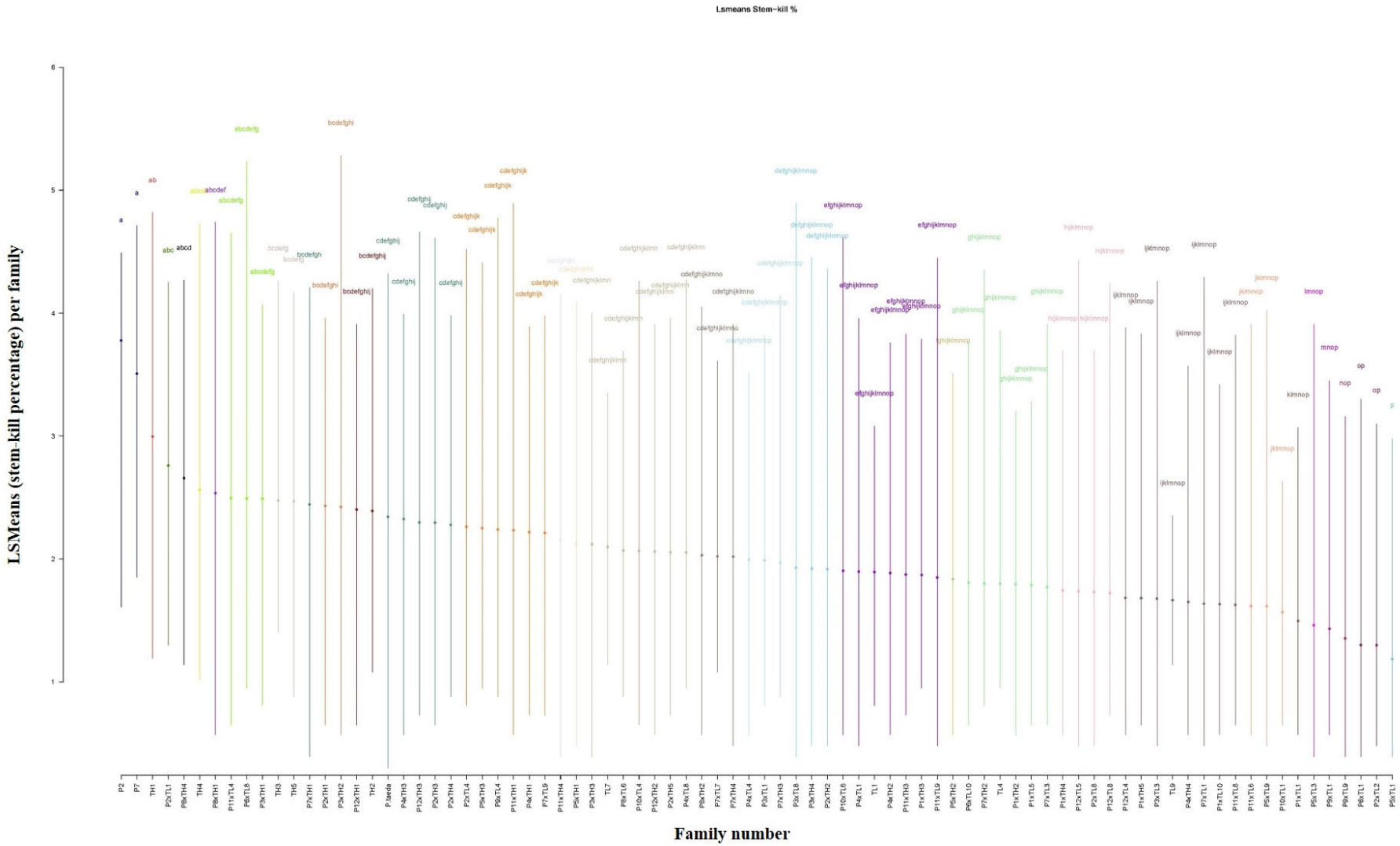

Figure 4.2: *Fusarium circinatum* LSMeans rankings for stem-kill percentage from high to low of *P. patula* × *P. tecunumanii* (HE and LE), *P. patula*, *P. tecunumanii* (HE and LE) and *P. taeda* (families with same letters are not significantly different at P < 0.05) (Table 4.11 for clarity)

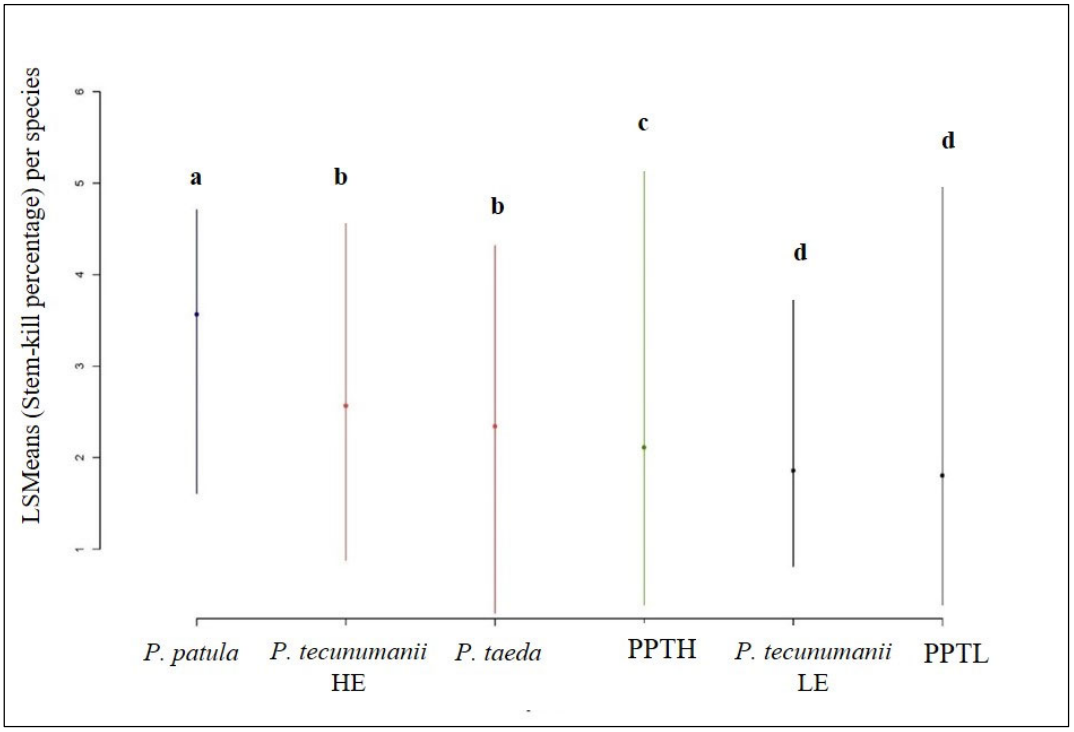

Figure 4.3: LSMeans summarised by hybrid treatment (PPTH and PPTL) and parental controls ranked from most to least *F. circinatum* tolerance based on stem-kill percentage

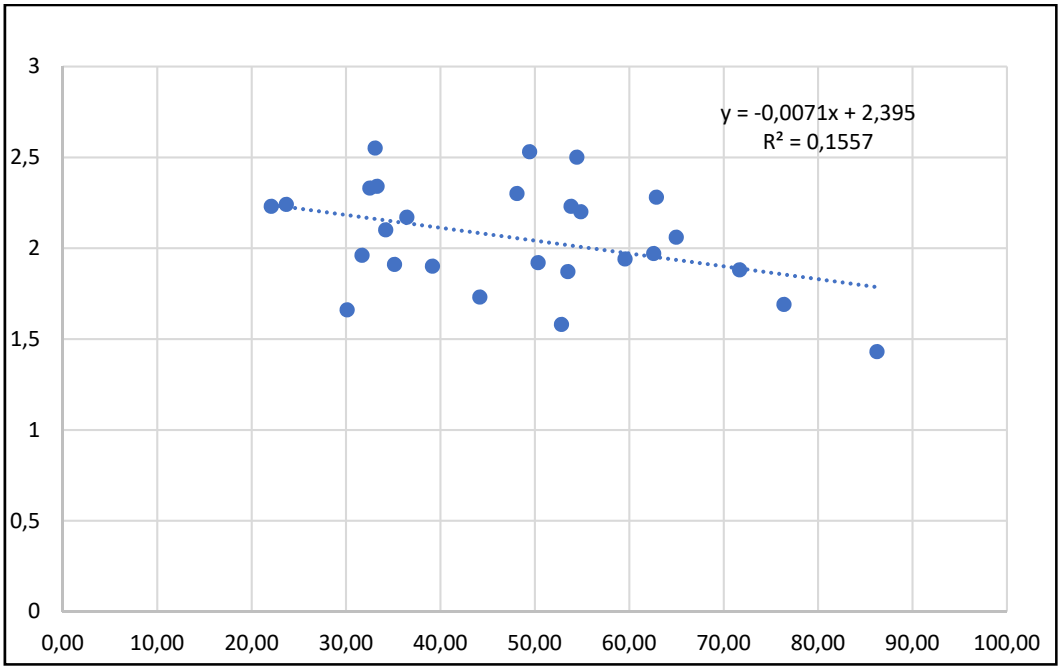

Figure 4.4: Correlation between *F. circinatum* experiment 1 (based on LSMeans for stem-kill percentage) and frost tolerance at -6 ˚C temperature (based on the LSMeans injury percentage) for PPTL and PPTH indicating the level of tolerance between the tested hybrid families

