Maternal effects on phenotype, resistance and the structuring of fungal communities in *Eucalyptus grandis*

María Vivas[1,*], Martin Kemler[1,2], Makobatjatji M. Mphahlele[3], Michael J. Wingfield[4] and Bernard Slippers[1]

[1]Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Cnr Lynnwood and University Road, Hatfield 0083, Pretoria, South Africa

[2]Geobotany, Ruhr-University Bochum, Universitätsstrasse 150, 44780 Bochum, Germany

[3]Mondi Forests, Tree Improvement Technology, Trahar Technology Centre - TTC, Mountain Home Estate, Off Dennis Shepstone Dr, Hilton, 3245, South Africa

[4]Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Cnr Lynnwood and University Road, Hatfield 0083, Pretoria, South Africa

[*] Corresponding author:
María Vivas
Tel: +27 12 420 5814
Email: maria.vivas@fabi.up.ac.za

Highlights

- Seedling germination and height were influenced by different maternal environments.
- Seedling resistance to biotic stresses differed depending on the maternal environment.
- Maternal environment can influence the structure of fungal communities in the progeny.

Abstract

The environmental experience of plants can modulate the development of the offspring and their interactions with other organisms. These effects, generally known as maternal effects, occur through seed provisioning and epigenetic modifications. This study considers the influence of differing environments of maternal plants on their progeny and their biotic interactions. Seeds were collected from two *Eucalyptus grandis* clonal seed orchards having different abiotic and biotic conditions. Seed and seedling development, and seedling responses to pest infestation and pathogen inoculation were measured. Finally, fungal communities in the foliage of the seedlings were assessed using a metabarcoding approach. The percentage seed germination and height of seedlings were influenced by the maternal environments. Seedlings from one of the maternal environments were significantly more resistant to a pathogen than seedlings from the other. The composition and diversity of fungal communities also differed between the offspring from the two maternal environments. We found that the differences in the maternal environment affected the progeny performance and resistance. Moreover, we show for the first time that the maternal environment can influence the structure of fungal communities in the foliage in the subsequent generation.

Keywords: Epigenetic changes, fungal microbiome, maternal environmental effects, phenotypic plasticity, resistance, seed mass.

1. Introduction

The environmental experience of plants can influence the phenotype and stress tolerance of their offspring (Agrawal et al., 1999; Holeski et al., 2012; Jablonka and Raz, 2009; Roach and Wulff, 1987). This transgenerational phenotypic plasticity, or parental effect, is not caused by modifications in the DNA sequence, but rather by the parental environment (Herman and Sultan, 2011; Roach and Wulff, 1987). In general, maternal plants are assumed to have a greater influence on the phenotype and resistance of their offspring than paternal plants. For instance, maternal plants directly provide seeds with substantial resources such as plastids, the seed endosperm and the seed tissues surrounding the embryo (Linkies et al., 2010; Rix et al., 2012).

The transmission of maternal effects to subsequent generations is not only related to seed provisioning, but also to epigenetic mechanisms. These epigenetic mechanisms are heritable transgenerational effects driven by reversible DNA methylation, histone modifications and small RNAs that alter the regulatory states of genomic regions (Hauser et al., 2011). In contrast, seed provisioning mechanisms refer to non-heritable transgenerational effects associated with carbohydrate, lipid, protein, and mineral nutrient reserves allocated to the seed by the maternal plant (Boyko and Kovalchuk, 2011; Herman and Sultan, 2011).

Maternal effects have been reported in many different plant species and traits. For example, differences in the seed traits of *Bromus madritensis* offspring can be the result of resource deficiencies due to competition in the maternal environment (Violle et al., 2009). In *Arabidopsis thaliana*, the maternal environment can affect the germination and flowering time of the progeny, as well as on the seed mass produced by the progeny (Elwell et al., 2011). In other *Arabidopsis* studies, the progeny of plants inoculated with *Pseudomonas syringae* or exposed to caterpillar herbivory have been shown to increase resistance in comparison to progeny from unthreatened parent plants (Luna et al., 2012; Rasmann et al., 2012). For these reasons, maternal effects have been considered to be an adaptive strategy, offering an advantage to the progeny when it experiences similar conditions to the maternal environment (Galloway and Etterson, 2007; Mousseau and Fox, 1998). Consequently, a deeper understanding of these maternal effects could provide important predictions regarding, for example, stress tolerance of progeny based on the environmental experience of their maternal plants.

Seedling resistance against certain fungal pathogens varies depending on the maternal conditions (Vivas et al., 2013). This suggests that the association of the progeny with a broader fungal microbiome could vary depending on the maternal environment (Vivas et al., 2015). Such effects could be significant, given that microbial communities in the foliage of plants are thought to play crucial roles in the promotion of plant growth and protection (Peñuelas and Terradas, 2014; Vannier et al. 2015; Vorholt, 2012). Opportunities to study such maternal effects on plant microbiomes are increasing, because the in-depth characterization of microbial communities across a large number of samples is now feasible using high-throughput DNA sequencing (Rastogi et al., 2013). Such information could be used to understand plant ecology from the perspective of the extended genotype, which also includes the associated microbes and maternal effects.