Chapter 5
Discussion

5.1 Introduction

Fusarium circinatum can have a negative impact on some *Pinus* species. It is important to manage and control this disease by selecting tolerant parents for breeding purposes. In general, breeding in the forestry industry is important for producing species with good stem form, wood quality, high yield and tolerance (frost, drought, pest and diseases). Therefore, pre-screening of seedlings and cuttings in the nursery were conducted to identify the level of *F. circinatum* tolerance and genetic heritability amongst hybrid families and pure parental species. This will enable the breeder to identify specific hybrid families that are tolerant to *F. circinatum* for further commercial deployment or breeding purposes.

Stem-kill percentage was very low, indicating that the hybrid material being tested had uniformly high tolerance to the disease, and this resulted in the distribution of the data being skewed to the right (Figure 4.1 B). To stabilise variances and normalise proportional datasets, Arcsine data transformation was employed as there was not enough evidence to state that the data was normally distributed. Lesion length had a positive influence on stem-kill percentage with an *r*-fit of 93.6%. Previous *F. circinatum* tolerance studies (Mitchell *et al.,* 2013) also indicated a smaller lesion length with a positive *r*-fit (92%) between lesion length and stem-kill percentage. Despite the small lesion lengths observed within the mean values of the different treatments, the ANOVA indicated significant differences in the families and species (Table 4.5). Therefore, families and species differed in their *F. circinatum* tolerance.

5.2 Plant material and experiment design

Environmental conditions such as an interaction between temperature, humidity, temperature and rainfall are crucial during spore release and infection of pine plants by *F. circinatum* (Sakamoto and Gordon, 2006; Garbelotto *et al.,* 2008). Nursery inoculation screening for *F. circinatum* was conducted through two experiments. Experiment 1 was performed at Stellenbosch University in a commercial grow tunnel with a polycarbonate roof, brick walls, irrigation and temperature control (between 25 and 27 °C). The second experiment was performed at Shaw Research Centre (Pietermaritzburg) in an open structure with a plastic covered roof, manual irrigation and no temperature control (average of 12 °C). Lesion length of experiment 2 was affected by the low average temperature and resulted in unreliable data. Therefore, only the results of experiment 1 was analysed and reported.

Previous studies by Mitchell *et al.* (2011, 2012, 2013 and 2014) and Nel *et al.* (2014) were done in enclosed structures (roof and walls) with temperature control (between 24 and 25 °C) and compared well to that of experiment 1. Nel *et al*. (2014) and Mitchell *et al*. (2011 and 2012) screened *P. patula* and *P. tecunumanii* (LE and HE), while Mitchell *et al*. (2013 and 2014) screened PPTL, PPTH and *P. patula.* Experiment 1 included all these species and hybrids with the same inoculum (FCC 3577, FCC 3578 and FCC 3579) for comparison between these studies.

Lesion length, in this study, was already visible after one week of inoculation and correlated with Mitchell *et al.* (2012). Although there were some differences between the lesion length of this study and previous studies, it has been reported that species and hybrids reacts differently to *F. circinatum* inoculum. The more susceptible the species or hybrid is, the longer the lesion length, but plant length might have an impact as well (Hodge and Dvorak 2000). Stem-kill percentage does correct for the difference in plant length, as it is the proportion of the total plant that has formed a lesion.

Reported variables measured (plant length, lesion length and stem-kill percentage) in this study were based on untransformed data to give a better reflection of the dataset. For this study, *P. patula* had a mean lesion length of 13.44 mm and mean plant length of 54.15 mm, which was found intermediate compared to previous studies. *Pinus patula* had a lesion length of 22.0 mm and 136.77 mm plant length (Mitchell *et al*., 2011), 30.5 mm lesion length and 86.0 mm plant length (Mitchell, 2012), 29.65 mm lesion length and 118.30 mm plant length (Mitchell *et al*., 2013), 24.6 mm lesion length and 124.5 mm plant length (Mitchell *et al*., 2014), whereas (Nel *et al*., 2014) had a 31.0 mm lesion length and 11.54 mm plant length. The differences in lesion length and plant length could be due to variation in total number of *P. patula* families tested; for instance, this study tested two families of *P. patula* as controls, whereas Mitchell *et al.* (2014) tested 63 families of *P. patula* and Nel *et al.* (2014) tested 14 families of *P. patula*.

Pinus tecunumanii LE, in this study, had a lesion length of 3.29 mm and plant length of 97.36 mm, which was also found intermediate compared to previous studies. Lesion length of 2.78 mm and plant length of 147.68 mm for *P. tecunumanii* LE was reported by Mitchell *et al*. (2011). However, Mitchell *et al*. (2013) reported a lesion length of 5.07 mm and plant length of 150.8 mm. As for *P. tecunumanii* HE, this study indicated 6.29 mm lesion length and 73.72 mm plant length, which was lower compared to previous studies. Mitchell *et al.* (2011) reported a lesion length of 9.61 mm and plant length of 124.24 mm, nevertheless, Mitchell *et al*. (2013) reported 9.16 mm lesion length and 177.9 mm plant length for *P. tecunumanii* HE. In terms of hybrid families, PPTL in this study had a lesion length of 5.7 mm and plant length of 132.45 mm. Previous studies indicated 7.4 mm lesion length and 1 775.5 mm plant length by Mitchell *et al*. (2013), which was a greater than in this study. PPTH lesion length in this study was 7.86 mm and plant length 129.41 mm, compared to a lesion length of 16.1 mm and plant length of 155.9 mm reported by Mitchell *et al*. (2013).

The differences observed in plant length and lesion length, might be attributed to different genetic material (clones) used in the different studies. Even though same species and hybrid where compared, there are still differences in terms of the genetic make-up of different individual varieties. For example, different individual varieties sown and raised by different forestry companies in a different environment. A total number of families tested per variety per study could also influence the measured variables (plant length and lesion length) because of differences in population size. The altitude of different areas where seedlings were propagated could also contribute to the differences observed.

Stem-kill percentage was calculated as lesion length divided by plant length. Therefore, lesion length influenced stem-kill percentage in the sense that the longer the lesion development, the more it is prone to *F. circinatum.* A weak negative relationship (r = -23.3%) was observed between stem-kill percentage and plant length. This indicated that plant length does not have an effect on the tolerance of *F. circinatum* and correlated with findings of Mitchell *et.al*. (2012 and 2013) as -63.0% and -21.7% respectively. Therefore, genetic analyses concentrated only on stem-kill percentage. Furthermore, this study indicated a large variation of stem-kill percentage at species level.

The stem-kill percentage of *P. tecunumanii* LE (3.55%) were slightly higher than that of Mitchell *et al*. (2011) at 2.37%, but lower than that of Mitchell *et al*. (2013) at 3.91%. *Pinus tecunumanii* HE stem-kill percentage for this study was 9.88%, more or less the same as 9.61% reported by Mitchell *et al*. (2011) and slightly higher than that of Mitchell *et al*. (2013) at 7.1%. For *P. patula* (this study), stem-kill percentage was 24.38% lower than that of Nel *et al.* (2014) at 30.0% but correlated with that of Mitchell *et al*. (2013) at 23.38%. Even though the stem-kill percentage of *P. patula* in this study found to be lower compared to Nel *et al*. (2014), *P. patula* was still non-tolerant to *F. circinatum* because the obtained value was very high compared to other species in that specific study. The hybrids of PPTH had stem-kill percentage of 6.7% lower than that of Mitchell *et al*. (2013) at 12.1%. However, PPTL (this study) was 4.69%, which correlated with the 5.0% obtained by Mitchell *et al*. (2013), but was slightly lower than the 7.6% reported by Mitchell *et al*. (2014). In general, the lower values of stem-kill percentage indicated the high level of *F. circinatum* tolerance of specific species or hybrid tested.

5.3 Heritability and genetic parameters

Heritability (h^2) is a useful statistical tool to measure the level of genetic improvement, which can be expected in a certain trait of interest within the experimental population (Verryn, 2019). Both h^2 and genetic variance components of a trait measure how strong the observed variation of a trait is influenced by the genetic and environmental components in a population (Falconer *et al.,* 1996). Therefore, results can assist tree breeders to develop effective breeding strategies based on selective traits that indicate strong and positive genetic influences.