To the best of our knowledge, no study has simultaneously tested biotic and abiotic maternal effects on seed traits, seedling performance and plant-pathogen \ plant-insect interactions in the associated plant progeny. Furthermore, nothing is known regarding maternal effects on fungal communities of these progeny populations. This study was consequently designed to consider the influence of maternal plants, naturally exposed to abiotic and biotic stressors, on their progeny and their biotic interactions. We examined the influence of maternal effects, different genetic backgrounds and their interactions using a *Eucalyptus grandis* W. Hill ex Maiden case study. *Eucalyptus* species are amongst the most widely utilized trees for the establishment of plantations globally, with major ecological and economic importance (Wingfield et al., 2015). Specifically, we considered these maternal effects and different genetic backgrounds on (i) seed and seedling performance, (ii) seedling resistance to a pest and a pathogen, and (iii) fungal communities in the foliage of seedlings (as proposed by Vivas et al. (2015)).

2. Materials and methods

2.1. Plant material

Eucalyptus grandis trees used in this study were located in two clonal seed orchards in South Africa, Greytown (29°11'56.73"S, 30°39'34.46"E) and Kwambonambi (28°35'41.45"S, 32°11'38.98"E). The orchards were planted in 2005 within a commercial tree breeding program and included a selection of 3rd generation *E. grandis*

clones used for commercial seed production. The spatial design of the orchards was identical at both locations and, therefore, the pollen contribution in both orchards was expected to be the same. However, abiotic and biotic conditions differed among orchards. Kwambonambi orchard, offered more conducive abiotic conditions for *E. grandis* growth than Greytown orchard. Specifically, Kwambonambi is situated in a low altitude sub-tropical region with higher rainfal and more effective tree root depth than Greytown, which is situated in a temperate region with lower rainfal and temperatures (Table 1). However, the Kwambonambi orchard was more heavily affected by pests and diseases as compared to Greytown orchard (Table 1). Three *Eucalyptus* genotypes (G1, G2 and G3) present in both seed orchards were selected. Each genotype was represented by three ramets in each orchard (2 maternal environments × 3 genotypes × 3 ramets). Ramet identity was confirmed and the possibility of pollen contamination discarded through DNA fingerprinting of six to seven seedlings per ramet (n = 120), using previously designed microsatellite markers and STRUCTURE analyses (Pritchard et al., 2000) (Methods S1).

Table 1. Characteristic of the *Eucalyptus grandis* orchards.

Conditions	Characteristics	Orchard	
		Greytown	Kwambonambi
Abiotic	Climate	Temperate	Sub-tropical
	Average Annual Temperature (ºC)	17	21
	Minimum Annual Temperature (ºC)	5	11
	Maximum Annual Temperature (ºC)	25	29
	Average Annual Rainfall (mm)	832	1201
	Altitude (m)	1023	55
	Aspect	South	West
	Effective root depth (cm)	151	151 - 310
Biotic	*Leptocybe invasa*	Moderate	High
(pests)	*Glycaspis brimblecombei*	Absence- Low	Low-High
	Thaumastocoris peregrinus	Absence	Low
	Gonipterus scutellatus	High	High
	Phoracantha semipunctata	Absence	Absence
Biotic	Coniotyrium canker	Moderate	High
(pathogens)	(*Teratosphaeria zuluense*)		

	Botryosphaeria canker and die-back (*Neofusicoccum* spp. and others in the Botryosphaeriaceae)	Moderate	High

Data provided by the forest company (Temperature and precipitation cover the years 1957-2007).

2.2. Experiment 1: Maternal effects on seed and seedling performance

Seed capsules were collected from three ramets of each *Eucalyptus* genotype in each of the orchards. Seed mass was estimated as the total mass of seeds per capsule divided by the total number of seeds per capsule.

In July 2014, a factorial design of blocks was prepared with the seeds from the maternal environments and genotypes randomly distributed within each block. A total of 1620 pre-weighed seeds (2 maternal environments × 3 genotypes × 3 ramets × 90 seeds) were sown in Jiffypots® filled with vermiculite substrate in trays. The trays were placed in a common growth chamber with a 16 h day : 8 h night regime, 24°C and 80% relative humidity, and watered as needed for 3 months. Individual seedling germination was assessed daily for two weeks and seedling height was measured weekly.

Seedlings were transplanted and moved to a common greenhouse in October 2014 and their height was measured once each month. At the end of the study, the diameters of the main stems of plants were measured directly above the root collars.

2.3. Experiment 2: Maternal effects on seedling pest and pathogen response

Seedlings in the greenhouse were naturally infested (no forced infestation) with the Eucalyptus gall wasp *Leptocybe invasa* (Hymenoptera) in January 2015. *L. invasa* is one of the most damaging pests of *Eucalyptus* plantations outside its native range in Australia (Mendel et al., 2004). This wasp affects the new growth of all tree ages, causing galls and tree death in extreme cases (Dittrich-Schröder et al., 2012; Mendel et al., 2004). The number of galled leaves and the number of total leaves per plant was recorded to score the damage caused by *L. invasa* utilizing the rating scale of Dittrich-Schröder et al. (2012). In June 2015, all the seedlings were treated with a systemic insecticide (Imidacloprid 350 g l^{-1}) to prevent new infestations by *L. invasa*.