Various levels of *F. circinatum* tolerance were observed in the combined 32 PPTH and 37 PPTL hybrid families. The combined hybrid family level (h^2_{family}) estimates were stronger (ranging between 0.21 and 0.78) than that of separated hybrid families ranging between 0.07 and.0.54. This indicated a weaker level of *F. circinatum* tolerance compared to the combined families. These results correlated well with an average h^2_{family} estimate of 0.81 reported by Nel *et al.* (2014).

A genetic variation was also observed in male and female parents (Table 4.6) analysed as separate parents. The level of genetic control on separated male parents (*P. tecunumanii* LE and HE) was weak to strong with h^2_{male} ranging between 0.0 and 0.18. This indicated that male parents had a positive contribution towards *F. circinatum* tolerance. The closer the h^2_{male} is to 0.18 the better the *F. circinatum* tolerance. In comparison with the female parent (*P. patula*) results indicated that there was weak to moderate h^2_{female} ranging between 0.05 and 0.10. The closer the h^2_{female} is to 0.10 the better the *F. circinatum* tolerance. A weak h^2_{female} might be caused by errors occurring in the estimation of variances with the mixed models and small number of the *P. patula* population tested. These weak h^2_{female} estimates in *P. patula* was also reported by Mitchell *et al.* (2012) at 0.06. In general, the strong h^2_{male} (*P. tecunumanii* LE and HE) contributes more to *F. circinatum* tolerance than the female parent (*P. patula*).

5.4 Genetic Variance Components *(V)*

The genetic variance components indicated that the male parents (*P. tecunumanii* LE and HE) were additive (V_{male} varied between 0.0 and 92.71) compared to the V_{female} (*P. patula* varied between 0.39 and 39.29) as non-additive. Six of the 12 *P. patula* families had negative effects, resulting in non-additive components (Table 4.7). A low genetic variation in *P. patula* families was also reported by Mitchell *et al*. (2013). Furthermore, eight *P. tecunumanii* LE and one *P. tecunumanii* HE male parents were additive. This confirms that *P. tecunumanii* LE performed better than *P. tecunumanii* HE in terms of *F. circinatum* tolerance.

5.5 General Combining Ability and Specific Combining Ability (GCA and SCA)

The correlation between GCA and SCA indicates the probability whether parental species will produce offspring with *F. circinatum* tolerance (Retief and Stranger, 2009). Interpretation of the combining ability effects and variance are influenced by the particular mating design used, assumptions regarding the experimental material and the conditions implemented on the combining ability effects (Arashida *et al*., 2017). In this study, positive and negative GCA and SCA values were obtained. Negative values of GCA and SCA are of interest for genetic improvement since a lower stem-kill percentage indicated a higher level of *F. circinatum* tolerance as compared to the experiment mean. A large family variation was observed among the

male and female families tested. This is in agreement with the study by Nel *et al.* (2014) as the GCA for a group of *P. patula* families were clearly tolerant to *F. circinatum* (GCA -6.5) and another group performed below average (GCA +3.8). In generally, *P. tecunumanii* LE had a higher negative GCA value than *P. tecunumanii* HE. This was also evident with SCA as most PPTL hybrid families had a negative SCA compared to the PPTH hybrid families. This explains that the contribution of *P. tecunumanii* LE towards *F. circinatum* tolerance is stronger than that of *P. tecunumanii* HE. Previous studies did not use the same genetic material and comparisons are thus irrelevant.

Results from this study indicated significant differences between the ranking of PPTH and PPTL hybrid families. Significance difference observed might be because some of the known to be tolerant hybrid families indicated susceptibility to *F. circinatum* in this study. Previous studies indicated that PPTL is a possible hybrid to replace *P. patula* on commercial scale (Kanzler *et al*., 2014). However, results indicated some PPTL hybrid families was susceptible to *F. circinatum.* For example P2 × TL4, P4 × TL4, P4 × TL8 and P7 × TL3. This might be because of genetic components of the female parents that could have been dominant to the male parents. Nonetheless, some of the PPTH hybrid families indicated low to intermediate *F. circinatum* tolerance (P2 × TH2, P2 × TH5, P3 × TH4, P4 × TH2 and P4 × TH4), while other families were susceptible (P4 × TH1, P4 × TH3, P7 × TH3 etc.). This correlates with results obtained by Mitchell *et al*. (2013).

In order to increase the probability of *F. circinatum* tolerant hybrids, breeding strategies resulting in non-additive genetic effects can be employed (Retief and Stranger 2009). This indicates that a tolerant parent does not necessarily produce a tolerant hybrid and *vice versa*. However, a cross combination between parents that produced a tolerant hybrid could be repeated to produce offspring with a higher level of *F. circinatum* tolerance, known as epistasis interaction. Epistasis is basically the interaction between genes that influences a phenotype (Forsberg and Carlborg, 2017) in a way that genes can either mask each other and be considered as dominant or they can combine to produce a new trait (Priyadarshan, 2019). This interactions can be additive × non additive, additive × additive and non additive × non additive (Gao *et al.* 2013).

Different GCA's (Table 4.7) can be obtained with crossing additive and non-additive parents. For example:

- P4 (non-tolerant female) crossed with TH1 (non-tolerant male) produced a tolerant hybrid (P4 × TH1);
- P1 (tolerant female) crossed with TH4 (non-tolerant male) produced a tolerant hybrid (P1 × TH4);
- P1 (tolerant female) crossed with TL1 (tolerant male) produced a tolerant hybrid (P1 × TL1);
- P6 (non-tolerant female) crossed with TL10 (tolerant male) produced a tolerant hybrids (P6 × TL10);
- P7 (non-tolerant female) crossed with TL3 (tolerant male) produced a non-tolerant hybrid (P7 × TL3), and
- P2 (non-tolerant female) crossed with TH1 (non-tolerant male) produced a non-tolerant hybrid (P2 × TH4).

All three types of epistatic interaction components were present in the above combining abilities. This indicated that improvement of PPTH and PPTL hybrid families for *F. circinatum* tolerance might be possible

through identification of superior families. Continuous screening of next-generation hybrids is important because the hybrid treatments known not to be tolerant to *F. circinatum* could be tolerant in later generations.

5.6 GCA and SCA for combined hybrid families of PPTH and PPTL

Hybrid and parental families react differently to *F. circinatum* infestation as indicated in Tables 4.7, 4.8 and 4.9. Parental families with a positive GCA indicated less tolerance to *F. circinatum,* while a negative GCA indicated a high level of tolerance. These results are consistent with findings of Nel *et al.* (2014). They indicated that a group of families was more tolerant (i.e. negative predicted GCA values with a lower stem-kill percentage) than another group of families that were average or below average in tolerance to *F. circinatum* (positive GCA with a higher stem-kill percentage).

An inconsistence ranking of families was observed for combined hybrid families and separated families. Families were separated to prevent the assumption that *P. tecunumanii* HE (lower tolerance as a pure species) might mask *P. tecunumanii* LE (higher tolerance as a pure species) effects when hybrid families are combined. Results indicated the reverse as eight combined hybrid families of *P. tecunumanii* LE indicated high level of tolerance, opposed to separated analysis *P. tecunumanii* LE indicating six families with a high level of tolerance. No previous study focussing on these specific hybrids could be found in literature to compare results with.

The tendency of some parental families not being able to produce hybrids tolerant to *F. circinatum* is due to heritability. Genetic diversity and capacity are important for inbreeding of *F. circinatum* resistance (Morris, 2010). In this study, the contribution of *F. circinatum* tolerance by the female parent (*P. patula*) was indicated as negative, reducing the tolerance probability of hybrids. This might be attributed to that *P. patula* is highly susceptible to *F. circinatum* (Hodge and Dvorak, 2007). For instance (Table 4.7):

- P7 × TL3: P7 had a GCA of 1.15 compared to -8.12 for TL3, resulting in a non-tolerant hybrid with a SCA of 0.32; while
- P2 × TH1: P2 had a GCA of 3.88 compared to 17.06 for TH1, resulting in a non-tolerant hybrid with a SCA of 4.65.

5.7 GCA and SCA for separated families of PPTH and PPTL

PPTH hybrid families as male parent (Table 4.7) indicated that TH2 had the highest level of tolerance with a GCA of-5.82. PPTL hybrid families (Table 4.9) indicated that TL1, as male parent, had the highest level of tolerance with a GCA of -3.12. TL1 was also the second best male parent with the combined analyses (GCA

of -9.14). This is consistent with Mitchell *et al.* (2012) that *P. tecunumanii* LE had the highest level of *F. circinatum* tolerance.

In general, *P. patula* (female parent) were susceptible to *F. circinatum.* However, P1 with a GCA of -8.97 and P5 with a GCA of -7.69 seemed to be tolerant. This was consistent with Hodge and Dvorak (2007) indicating that in general, *P. patula* has a high level of susceptibility to *F. circinatum*. Mitchell *et al*. (2014) also reported the tendency of some *P. patula* families to have *F. circinatum* tolerance. Ford *et al*. (2014) suggested that *P. patula* families that showed a high level of *F. circinatum* tolerance could be deployed as rooted cuttings.

5.8 Least square means ranking (LSMeans)

LSMeans results indicated that a higher number of families of PPTL was tolerant to *F. circinatum* than PPTH families. In particular, P2 × TL2. Previous studies also reported difference of tolerance at family level (Mitchell *et al*. 2011, 2012; Hodge and Dvorak 2000, 2007). *Pinus tecunumanii* LE indicated a higher level of tolerance than the female parent *P. patula* as indicated in previous studies (Ford *et. al*., 2014; Nel *et al*., 2014; Mitchell *et. al*., 2012, 2013; Steenkamp *et al.,* 2012; Hodge and Dvorak, 2007). Ford *et al.* (2014) also reported that the high survival rate of hedges of PPTL indicates a consistent tolerance to *F. circinatum*.

5.9 Correlation between *Fusarium circinatum* and frost tolerance in PPTL and PPTH

A novelty of this study was the attempted correlation between *F. circinatum* and frost tolerances of the same genetic material (see Chapter 4, section 4.9). Unfortunately, a low, negative correlation (r = - 0.39) was observed between frost and *F. circinatum* tolerance. This indicated that there might be different gene interaction between hybrid families for *F. circinatum* and frost tolerance. Nevertheless, gene reaction proportion in the hybrid family quantifies for the intermediate tolerance of the hybrid to both frost and *F. circinatum*. This means that breeders need to identify and select parents that are both frost and *F. circinatum* tolerant. The combination or interaction between frost and *F. circinatum* tolerant parents should, therefore, result in an intermediate hybrid tolerant to both frost and *F. circinatum*. For example, a combination between a frost tolerant *P. patula* and *P. tecunumanii* LE families might result in an intermediate PPTL hybrid tolerant to both frost and *F. circinatum.* The *P. patula* families that indicated to be *F. circinatum* tolerant might be a good possibility as both female and male parent as *P. patula* is more prone to frost tolerance than *P. tecunumanii* (HE and LE) (Malinga, 2018). Possible hybrid combinations from this study and Malinga (2018) to be considered are: P2 × TH2; P3 × TL1; P7 × TL7; P7 × TL9; P2 × TL2; and P3 × TL3.

Chapter 6
Conclusion and recommendations

The purpose of this study was to determine the level of genetic control of *F. circinatum* tolerance in PPTL and PPTH interspecific hybrid families. Limited evidence from literature indicated that the PPTH hybrid has lower *F. circinatum* tolerance compared to PPTL. Therefore, this study offered the first opportunity to screen many different PPTH and PPTH hybrid families for *F. circinatum* tolerance. Greenhouse inoculation screening methods were used in the study to determine *F. circinatum* tolerance of hybrid families in order to assist breeders to select tolerant hybrid parents and families for future cross combinations. Various statistical parameters, such as heritability (h^2), general combining ability (GCA) and specific combining ability (SCA) were calculated to better understand the genetic control of *F. circinatum* tolerance. These results will improve the understanding of the level of *F. circinatum* tolerance present in the two hybrid groups (PPTH and PPTL) investigated and how parents can be utilised to impart *F. circinatum* tolerance in future hybrid progeny.

Results indicated that the level of *F. circinatum* tolerance of the PPTL hybrid was superior to that of the PPTH hybrid. This study also confirmed that *P. tecunumanii* LE as male parent imparted more *F. circinatum* tolerance to hybrid families compared to *P. tecunumanii* HE male parents. *Pinus patula* as female parent, known to have little *F. circinatum* tolerance, imparted low levels of *F. circinatum* tolerance to hybrid families. There was some evidence that specific families of the PPTH hybrid could be produced containing higher levels of tolerance than the mean for the PPTH group. This could allow for the combination of cold and *F. circinatum* tolerance in single specific PPTH hybrid families.

Incorporating the results from this study with current breeding strategies will increase the knowledge of the hybrid parent species and specific hybrid families used in this study. These results can be used to identify parents with good GCA and employed in future mating designs to produce controlled cross seed for commercial hedges.

Results from this study indicated that the gene action for the PPTH hybrid is more non-additive and can be used to identify specific crosses with higher *F. circinatum* tolerance. For the PPTL hybrid, the results indicated that GCA ability could be used to identify parents for future controlled crosses. Thirty-nine (PPTL and PPTH) hybrid families were identified with higher levels of *F. circinatum* tolerance than *P. patula* and can thus be considered for commercial deployment.

Overall, there was evidence from this study that *F. circinatum* tolerance of PPTH and PPTL hybrid families are under genetic control. Therefore, *F. circinatum* tolerance can be further improved by selecting specific

parents and families that are more tolerant to *F. circinatum*. From this study, the following recommendations are proposed:

- Breeders should consider crossing *P. patula* with *P. tecunumanii* LE to develop *F. circinatum* resistant commercial hybrids. However, this hybrid (PPTL) could be vulnerable to frost and should be restricted to the warm temperate zone of South Africa.
- Based on the GCA and SCA values, *P. patula* as female parent (P1) could also be used to improve *F. circinatum* tolerance if crossed with a high tolerant *P. tecunumanii* male parent.
- Five *P. patula* female parents (P5, P9, P10, P11 and P12) need to be re-tested to confirm results and possible *F. circinatum* tolerance. If the results persist, these parents could also be crossed with a tolerant *P. tecunumanii* (LE or HE) male parent.
- Specific cross combinations or families that had a high level of *F. circinatum* tolerance could be propagated as cuttings and tested for commercial deployment. Previous studies confirmed this statement as a possibility.
- As the GCA indicated some *P. patula* female parents displayed *F. circinatum* tolerance, intraspecific hybrid crossing could also be an option. This can also be tested for *P. tecunumanii* LE male parents that had a high level of *F. circinatum* tolerance based on GCA.
- To produce interspecific hybrids with both frost and *F. circinatum* tolerance, a three-way cross between families with intermediate (P2 × TH2, P3 × TL1, P7 × TL7 and P7 × TL9) and a high level (P2 × TL2 and P3 × TL3) of tolerance are suggested.

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Appendices

Appendix A

Mean values for variables measured (R output)

```
exp2 untrans

> numSummary(Exp2Untrans[,c("Lesion.length", "Plant.length", "Stem.kill"),
+   drop=FALSE], groups=Exp2Untrans$Species, statistics=c("mean", "quantiles"),
+   quantiles=c(0,.25,.5,.75,1))

Variable: Lesion.length
                                    mean 0% 25% 50% 75% 100%    n NA
                                     NaN NA  NA  NA  NA   NA    0  1
P. patula x P. tecunumanii HE 12.299851  1   2   4  15  100 1344  0
P. patula x P. tecunumanii LE  8.537921  1   2   3   9   98 1424  0
P. tecunumani HE              13.086957  1   3   6  17   60   23  0
P. tecunumani LE               8.125000  2   3   6  10   32   40  0
P.patula                      20.130435  1   2   6  22   90   23  0

Variable: Plant.length
                                   mean      0%      25%      50%      75%      100%
                                218.6990 218.699 218.699 218.699 218.699  218.699
P. patula x P. tecunumanii HE 206.9457  30.000 164.000 201.000 248.250  936.000
P. patula x P. tecunumanii LE 226.1067  53.000 178.000 220.000 270.000 2018.000
P. tecunumani HE              269.2174 100.000 180.000 260.000 340.000  424.000
P. tecunumani LE              347.4250 100.000 247.500 350.000 470.000  555.000
P.patula                      172.4783  90.000 120.500 148.000 210.000  332.000
                                 n NA
                                 1  0
P. patula x P. tecunumanii HE 1344  0
P. patula x P. tecunumanii LE 1424  0
P. tecunumani HE                23  0
P. tecunumani LE                40  0
P.patula                        23  0

Variable: Stem.kill
                                    mean   0%   25%  50%     75%  100%    n NA
                                     NaN   NA    NA   NA      NA    NA    0  1
P. patula x P. tecunumanii HE  6.604591 0.26 1.110 2.08  7.6900 66.67 1344  0
P. patula x P. tecunumanii LE  4.423455 0.06 0.940 1.58  4.2525 59.38 1424  0
P. tecunumani HE               4.180000 0.40 1.840 3.00  5.0450 18.18   23  0
P. tecunumani LE               2.910750 0.62 1.125 1.63  2.8075 14.29   40  0
P.patula                      13.552609 0.68 1.025 2.65 17.3900 74.38   23  0
########################################
----------------------------------------
EXP2 TRANS
> numSummary(Exp2Trans[,c("LL", "LL.PL", "PL"), drop=FALSE],
+   groups=Exp2Trans$Species, statistics=c("mean"), quantiles=c(0,.25,.5,.75,1))
                                      LL    LL.PL       PL LL:n LL.PL:n PL:n
P. patula                       2.565217 2.208696 5.652174   23      23   23
P. patula x P. tecunumanii HE   2.417411 1.867307 5.941220 1344    1344 1344
P. patula x P. tecunumanii LE   2.165028 1.574354 5.991573 1424    1424 1424
P. tecunumanii HE               2.739130 1.845652 6.217391   23      23   23
P. tecunumanii LE               2.425000 1.459000 6.475000   40      40   40
################################################
```