Isolate CMW2113 of the *Eucalyptus* canker pathogen *Chrysoporthe austroafricana* (Nakabonge et al., 2006; Wingfield et al., 2013) was selected to examine the influence of the different maternal environments on seedling resistance to disease. The identity of the *C. austroafricana* isolate was verified based on DNA sequencing (Chen et al., 2016). Its aggressiveness was confirmed in a pilot trial where stems of *E. grandis* seedlings were inoculated, and where lesions were longer on inoculated than in control seedlings (n = 20).

In November 2015, when the plants were ~ 16-months-old, a total of 714 seedlings that were ≥ 70 cm tall were inoculated with *C. austroafricana*. The cultures used for inoculation were grown on 2 % Malt extract agar (MEA) for 5 days at 28 °C. Seedlings were inoculated on the main stems, approximately 10 cm above the root collar. Wounds were made on the stems using a 5 mm cork borer to remove the bark and to expose the vascular cambium. An agar plug overgrown with *C. austroafricana* was placed, mycelium side facing the cambium, on the stem-wounds and the inoculation site was sealed with Parafilm®. A total of 18 seedlings were inoculated with sterile 2 % MEA agar plugs to serve as controls. Lesion lengths were measured 6 weeks after inoculation by removing the bark around the inoculation sites. *C. austroafricana* infection was confirmed by isolating from a sub-set of the inoculated wounds on 2 % MEA and confirming that the culture morphology of the re-isolated fungus was the same as that of the inoculum.

2.4. Experiment 3: Maternal effects on the community of foliar fungi

Mature leaves of seedlings were collected (2 maternal environments × 3 genotypes × 3 ramets), giving a total of 18 samples. Sampling was done at two-time points when the seedlings were (i) 3-months-old (growing in the growth chamber, October 2014) and (ii) 8-months-old (growing in an open greenhouse, March 2015). The samples were placed in individual centrifuge tubes, covered with 99 % ethanol and maintained at 4 ºC until DNA extraction.

The fungal community of the foliage was characterized at the seedling level for each sampling time. Total DNA, including plant and fungal DNA, was isolated from leaves. For the plants in the growth chambers (3-months-old), 5 × 5 mm pieces were cut from ten leaves per tree with a sterile scalpel and crushed. DNA was isolated using the

QIAGEN EasyDNA PlantMini preparation kit, following the manufacturer's instructions. No surface sterilization was applied, as we were interested in the whole fungal community associated with the trees. In the case of the seedlings in the open greenhouse (8-months-old), sample aliquots from three DNA isolations from the DNA fingerprinting analysis were used. Each of the DNA fingerprinting samples originated from 5 mg of leaves per ramet. The ITS1 gene region was subsequently amplified and sequenced bidirectionally using an Illumina MiSeq at MrDNA (Shallowater, USA) using the fungal specific primer ITS1F (Gardes and Bruns, 1993) and ITS2 (White et al., 1990). The sequence data was deposited in the Short Read Archive of the European Nucleotide Archive: PRJEB15631 (http://www.ebi.ac.uk/ena).

The obtained forward and reverse reads were merged using PEAR (Zhang et al., 2014) under default settings. The resulting sequence files were processed using the QIIME pipeline (Caporaso et al., 2010). Sequence files were de-multiplexed based on their sample identity according to a molecular barcode (Table S1) and sequence quality. Only sequences longer than 200 bp, with a mean PHRED quality score over 30, a perfect matching forward barcode sequence, and a maximum of 6 homopolymers were used in subsequent steps. Sequence chimeras were removed using usearch61 (Edgar, 2010; Edgar et al., 2011). The sequences retained from the previous step were clustered into OTUs at 95 % sequence similarity using uclust (Edgar, 2010). Taxonomic identities were assigned to each OTU with > 10 sequences using blastn at an e-value of $1e^{-30}$ against the UNITE database (v7_99_s_31.01.2016, Abarenkov et al., 2010).

2.5. Data analysis

The effect of the maternal environments, the genotypes and their interactions on seed and seedling performance (seed mass, germination, seedling height and diameter; Experiment 1) and seedling resistance (insect pest and fungal pathogen; Experiment 2) were analyzed using mixed models (Bolker et al., 2009). The dependent variables seed mass, seedling height and diameter fitted a normal distribution, germination fitted a binomial distribution, number of leaves galled by *L. invasa* infestation fitted a Poisson distribution and lesion length resulting from inoculation with *C. austroafricana* fitted a gamma distribution. The maternal environments, the genotypes and their interactions were included as explanatory variables. The ramet nested within a single maternal environment was considered as a random effect in the models. To account for variation

in ontogeny among seedlings and greenhouse heterogeneity, the mixed models included the covariation with seed mass and block. For height growth analysis, we also fitted a repeated measures mixed model accounting for the same effects. In all cases, model validity was checked by visual examination of residual plots and by assessment of dispersion parameters (Bolker et al., 2009). The 'lme4' package of the R software (R Core Team, 2014) was used for mixed models.