EXP1 UNTRANS

```
> numSummary(Exp1Untra[,c("LL", "LL.PL", "PL"), drop=FALSE],
+   groups=Exp1Untra$Species, statistics=c("mean"), quantiles=c(0,.25,.5,.75,1))
                         LL      LL.PL        PL LL:n LL.PL:n PL:n LL:NA LL.PL:NA
                        NaN        NaN 122.88787    0       0    1     1        1
P.pat x P. tecHE   7.860838   6.471251 129.40602 1527    1527 1527     0        0
P.pat x P. tecLE   5.378805   4.690361 132.44532 1774    1774 1774     0        0
P.patula          13.439024  24.378049  54.14634   41      41   41     0        0
P.taeda            7.604061   7.694416  94.98985  197     197  197     0        0
P.tecHE            6.950943   9.876604  73.72453  265     265  265     0        0
P.tecLE            3.293578   3.549083  97.35780  218     218  218     0        0
                  PL:NA
                      0
P.pat x P. tecHE      0
P.pat x P. tecLE      0
P.patula              0
P.taeda               0
P.tecHE               0
P.tecLE               0
```

EXP1tRANS

```
> numSummary(Exp1trans[,c("LL", "PL", "SK"), drop=FALSE],
+   groups=Exp1trans$Species, statistics=c("mean"), quantiles=c(0,.25,.5,.75,1))
                         LL       PL       SK LL:n PL:n SK:n LL:NA PL:NA SK:NA
                        NaN      NaN      NaN    0    0    0     1     1     1
P.pat x P. tecHE  2.334304 5.541350 2.114112 1491 1491 1491     0     0     0
P.pat x P. tecLE  2.016579 5.536335 1.807990 1755 1755 1755     0     0     0
P.patula          2.948862 4.666431 3.568457   41   41   41     0     0     0
P.taeda           2.348687 5.294799 2.344378  177  177  177     0     0     0
P.tecHE           2.254632 4.972709 2.569055  264  264  264     0     0     0
P.tecLE           1.810803 5.250616 1.860751  216  216  216     0     0     0
```

Appendix B

Descriptive statistics summary (R output)

```
EXPERI1 TRANSFORMED
> numSummary(Exp1trans[,"SK", drop=FALSE], groups=Exp1trans$Species,
+   statistics=c("mean", "sd", "se(mean)", "IQR", "quantiles", "cv", "skewness",
+    "kurtosis"), quantiles=c(0,.25,.5,.75,1), type="1")
                       mean        sd   se(mean)        IQR         cv    skewness
                        NaN        NA         NA         NA         NA         NaN
P.pat × P. tecHE 2.114112 0.9659002 0.02501459 1.5702913 0.4568822  0.41704723
P.pat × P. tecLE 1.807990 0.8587528 0.02049886 1.0570764 0.4749766  0.98386366
P.patula         3.568457 0.9103459 0.14217215 1.5897146 0.2551091 -0.61369199
P.taeda          2.344378 1.0425824 0.07836529 1.7768365 0.4447160  0.09636082
P.tecHE          2.569055 0.8735103 0.05376084 1.2289324 0.3400123  0.60928367
P.tecLE          1.860751 0.4876280 0.03317888 0.6775339 0.2620597  0.58247747
                   kurtosis        0%       25%       50%       75%      100% SK:n
                        NaN        NA        NA        NA        NA        NA    0
P.pat × P. tecHE -0.7822731 0.3900353 1.300820 1.909274 2.871112 5.282214 1491
P.pat × P. tecLE  0.5729387 0.3900353 1.194763 1.609438 2.251840 5.234340 1755
P.patula         -0.9735362 1.6094379 2.738558 3.761741 4.328272 4.711411   41
P.taeda          -1.1995679 0.2956730 1.443635 2.312438 3.220472 4.317666  177
P.tecHE          -0.5170025 0.8813736 1.901921 2.312438 3.130854 4.820347  264
P.tecLE           0.9060915 0.8088669 1.487483 1.879864 2.165017 3.863274  216
                 SK:NA
                     1
P.pat × P. tecHE     0
P.pat × P. tecLE     0
P.patula             0
P.taeda              0
P.tecHE              0
P.tecLE              0
```

```
Exp2Trans
> Exp2Trans <-
+   read.table("E:/LEBODATA/Trial 2/PDS026T TSK combined dataset_xlsx.csv",
+   header=TRUE, sep=",", na.strings="NA", dec=".", strip.white=TRUE)

> numSummary(Exp2Trans[,"LL.PL", drop=FALSE], groups=Exp2Trans$Species,
+   statistics=c("mean", "sd", "se(mean)", "IQR", "quantiles", "cv", "skewness",
+    "kurtosis"), quantiles=c(0,.25,.5,.75,1), type="2")
                                    mean        sd   se(mean)    IQR        cv
P. patula                       2.208696 1.4896989 0.31062369 2.5000 0.6744700
P. patula × P. tecunumanii HE   1.867307 1.1442056 0.03121077 1.7800 0.6127572
P. patula × P. tecunumanii LE   1.574354 1.0025347 0.02656712 1.3125 0.6367912
P. tecunumanii HE               1.845652 0.7750298 0.16160488 0.9550 0.4199219
P. tecunumanii LE               1.459000 0.7547348 0.11933405 0.7875 0.5172960
                                 skewness    kurtosis   0%    25%   50%    75%
P. patula                       0.6960347 -0.97390775 0.63 0.8950 1.700 3.3950
P. patula × P. tecunumanii HE   0.6854634 -0.68369833 0.26 0.9600 1.480 2.7400
P. patula × P. tecunumanii LE   1.0151857  0.17605275 0.05 0.8400 1.240 2.1525
P. tecunumanii HE               0.2723596  0.03300293 0.39 1.3650 1.820 2.3200
P. tecunumanii LE               1.1888147  0.73596647 0.59 0.9675 1.265 1.7550
                                100% LL.PL:n
P. patula                       5.00      23
P. patula × P. tecunumanii HE   4.89    1344
P. patula × P. tecunumanii LE   4.78    1424
P. tecunumanii HE               3.59      23
P. tecunumanii LE               3.35      40
```

```
> Exp1UN <- read.table("E:/LEBODATA/exp1/Combined_dataset-25.csv",
+   header=TRUE, sep=",", na.strings="NA", dec=".", strip.white=TRUE)

> numSummary(Exp1UN[,"LL.PL", drop=FALSE], groups=Exp1UN$Species,
+   statistics=c("mean", "sd", "se(mean)", "IQR", "quantiles", "cv", "skewness",
+    "kurtosis"), quantiles=c(0,.25,.5,.75,1), type="1")
                       mean         sd  se(mean)  IQR        cv  skewness
                        NaN         NA        NA   NA        NA       NaN
P.pat × P. tecHE   6.471251  7.665159 0.1961560  7.1 1.1844941 2.6090377
P.pat × P. tecLE   4.690361  6.937794 0.1647193  3.1 1.4791600 4.5988167
P.patula          24.378049 16.142622 2.5210541 30.2 0.6621786 0.1770192
P.taeda            7.694416  8.573352 0.6108260 10.3 1.1142304 1.4303104
P.tecHE            9.876604 10.829518 0.6652520  8.2 1.0964820 2.1623434
P.tecLE            3.549083  2.414744 0.1635471  2.2 0.6803855 3.7679727
                   kurtosis  0% 25%  50%  75% 100% LL.PL:n LL.PL:NA
                        NaN  NA  NA   NA   NA   NA       0        1
P.pat × P. tecHE 10.271710 0.0 1.6  3.3  8.7 69.0    1527        0
P.pat × P. tecLE 32.387999 0.0 1.4  2.4  4.5 93.8    1774        0
P.patula         -1.284094 2.4 7.7 21.5 37.9 55.6      41        0
P.taeda           1.383451 0.0 1.7  3.8 12.0 37.5     197        0
P.tecHE           5.004353 0.0 3.2  5.0 11.4 62.0     265        0
P.tecLE          24.135876 0.0 2.1  3.2  4.3 23.8     218        0

"""""""""""""""""""""""""""""""""""""""""

> Exp2UN <- read.table("E:/LEBODATA/Trial 2/PDS026T_SK_dataset_.csv",
+   header=TRUE, sep=",", na.strings="NA", dec=".", strip.white=TRUE)

> numSummary(Exp2UN[,"Stem.kill", drop=FALSE], groups=Exp2UN$Species,
+   statistics=c("mean", "sd", "se(mean)", "IQR", "quantiles", "cv", "skewness",
+    "kurtosis"), quantiles=c(0,.25,.5,.75,1), type="1")
                                    mean         sd  se(mean)     IQR         cv
                                     NaN         NA        NA      NA         NA
P. patula × P. tecunumanii HE   6.604591  9.744677 0.2658079  6.5800 1.4754399
P. patula × P. tecunumanii LE   4.423455  7.164581 0.1898610  3.3125 1.6196799
P. tecunumani HE                4.180000  4.008935 0.8359208  3.2050 0.9590755
P. tecunumani LE                2.910750  3.427740 0.5419733  1.6825 1.1776140
P.patula                       13.552609 21.124166 4.4046930 16.3650 1.5586789
                               skewness  kurtosis   0%   25%  50%     75%  100%
                                    NaN       NaN   NA    NA   NA      NA    NA
P. patula × P. tecunumanii HE 2.520340  7.182700 0.26 1.110 2.08  7.6900 66.67
P. patula × P. tecunumanii LE 3.268286 12.704111 0.06 0.940 1.58  4.2525 59.38
P. tecunumani HE              2.180069  4.845797 0.40 1.840 3.00  5.0450 18.18
P. tecunumani LE              2.258084  4.032679 0.62 1.125 1.63  2.8075 14.29
P.patula                      1.726049  1.846004 0.68 1.025 2.65 17.3900 74.38
                              Stem.kill:n Stem.kill:NA
                                        0           1
P. patula × P. tecunumanii HE        1344           0
P. patula × P. tecunumanii LE        1424           0
P. tecunumani HE                       23           0
P. tecunumani LE                       40           0
P.patula                               23           0
"""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""""
```