Community composition and diversity were analyzed to assess differences in the fungal communities of the foliage among the maternal environments, the genotypes, the sampling times and their interactions (Experiment 3). Community analyses were made using individual OTUs (i.e. without considering taxonomic identity) and taxonomic units (i.e. with genus, family or order identities assigned). Only fungal OTUs occurring in more than one sample and containing > 10 reads were included in the analysis. To visualize variation in fungal community composition among the sampled seedlings, a classical (metric) multidimensional scaling (MDS) was conducted using Bray-Curtis distance. To assess differences among the maternal environments, the genotypes, the sampling times and their interactions on community composition, a permutational multivariate analysis of variance (PERMANOVA) was used. To analyze fungal diversity, individual OTUs and taxonomic unit richness and Shannon's diversity index were calculated for each seedling. The effects of the maternal environment, the genotype, the sampling time and their interactions on OTUs and taxonomic units richness and Shannon's index were analyzed with linear models. The "vegan" package (Oksanen et al., 2005) of the R software (R Core Team, 2014) was used for classical MDS, and PERMANOVA and to calculate richness and Shannon's index.

3. Results

3.1. Experiment 1: Maternal effects on seed and seedling performance

Seed mass was not influenced by the maternal environments (Greytown and Kwambonambi orchards) (F = 0.66, df = 1, P =0.606), but there was a significant effect of the maternal *Eucalyptus* genotypes (G1, G2 and G3) (F = 21.38, df = 2, P < 0.001) and of the interaction between maternal environments and genotypes (F = 3.82, df = 2, P < 0.05) (Fig. S1). The percentage of germination was 1 % higher in seeds from the Greytown orchard than from Kwambonambi (F = 43.23, df = 1, P < 0.05) for all three maternal genotypes (F = 30.44, df = 2, P < 0.001) (maternal environment × genotype

interaction F = 13.42, df = 2, P < 0.001) (Fig. S2A). However, of the seeds that germinated, all germinated during two consecutive days and there were no differences in germination time between the maternal environments or the genotypes (Fig. S2B). There were also no differences in stem diameters of seedlings at 18-months-old linked with the maternal environments or the genotypes (P > 0.05).

The repeated measures mixed model analysis showed that height in the greenhouse was greater in seedlings grown from the Greytown orchard than from the Kwambonambi orchard (F = 28.06, df = 1, P < 0.05) and there were no significant differences between the maternal genotypes (F = 6.04, df = 2, P = 0.106) (maternal environment × genotype interaction F = 9.53, df = 2, P < 0.01) (Fig. 1). Seedling height data measured weekly in the growth chamber also showed significant differences between the maternal environments (Fig. S3). However, the individual monthly height analysis showed contrasting effects of the maternal environments and the genotypes over the course of the study period (Table 2). Seedling height was influenced by the maternal genotypes up to 5-months-old. The maternal environments influenced seedling height the first month and from 8-months-old to the end of the study. We also observed that the magnitude of the covariate seed mass on seedling height diminished with seedling age (up to 8-months-old), as shown in the decreasing F ratios and P values (Table 2).

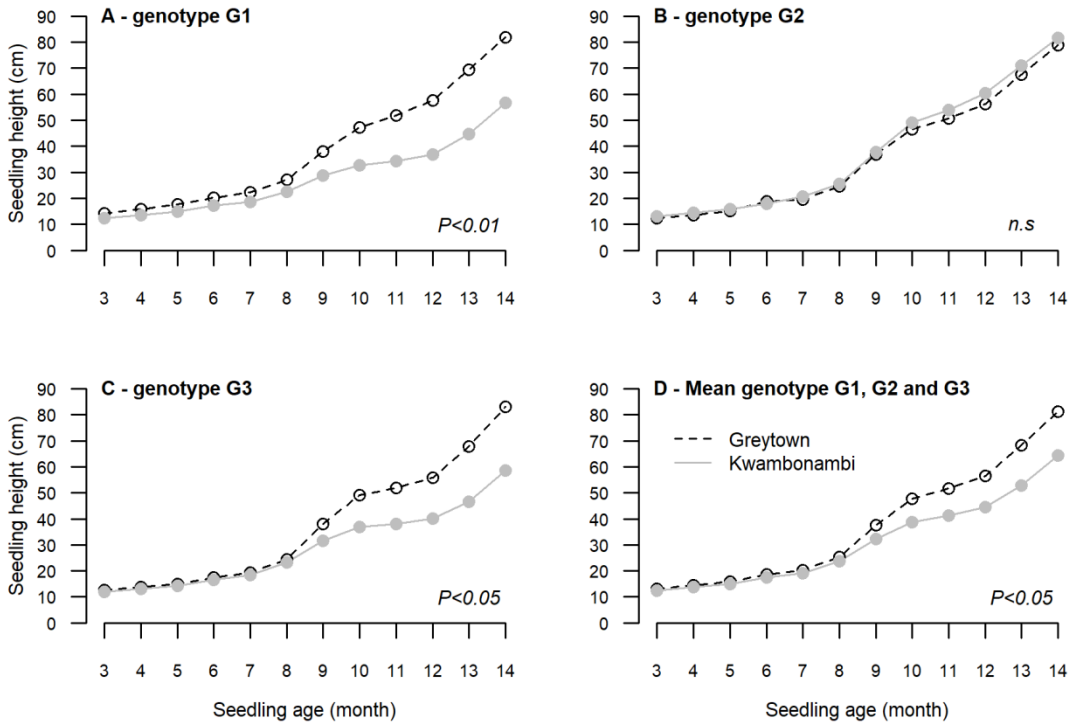

Fig. 1. Changes in total height of *Eucalyptus grandis* seedlings derived from three genotypes clonally replicated in two maternal environments. Seedling height for: (A) genotype G1, (B) genotype G2, (C) genotype G3 and (D) the mean of the three genotypes.