Appendix C

ANOVA per family (R output)

```
> Exp1Trans <- read.table("E:/LEBODATA/exp1/TSK Combined dataset.csv",
+    header=TRUE, sep=",", na.strings="NA", dec=".", strip.white=TRUE)

> LinearModel.1 <- lm(SK ~ Rep + Family, data=Exp1Trans)

> summary(LinearModel.1)

Call:
lm(formula = SK ~ Rep + Family, data = Exp1Trans)

Residuals:
    Min      1Q  Median      3Q     Max
-2.1517 -0.6091 -0.1380  0.5691  2.9765

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)        2.28001    0.07230  31.536  < 2e-16 ***
Rep                0.02445    0.01242   1.968 0.049112 *
Family[T.P1 x TH2] -0.54377    0.13533  -4.018 5.98e-05 ***
Family[T.P1 x TH3] -0.46754    0.14079  -3.321 0.000906 ***
Family[T.P1 x TH4] -0.59703    0.13337  -4.477 7.80e-06 ***
Family[T.P1 x TH5] -0.65791    0.14452  -4.552 5.47e-06 ***
Family[T.P1 X TL1] -0.84284    0.14454  -5.831 5.95e-09 ***
Family[T.P1 X TL10] -0.69699    0.15521  -4.491 7.30e-06 ***
Family[T.P1 X TL5] -0.55243    0.13337  -4.142 3.51e-05 ***
Family[T.P10 X TL1] -0.77501    0.14197  -5.459 5.09e-08 ***
Family[T.P10 X TL4] -0.27354    0.13532  -2.021 0.043305 *
Family[T.P10 X TL6] -0.43629    0.13153  -3.317 0.000919 ***
Family[T.P11 x TH1] -0.10607    0.13961  -0.760 0.447472
Family[T.P11 x TH3] -0.45558    0.20720  -2.199 0.027953 *
Family[T.P11 x TH4] -0.18372    0.14197  -1.294 0.195723
Family[T.P11 X TL4]  0.15204    0.14076   1.080 0.280157
Family[T.P11 X TL6] -0.72412    0.13739  -5.271 1.43e-07 ***
Family[T.P11 X TL8] -0.71355    0.13848  -5.153 2.70e-07 ***
Family[T.P11 X TL9] -0.49007    0.14078  -3.481 0.000505 ***
Family[T.P12 x TH1]  0.07835    0.16727   0.468 0.639519
Family[T.P12 x TH2] -0.28058    0.13244  -2.119 0.034188 *
Family[T.P12 x TH3] -0.04485    0.13531  -0.331 0.740316
Family[T.P12 X TL4] -0.65691    0.13153  -4.994 6.17e-07 ***
Family[T.P12 X TL5] -0.60445    0.13337  -4.532 6.01e-06 ***
Family[T.P12 X TL8] -0.61687    0.13338  -4.625 3.87e-06 ***
Family[T.P2]        1.43225    0.29312   4.886 1.07e-06 ***
```

```
Family[T.P12 X TL8] -0.61687    0.13338  -4.625 3.87e-06 ***
Family[T.P2]         1.43225    0.29312   4.886 1.07e-06 ***
Family[T.P2 x TH1]   0.10124    0.16080   0.630 0.529004
Family[T.P2 x TH2]  -0.41660    0.14458  -2.882 0.003980 **
Family[T.P2 x TH3]  -0.05168    0.14452  -0.358 0.720690
Family[T.P2 x TH4]  -0.06291    0.13634  -0.461 0.644521
Family[T.P2 x TH5]  -0.28623    0.13532  -2.115 0.034471 *
Family[T.P2 X TL1]   0.42959    0.16278   2.639 0.008346 **
Family[T.P2 X TL2]  -1.04152    0.13848  -7.521 6.73e-14 ***
Family[T.P2 X TL4]  -0.07857    0.14869  -0.528 0.597219
Family[T.P2 X TL8]  -0.60787    0.13434  -4.525 6.23e-06 ***
Family[T.P3 x TH1]   0.14885    0.14197   1.048 0.294520
Family[T.P3 x TH2]   0.07894    0.15337   0.515 0.606774
Family[T.P3 x TH3]  -0.23157    0.15180  -1.525 0.127234
Family[T.P3 x TH4]  -0.41467    0.14081  -2.945 0.003250 **
Family[T.P3 X TL1]  -0.35199    0.13740  -2.562 0.010449 *
Family[T.P3 X TL3]  -0.66187    0.13338  -4.962 7.26e-07 ***
Family[T.P3 X TL8]  -0.41204    0.14077  -2.927 0.003443 **
Family[T.P4 x TH1]  -0.12613    0.13960  -0.904 0.366304
Family[T.P4 x TH2]  -0.44435    0.15190  -2.925 0.003462 **
Family[T.P4 x TH3]  -0.01754    0.14197  -0.124 0.901692
Family[T.P4 x TH4]  -0.68983    0.14078  -4.900 9.97e-07 ***
Family[T.P4 X TL1]  -0.44204    0.14079  -3.140 0.001704 **
Family[T.P4 X TL4]  -0.33598    0.16952  -1.982 0.047554 *
Family[T.P4 X TL8]  -0.28749    0.14197  -2.025 0.042945 *
Family[T.P5 x TH1]  -0.21725    0.14585  -1.489 0.136443
Family[T.P5 x TH2]  -0.50403    0.14078  -3.580 0.000347 ***
Family[T.P5 x TH3]  -0.08808    0.14198  -0.620 0.535075
Family[T.P5 X TL1]  -1.16567    0.14873  -7.838 5.89e-15 ***
Family[T.P5 X TL3]  -0.87820    0.13634  -6.441 1.33e-10 ***
Family[T.P5 X TL9]  -0.72641    0.13634  -5.328 1.05e-07 ***
Family[T.P6 X TL1]  -1.03930    0.13850  -7.504 7.64e-14 ***
Family[T.P6 X TL10] -0.53070    0.14080  -3.769 0.000166 ***
Family[T.P6 X TL8]   0.14779    0.14585   1.013 0.310982
Family[T.P7]         1.16702    0.16479   7.082 1.68e-12 ***
Family[T.P7 x TH1]   0.09860    0.14451   0.682 0.495080
Family[T.P7 x TH2]  -0.55272    0.15346  -3.602 0.000320 ***
Family[T.P7 x TH3]  -0.37173    0.13532  -2.747 0.006039 **
Family[T.P7 x TH4]  -0.32141    0.13336  -2.410 0.015999 *
Family[T.P7 X TL1]  -0.69190    0.15889  -4.355 1.37e-05 ***
Family[T.P7 X TL3]  -0.57303    0.14076  -4.071 4.78e-05 ***
Family[T.P7 X TL7]  -0.31969    0.14077  -2.271 0.023204 *
Family[T.P7 X TL9]  -0.12990    0.13531  -0.960 0.337116
```

```
Family[T.P7 X TL9]  -0.12990    0.13531  -0.960 0.337116
Family[T.P8 x TH1]   0.19470    0.13153   1.480 0.138889
Family[T.P8 x TH2]  -0.31641    0.14452  -2.189 0.028625 *
Family[T.P8 x TH4]   0.31210    0.14451   2.160 0.030854 *
Family[T.P8 X TL6]  -0.26884    0.13742  -1.956 0.050494 .
Family[T.P9 X TL1]  -0.90879    0.13244  -6.862 7.88e-12 ***
Family[T.P9 X TL4]  -0.09442    0.14875  -0.635 0.525641
Family[T.P9 X TL9]  -0.98445    0.13742  -7.164 9.35e-13 ***
Family[T.TH1]        0.65630    0.13962   4.701 2.68e-06 ***
Family[T.TH2]        0.05018    0.13153   0.382 0.702850
Family[T.TH3]        0.13572    0.13337   1.018 0.308927
Family[T.TH4]        0.22239    0.13434   1.655 0.097911 .
Family[T.TH5]        0.12955    0.13434   0.964 0.334926
Family[T.TL1]       -0.44570    0.13244  -3.365 0.000772 ***
Family[T.TL4]       -0.54233    0.13433  -4.037 5.51e-05 ***
Family[T.TL7]       -0.24353    0.13532  -1.800 0.071999 .
Family[T.TL9]       -0.67574    0.13153  -5.137 2.92e-07 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.8578 on 3861 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.1774,    Adjusted R-squared:  0.1599
F-statistic: 10.15 on 82 and 3861 DF,  p-value: < 2.2e-16


> Anova(LinearModel.1, type="II")
Anova Table (Type II tests)

Response: SK
           Sum Sq   Df F value  Pr(>F)
Rep          2.85    1   3.874 0.04911 *
Family     608.80   81  10.214 < 2e-16 ***
Residuals 2841.23 3861
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Appendix D

ANOVA per species (R output)