Table 2. Results of the general linear mixed model for analysis of monthly height (November 2014 - October 2015) of the *Eucalyptus grandis* seedlings derived from three genotypes clonally replicated in two maternal environments. Bold *P* value indicated $P<0.05$, darker colours indicate smaller *P* values.

		1 months		2 months		3 months		4 months		5 months		6 months		7 months		8 months		9 months		10 months		11 months		12 months		13 months		14 months	
Fixed effects	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
ME[1]	1	53.1	**<0.01**	1.6	0.250	2.8	0.081	2.2	0.101	2.7	0.077	3.4	0.083	3.1	0.0854	8.1	**<0.05**	45.4	**<0.05**	59.1	**<0.05**	53.0	**<0.05**	46.8	**<0.05**	59.5	**<0.05**	70.3	**<0.05**
G[2]	2	35.8	**<0.001**	11.5	**<0.001**	2.4	**<0.05**	2.6	**<0.05**	3.3	**<0.05**	3.2	0.057	2.5	0.094	1.7	0.128	8.1	0.700	16.0	0.424	16.3	0.204	18.1	0.080	17.2	0.089	11.7	0.378
ME × G	2	3.7	**<0.05**	0.5	0.584	3.2	0.063	3.1	0.0687	2.9	0.079	3.7	**<0.05**	4.4	**<0.05**	6.9	**<0.01**	8.8	**<0.01**	10.4	**<0.001**	10.7	**<0.001**	11.4	**<0.001**	9.9	**<0.01**	8.8	**<0.01**
Seed mass	1	213.3	**<0.001**	80.9	**<0.001**	71.9	**<0.001**	64.5	**<0.001**	53.7	**<0.001**	39.7	**<0.001**	32.9	**<0.001**	35.6	**<0.001**	8.1	0.072	0.5	0.822	0.2	0.640	0.1	0.316	0.2	0.264	0.1	0.520
Block	1					12.0	**<0.001**	16.9	**<0.001**	26.2	**<0.001**	9.4	**<0.001**	16.8	**<0.001**	8.1	**<0.001**	125.0	**<0.001**	302.7	**<0.001**	295.1	**<0.001**	297.2	**<0.001**	369.9	**<0.001**	394.6	**<0.001**

[1]Maternal environment

[2]Maternal genotype

3.2. Experiment 2: Maternal effects on seedling pest and pathogen response

Natural infestation by *L. invasa* was not influenced by the maternal environments (F = 0.62, df = 1, P = 0.453), or the maternal *Eucalyptus* genotypes (F = 1.61, df = 2, P = 0.472). The interaction between the maternal environments and the genotypes for infestation by the insect pest was not significant (F = 0.57, df = 2, P = 0.575) (Fig. 2).

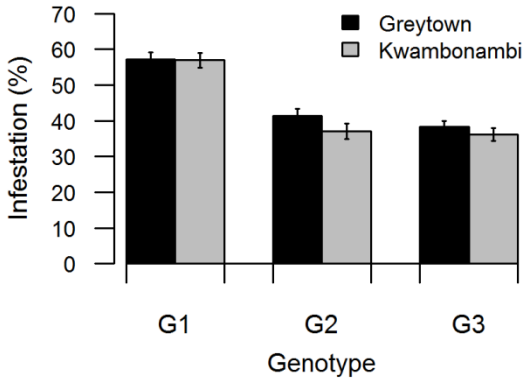

Fig. 2. Infestation percentage of *Eucalyptus grandis* seedlings after natural infestation with *Leptocybe invasa*. Seedlings were derived from three genotypes clonally replicated in two maternal environments, Greytown and Kwambonambi. Data are the mean ± 1 SE (n=270).

Lesions resulting from infection by *C. austroafricana* were influenced by the maternal environments and the genotypes. The mean lesion length on seedlings inoculated with the pathogen was 23 % greater in seedlings from the Greytown orchard than in those from Kwambonambi (F = 10.90, df = 1, P < 0.05). Inoculated seedlings from the maternal *Eucalyptus* genotype G1 had the longest lesions (6.54 ± 0.15 cm) followed by G3 (5.64 ± 0.25 cm) and G2 (4.05 ± 0.21 cm) (F = 20.63, df = 2, P < 0.001). There was, however, no significant interaction between the maternal environments and the genotypes for the lesion length (F = 1.93, df = 2, P = 0.154) (Fig. 3).

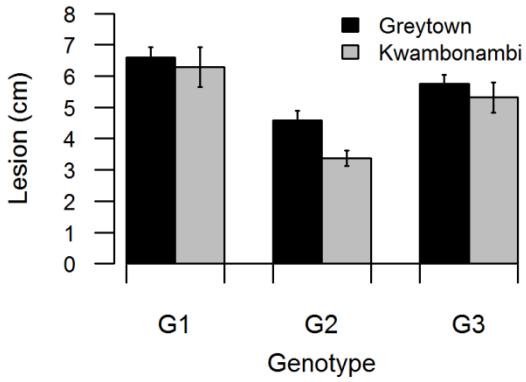

Fig. 3. Lesion length of *Eucalyptus grandis* seedlings six weeks after inoculations with the fungal pathogen *Chrysoporthe austroafricana*. Seedlings were derived from three genotypes clonally replicated in two maternal environments, Greytown and Kwambonambi. Data are the mean ± 1 SE (n=135).

3.3. Experiment 3: Maternal effects on the community of foliar fungi

There were 1969 individual OTUs taxonomically assigned to 275 different fungal genera. The datasets were analyzed separately as individual OTUs or as taxonomic units for genera. The composition of the fungal community from the foliage was different in seedlings from the two sampling times: 3-months-old (growing in the growth chamber) and 8-months-old (growing in an open greenhouse), using datasets for individual OTUs and taxonomic units for genera. The MDS plot supported the difference in fungal community composition between these two sampling times (OTUs Fig. 4A; genera Figure 4B). PERMANOVA confirmed that sampling time was the only factor significantly explaining the variation in fungal community composition (OTUs $F_{1,20}$ = 59.92, r^2 = 0.662, P < 0.001; genera $F_{1,20}$ = 124.30, r^2 = 0.772, P < 0.001).

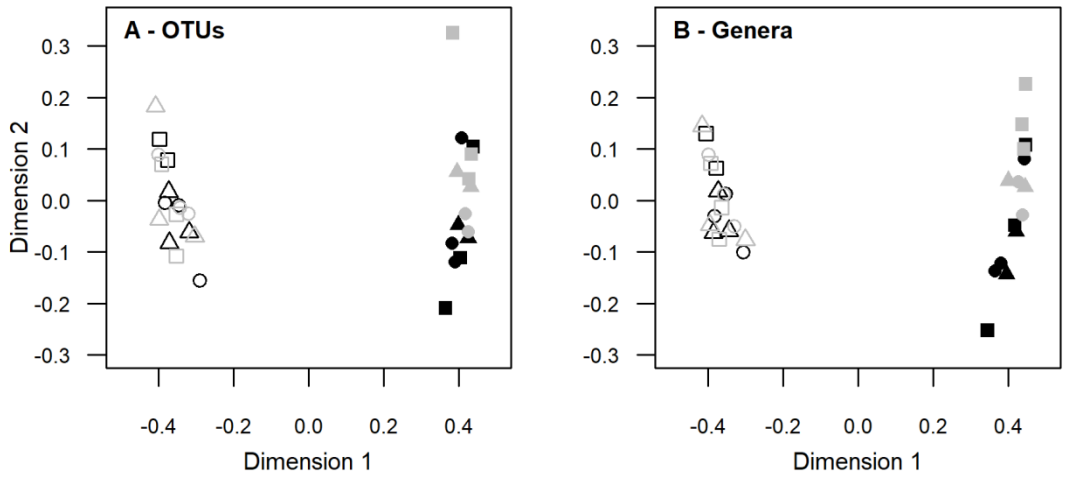

Fig. 4. Classical (metric) multidimensional scaling (MDS) diagram of fungal community composition in the foliage among the *Eucalyptus grandis* seedlings derived from three genotypes (■=G1, ●=G2, ▲=G3), replicated in two maternal environments (Greytown and Kwambonambi), collected when seedlings were 3-months-old (growth chamber) (open symbols) and 8-months-old (open greenhouse) (filled symbols). (A) MDS using individual OTUs. (B) MDS using taxonomic units for genera.

Fungal diversity also differed between sampling times. OTU richness was higher in 3-month-old seedlings than in 8-month-old seedlings (OTUs $F_{1,20} = 5.95$, $P < 0.05$; genera $F_{1,20} = 0.03$, $P > 0.05$). The Shannon's index was significantly lower in 3-month-old seedlings than in 8-month-old seedlings, using datasets for individual OTUs and taxonomic units for genera (OTUs $F_{1,20} = 13.29$, $P < 0.01$; genera $F_{1,20} = 128.35$, $P < 0.001$).

Fungal community composition and diversity varied to a greater extent between sampling times than among the maternal environments or the *Eucalyptus* genotypes. Therefore, to further consider the differences between the studied factors (maternal environment and genotype), we re-analyzed the data within sampling time. Community composition in 3-month-old seedlings was significantly different among maternal *Eucalyptus* genotypes, using data for taxonomic units for genera (Fig. 5A) (PERMANOVA genera $F_{1,11} = 1.99$, $r^2 = 0.216$, $P < 0.01$). Moreover, the richness of the taxonomics units for genera was significantly different between the two maternal environments (i.e. higher for seedlings from the Greytown that from the Kwambonambi orchard; genera $F_{1,1} = 5.53$, $P < 0.05$). Community composition in 8-month-old

seedlings was also significantly different between the seedlings from the two maternal environments (Greytown and Kwambonambi), using data taxonomic units for genera (Fig. 5B) (PERMANOVA genera: $F_{1,9} = 2.54$; $r^2 = 0.157$; $P < 0.05$). Individual OTU dataset did not show any pattern in fungal community composition or diversity when separating the data by sampling time.