```
> LinearModel.3 <- lm(SK ~ Rep +Species, data=Exp1Trans)

> summary(LinearModel.3)

Call:
lm(formula = SK ~ Rep + Species, data = Exp1Trans)

Residuals:
    Min      1Q  Median      3Q     Max
-2.0809 -0.6875 -0.1690  0.5976  3.4375

Coefficients:
                           Estimate Std. Error t value Pr(>|t|)
(Intercept)                 2.05477    0.03978  51.652  < 2e-16 ***
Rep                         0.02355    0.01282   1.836 0.066420 .
Species[T.P.pat x P. tecLE] -0.30499    0.03155  -9.668  < 2e-16 ***
Species[T.P.patula]         1.45282    0.14177  10.248  < 2e-16 ***
Species[T.P.taeda]          0.22762    0.07121   3.197 0.001402 **
Species[T.P.tecHE]          0.45587    0.05980   7.624 3.07e-14 ***
Species[T.P.tecLE]         -0.25288    0.06520  -3.879 0.000107 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.8955 on 3937 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.08593,   Adjusted R-squared:  0.08453
F-statistic: 61.68 on 6 and 3937 DF,  p-value: < 2.2e-16


> Anova(LinearModel.3, type="II")
Anova Table (Type II tests)

Response: SK
           Sum Sq   Df F value  Pr(>F)
Rep          2.70    1  3.3712 0.06642 .
Species    292.87    5 73.0425 < 2e-16 ***
Residuals 3157.16 3937
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```


```
> LinearModel.3 <- lm(SK ~ Rep +Species, data=Exp1Trans)

> summary(LinearModel.3)

Call:
lm(formula = SK ~ Rep + Species, data = Exp1Trans)

Residuals:
    Min      1Q  Median      3Q     Max
-2.0809 -0.6875 -0.1690  0.5976  3.4375

Coefficients:
                           Estimate Std. Error t value Pr(>|t|)
(Intercept)                 2.05477    0.03978  51.652  < 2e-16 ***
Rep                         0.02355    0.01282   1.836 0.066420 .
Species[T.P.pat x P. tecLE] -0.30499    0.03155  -9.668  < 2e-16 ***
Species[T.P.patula]         1.45282    0.14177  10.248  < 2e-16 ***
Species[T.P.taeda]          0.22762    0.07121   3.197 0.001402 **
Species[T.P.tecHE]          0.45587    0.05980   7.624 3.07e-14 ***
Species[T.P.tecLE]         -0.25288    0.06520  -3.879 0.000107 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.8955 on 3937 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.08593,   Adjusted R-squared:  0.08453
F-statistic: 61.68 on 6 and 3937 DF,  p-value: < 2.2e-16


> Anova(LinearModel.3, type="II")
Anova Table (Type II tests)

Response: SK
           Sum Sq   Df F value  Pr(>F)
Rep          2.70    1  3.3712 0.06642 .
Species    292.87    5 73.0425 < 2e-16 ***
Residuals 3157.16 3937
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Appendix E

LS Means (R output)

```
> # New code for agricolae
> library(agricolae)
> anova(aov1 <- aov(SK ~  REP + Family, data=fullDB))
Analysis of Variance Table

Response: SK
            Df  Sum Sq Mean Sq F value    Pr(>F)
REP          3   35.59 11.8640  16.306 1.573e-10 ***
Family      81  610.41  7.5359  10.358 < 2.2e-16 ***
Residuals 3859 2807.67  0.7276
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> summary(aov1 <- aov(SK ~  REP + Family, data=fullDB))
              Df Sum Sq Mean Sq F value   Pr(>F)
REP            3   35.6  11.864   16.31 1.57e-10 ***
Family        81  610.4   7.536   10.36  < 2e-16 ***
Residuals   3859 2807.7   0.728
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> model <- aov(SK ~  REP + Family, data=fullDB)
> out <- HSD.test(model,"Family", group=TRUE,console=TRUE)

Study: model ~ "Family"