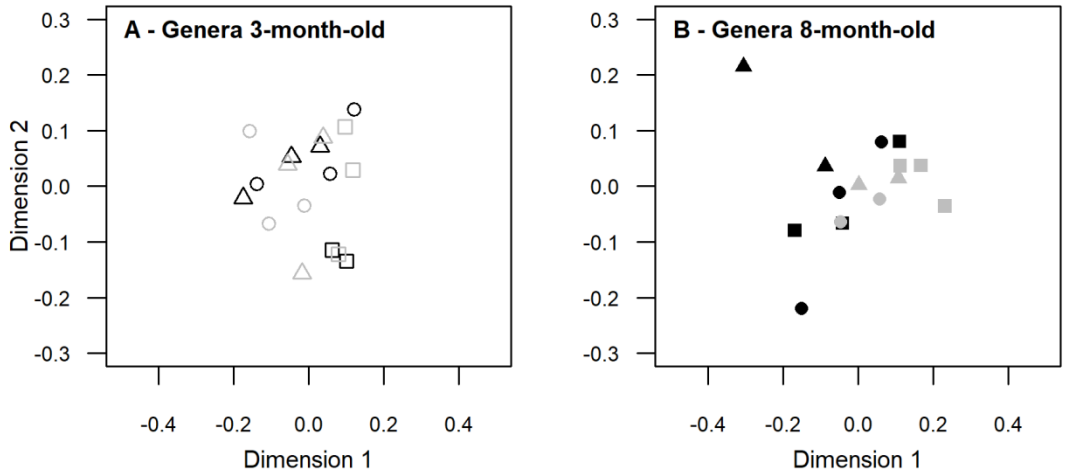

Fig. 5. Classical (metric) multidimensional scaling (MDS) diagram of fungal community composition in the foliage, using taxonomic units for genera, among the *Eucalyptus grandis* seedlings derived from three genotypes (■=G1, ●=G2, ▲=G3), replicated in two maternal environments (Greytown and Kwambonambi). (A) MDS using 3-month-old (growth chamber) seedling samples. (B) MDS using 8-month-old (open greenhouse) seedling samples.

Community analysis done using taxonomic units with order and family identities assigned to each OTU showed the same trend as that when the genera were considered as taxonomic units (Fig. S4-S5).

4. Discussion

4.1. Experiment 1: Maternal effects on seed and seedling performance

Evidence is provided that genetic effects influence seed mass in *E. grandis* and this would influence the reserves available for plant establishment. However, there were not only genetic effects, but also maternal effects responsible for differences in the germination rate of the seedlings. The results also indicate that seeds from the Greytown orchard had a higher viability (showing a higher germination rate) than seeds from

Kwambonambi. Contrasting maternal effects have been reported previously in the closely related species, *Eucalyptus globulus* (Lopez et al., 2003). In that study, maternal environment influenced the seed mass, but not the germination traits. In a more recent study on the same host, the genotype of the mother plant was shown to affect seed germination, but not seed mass (Rix et al., 2012).

Seedling growth in this study was influenced by the maternal environment. This effect increased with time, based on monthly growth measurements, for the seedlings from the two different maternal environments. To quantify whether the influence of the maternal environment was mediated by seed provisioning, seed mass was considered in the analysis. Differences in seedling growth from germination to 8-months-old could be explained by the influence of seed mass and thus resources allocated to the seed by the mother plants. However, from 8-months-old to the end of the study, the seed mass effect dissipated. These results confirm that seed provisioning had an ephemeral influence on *E. grandis* seedling growth and that its influence becomes less detectable with time. This suggests that maternal effects mediated by other (e.g. epigenetic) mechanisms were active. These findings are similar to those for seed mass variation when analyzing the influence of maternal effects on early seedling performance in other plant species (Halpern, 2005; Lopez et al., 2003; Vivas et al., 2013).

4.2. Experiment 2: Maternal effects on seedling pest and pathogen response

Both maternal environment and genotype influenced the resistance of the *E. grandis* seedlings to infection by the canker pathogen *C. austroafricana*. This influence was significant, even when considering the seed mass in the analysis. It was interesting that there was no interaction between the effects of the maternal environments and the genotypes on seedling resistance to the pathogen. This implies that the maternal effect was similar between the genotypes; with seedlings from the Greytown orchard more susceptible than those from Kwambonambi.

Although not significant ($P < 0.05$) *L. invasa* infestation was 2 % higher in seedlings from Greytown than from the orchard in Kwambonambi. Previous studies have shown substantial variation in susceptibility to the infestation by *L. invasa* between *Eucalyptus* genotypes, even amongst ramets of a genotype (e.g. Dittrich-Schröder et al., 2012; Mendel et al., 2004). This suggests that the variation in susceptibility to *L. invasa* within

E. grandis clones (between ramets) could mask the influence of the maternal environment. Nevertheless, seedlings from Kwambonambi appeared to be more resistant to both fungal infection and insect infestation than those from the Greytown orchard.

The mother plants in the Kwambonambi orchard were visibly more affected by pests and diseases than in Greytown. The fact that seedlings raised from Kwambonambi mother plants were more resistant could reflect transgenerational resistance to pests and pathogens (Galloway and Etterson, 2007). Such transgenerational effects have been suggested as a strategy by which plants can increase resistance when they are exposed to the same damage as those from previous generations (Galloway and Etterson, 2007). Such maternal effects have been demonstrated in many studies on short-lived annual plants (e.g. Agrawal, 2002; Rasmann et al., 2012), but have been less-well studied in trees (e.g. Holeski et al., 2013), possibly due the complexities of dealing with their longer life-spans.