HSD Test for SK

Mean Square Error:  0.7275638

Family,  means

               SK       std   r  Min  Max
P.taeda 2.344350 1.0427465 177 0.30 4.32
P10xTL1 1.568043 0.4761495  46 0.65 2.63
P10xTL4 2.067500 0.8715973  52 0.65 4.26
P10xTL6 1.905179 0.8606615  56 0.57 4.61
P11xTH1 2.234375 1.0779234  48 0.57 4.89
P11xTH3 1.874737 0.9069201  19 0.73 3.83
P11xTH4 2.158913 1.0498047  46 0.39 4.16
P11xTL4 2.496170 1.0983853  47 0.65 4.65
P11xTL6 1.618000 0.7327249  50 0.57 3.91
P11xTL8 1.628163 0.6244420  49 0.65 3.82
P11xTL9 1.849787 0.9349413  47 0.48 4.45
P12xTH1 2.403871 1.0659508  31 0.65 3.91
P12xTH2 2.061273 0.8637495  55 0.57 3.91
P12xTH3 2.298654 0.9689291  52 0.73 4.66
P12xTL4 1.683929 0.8244771  56 0.57 3.88
P12xTL5 1.737593 0.7587525  54 0.48 4.43
P12xTL8 1.723333 0.8245296  54 0.73 4.24
P1xTH2  1.795769 0.7726586  52 0.57 3.20
P1xTH3  1.871064 0.7336456  47 0.95 3.79
P1xTH4  1.744815 0.7786508  54 0.57 3.69
P1xTH5  1.682500 0.7549068  44 0.65 3.83
P1xTL1  1.496136 0.4741220  44 0.57 3.07
P1xTL10 1.634054 0.6931428  37 0.57 3.42
P1xTL5  1.789444 0.6326686  54 0.65 3.28
P2      3.778889 0.9348722   9 1.61 4.49
P2xTH1  2.431471 1.0495977  34 0.65 3.96
P2xTH2  1.918636 0.8997121  44 0.48 4.36
```

P2xTH3	2.297273	1.1203786	44	0.65	4.61
P2xTH4	2.278235	1.0275558	51	0.88	3.98
P2xTH5	2.055192	0.8426022	52	0.73	3.96
P2xTL1	2.760909	0.9032074	33	1.30	4.25
P2xTL2	1.299388	0.5926333	49	0.48	3.10
P2xTL4	2.263171	1.0712573	41	0.81	4.52
P2xTL8	1.732075	0.8689502	53	0.48	3.69
P3xTH1	2.490870	1.0139884	46	0.81	4.07
P3xTH2	2.424737	1.2871081	38	0.57	5.28
P3xTH3	2.121538	0.8380006	39	0.39	4.00
P3xTH4	1.922979	1.0643776	47	0.48	4.45
P3xTL1	1.989200	0.8614697	50	0.81	3.82
P3xTL3	1.678889	0.7049466	54	0.48	4.26
P3xTL8	1.929362	1.1305278	47	0.39	4.89
P4xTH1	2.218958	0.9514720	48	0.73	3.89
P4xTH2	1.886410	0.7578518	39	0.57	3.76
P4xTH3	2.326304	0.8573457	46	0.57	3.99
P4xTH4	1.650638	0.7508007	47	0.57	3.57
P4xTL1	1.897660	0.7445431	47	0.48	3.96
P4xTL4	1.995000	0.6537201	30	0.57	3.51
P4xTL8	2.054348	0.8182031	46	0.95	4.27
P5xTH1	2.124419	1.1829570	43	0.48	4.09
P5xTH2	1.837021	0.7896172	47	0.57	3.51
P5xTH3	2.251957	0.9072293	46	0.95	4.41
P5xTL1	1.185854	0.4995146	41	0.39	2.98
P5xTL3	1.462353	0.7432700	51	0.39	3.91
P5xTL9	1.616471	0.7239056	51	0.48	4.02
P6xTL1	1.300612	0.5878400	49	0.39	3.30
P6xTL10	1.808085	0.9121037	47	0.65	3.76
P6xTL8	2.493023	1.0997522	43	0.95	5.23
P7	3.509063	0.9103079	32	1.85	4.71
P7xTH1	2.444773	0.8998643	44	0.39	4.21
P7xTH2	1.802105	0.8717369	38	0.81	4.35
P7xTH3	1.970769	0.8754782	52	0.88	4.14
P7xTH4	2.020741	0.9963516	54	0.48	3.91
P7xTL1	1.636571	0.8205125	35	0.48	4.29
P7xTL3	1.770638	0.7926165	47	0.65	3.91
P7xTL7	2.021915	0.6939980	47	1.08	3.61
P7xTL9	2.212692	0.8236412	52	0.73	3.98
P8xTH1	2.536250	0.9563217	56	0.57	4.74
P8xTH2	2.031364	0.9805399	44	0.57	4.05
P8xTH4	2.658636	0.9583309	44	1.14	4.27
P8xTL6	2.069800	0.8072440	50	0.88	3.69
P9xTL1	1.432182	0.6516381	55	0.57	3.45
P9xTL4	2.240732	1.1032348	41	0.88	4.77
P9xTL9	1.354400	0.5707823	50	0.39	3.16
TH1	2.996458	0.9578066	48	1.19	4.82
TH2	2.391607	0.8003948	56	1.08	4.20
TH3	2.476852	0.7547880	54	1.40	4.26
TH4	2.562264	0.9868085	53	1.02	4.73
TH5	2.470566	0.7582582	53	0.88	4.17
TL1	1.895091	0.5296605	55	0.81	3.08
TL4	1.799623	0.5139177	53	0.95	3.86
TL7	2.098269	0.4514062	52	1.14	3.35
TL9	1.665893	0.3441183	56	1.14	2.35

Alpha: 0.05 ; DF Error: 3859
Critical Value of Studentized Range: 5.967763

Groups according to probability of means differences and alpha level(0.05)

Treatments with the same letter are not significantly different.

	SK	groups

P2	3.778889	a
P7	3.509063	a
TH1	2.996458	ab
P2xTL1	2.760909	abc
P8xTH4	2.658636	abcd
TH4	2.562264	abcde
P8xTH1	2.536250	abcdef
P11xTL4	2.496170	abcdefg
P6xTL8	2.493023	abcdefg
P3xTH1	2.490870	abcdefg
TH3	2.476852	bcdefg
TH5	2.470566	bcdefg
P7xTH1	2.444773	bcdefgh
P2xTH1	2.431471	bcdefghi
P3xTH2	2.424737	bcdefghi
P12xTH1	2.403871	bcdefghij
TH2	2.391607	bcdefghij
P.taeda	2.344350	cdefghij
P4xTH3	2.326304	cdefghij
P12xTH3	2.298654	cdefghij
P2xTH3	2.297273	cdefghij
P2xTH4	2.278235	cdefghij
P2xTL4	2.263171	cdefghijk
P5xTH3	2.251957	cdefghijk
P9xTL4	2.240732	cdefghijk
P11xTH1	2.234375	cdefghijk
P4xTH1	2.218958	cdefghijk
P7xTL9	2.212692	cdefghijk
P11xTH4	2.158913	cdefghijkl
P5xTH1	2.124419	cdefghijklm
P3xTH3	2.121538	cdefghijklmn
TL7	2.098269	cdefghijklmn
P8xTL6	2.069800	cdefghijklmn
P10xTL4	2.067500	cdefghijklmn
P12xTH2	2.061273	cdefghijklmn
P2xTH5	2.055192	cdefghijklmn
P4xTL8	2.054348	cdefghijklmn
P8xTH2	2.031364	cdefghijklmno
P7xTL7	2.021915	cdefghijklmno
P7xTH4	2.020741	cdefghijklmno
P4xTL4	1.995000	cdefghijklmnop
P3xTL1	1.989200	cdefghijklmnop
P7xTH3	1.970769	cdefghijklmnop
P3xTL8	1.929362	defghijklmnop
P3xTH4	1.922979	defghijklmnop
P2xTH2	1.918636	defghijklmnop
P10xTL6	1.905179	efghijklmnop
P4xTL1	1.897660	efghijklmnop
TL1	1.895091	efghijklmnop
P4xTH2	1.886410	efghijklmnop
P11xTH3	1.874737	efghijklmnop
P1xTH3	1.871064	efghijklmnop
P11xTL9	1.849787	efghijklmnop
P5xTH2	1.837021	fghijklmnop
P6xTL10	1.808085	ghijklmnop
P7xTH2	1.802105	ghijklmnop
TL4	1.799623	ghijklmnop
P1xTH2	1.795769	ghijklmnop
P1xTL5	1.789444	ghijklmnop
P7xTL3	1.770638	ghijklmnop
P1xTH4	1.744815	hijklmnop
P12xTL5	1.737593	hijklmnop
P2xTL8	1.732075	hijklmnop
P12xTL8	1.723333	hijklmnop

P12xTL4	1.683929	ijklmnop
P1xTH5	1.682500	ijklmnop
P3xTL3	1.678889	ijklmnop
TL9	1.665893	ijklmnop
P4xTH4	1.650638	ijklmnop
P7xTL1	1.636571	ijklmnop
P1xTL10	1.634054	ijklmnop
P11xTL8	1.628163	ijklmnop
P11xTL6	1.618000	jklmnop
P5xTL9	1.616471	jklmnop
P10xTL1	1.568043	jklmnop
P1xTL1	1.496136	klmnop
P5xTL3	1.462353	lmnop
P9xTL1	1.432182	mnop
P9xTL9	1.354400	nop
P6xTL1	1.300612	op
P2xTL2	1.299388	op
P5xTL1	1.185854	p

Appendix F

Correlation between frost and *F. circinatum* tolerance (R output)

```
> Exp2Trans <-
+   read.table("E:/LEBODATA/Trial 2/PDS026T TSK combined dataset_xlsx.csv",
+   header=TRUE, sep=",", na.strings="NA", dec=".", strip.white=TRUE)


> with(Exp2Trans, cor.test(LL, LL.PL, alternative="two.sided",
+   method="pearson"))

        Pearson's product-moment correlation

data:  LL and LL.PL
t = 143.19, df = 2852, p-value < 2.2e-16
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 0.9323157 0.9412872
sample estimates:
      cor
0.9369557


> with(Exp2Trans, cor.test(LL.PL, PL, alternative="two.sided",
+   method="pearson"))

        Pearson's product-moment correlation

data:  LL.PL and PL
t = -12.821, df = 2852, p-value < 2.2e-16
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 -0.2678332 -0.1984457
sample estimates:
       cor
-0.2334366
```

```
> Exp1trans <- read.table("E:/LEBODATA/exp1/TSK Combined dataset.csv",
+   header=TRUE, sep=",", na.strings="NA", dec=".", strip.white=TRUE)

> with(Exp1trans, cor.test(PL, SK, alternative="two.sided", method="pearson"))

        Pearson's product-moment correlation

data:  PL and SK
t = -27.929, df = 3942, p-value < 2.2e-16
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 -0.4321680 -0.3800498
sample estimates:
       cor
-0.4064395


> with(Exp1trans, cor.test(LL, SK, alternative="two.sided", method="pearson"))

        Pearson's product-moment correlation

data:  LL and SK
t = 166.44, df = 3942, p-value < 2.2e-16
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 0.9316358 0.9394186
sample estimates:
      cor
0.9356408
```