The results of this study suggest that the transgenerational resistance to pests and pathogens found in *E. grandis* seedlings from Kwambonambi orchard is likely a maternal effect mediated by epigenetic mechanisms. It has been shown in other studies that epigenetic changes in mother plants can lead to induction of transgenerational resistance (Zhu et al., 2016). It is also known that biotic stresses can generate epigenetic modifications that represent an important source of phenotypic plasticity, even between generations (Dowen et al., 2012). However, few studies have addressed epigenetic modifications associated with stresses in trees other than those considering *Populus* spp. (Gourcilleau et al., 2010; Lafon-Placette et al., 2013; Raj et al., 2011; Vining et al., 2012). It would clearly be worthwhile to consider the role of epigenetic modifications in *Eucalyptus* transgenerational resistance in the future.

4.3. Experiment 3: Maternal effects on the community of foliar fungi

The composition and diversity of the fungal community of the foliage was strongly correlated with sampling time. There was a higher OTU richness and a more homogenous distribution of diversity in 3-month-old seedlings (growth chamber) than in 8-month-old seedlings (open greenhouse). These results were influenced by the sampling time point (3-month-old *vs.* 8-month-old seedlings) and/or the sampling

location (growth chamber *vs.* open greenhouse). It was, however, not possible to disentangle the effects of these factors.

The fungal communities at the two sampling times could be differentiated from each other. This suggests a horizontal acquisition of these fungal communities of *E. grandis* seedlings. This is consistent with the fact that microorganisms are predominantly transmitted horizontally between tree hosts and generations (Ganley and Newcombe, 2006; Wilson, 1996). Consequently, a substantial variation of fungal communities depending on sites (related to different environmental factors), time of the year, plant age and phenological state of the plant has been found in previous studies (Eusemann et al., 2016; García et al., 2013; Zimmerman and Vitousek, 2012). The controlled environment of the growth chamber where the *E. grandis* seedlings were germinated was very different from the open greenhouse environment where the seedlings were grown from 3-months-old onwards surrounded by other plant species (see Materials and Methods). Since, the transmission of the fungal communities occurred horizontally in our study, to analyze the data within sampling time was essential to assess maternal effects.

When the sampling times were analyzed separately, fungal community composition as represented by genera differed significantly between 3-month-old seedlings from the different genotypes. This is in agreement with a growing body of literature showing that plant genotypes can define the structure of fungal communities (e.g. Agler et al., 2016; Lamit et al., 2015; Rajala et al., 2013). Our results extend this knowledge showing an intraspecific variation in the structure of fungal communities amongst *E. grandis* seedlings from mother plants that are closely related, and that grow along side each other.

The fungal community composition in seedlings sampled at 8-months-old from the open greenhouse differed depending on the maternal environments from which they had originated. We expected this significant maternal effect on the fungal communities of the seedlings, in accordance with the differing resistance of *E. grandis* seedling to an insect pest and a fungal pathogen between maternal environments (as discussed above). We suggest that if plant defense compounds provide important barriers for pathogens, this would likely also affect fungal endophytes (Agler et al., 2016; Saunders and Kohn,

2009). These results suggest that maternal effects can significantly influence the composition of fungal communities of *E. grandis* seedling. These different communities could induce changes to modify plant development and protection (Vannier et al., 2015).

5. Conclusions

This study supports the idea that the abiotic/biotic differences in the maternal environment influence the early phenotype of *E. grandis* seedlings and their biotic interactions. To our knowledge, this is the first study showing that the maternal environment can also influence the structure of fungal communities of the progeny. More detailed studies (cf. e.g. Yakovlev et al., 2014; Yakovlev et al., 2011; Yakovlev et al., 2010) are now needed to explain the transgenerational plasticity behind this maternal effect in *Eucalyptus* trees. Particularly in a changing climatic environment, a better understanding of maternal effects will be increasingly important to maximize plant growth while minimizing the negative effects associated with stressors in the offspring. The identification of maternal effects will also allow us to enhance the efficiency of breeding programs in the forestry sector.

Acknowledgements

We thank Melissa Reynolds, Christy Marais, Patience Ralikonyana, Maleshoane Selaocoe and Zander Myburg for the DNA fingerprinting analysis, MONDI Forests for providing the plant material. This work was supported by the Claude Leon Foundation, University of Pretoria, the Tree Protection Co-operative Programme, and the Genome Research Institute at the University of Pretoria.

Author's contributions

MV, MK and BS planned and designed the study; MV, MK and MM were responsible for fieldwork and MM for environmental data; MV performed experiments and MK characterized the fungal community; MV analysed the data; MV, MK, MJW and BS contributed to the interpretation and discussion of the results; MV wrote the manuscript with contributions from all authors.

References

Abarenkov K, Henrik Nilsson R, Larsson K-H, et al. 2010. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytologist 186, 281-285.

Agler MT, Ruhe J, Kroll S, Morhenn C, Kim S-T, Weigel D, Kemen EM. 2016. Microbial hub taxa link host and abiotic factors to plant microbiome variation. PLoS Biology doi: 10.1371/journal.pbio.1002352

Agrawal AA. 2002. Herbivory and maternal effects: Mechanisms and consequences of transgenerational induced plant resistance. Ecology 83, 3408-3415.

Agrawal AA, Laforsch C, Tollrian R. 1999. Transgenerational induction of defences in animals and plants. Nature 401, 60-63.

Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MHH, White JDS. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends in Ecology and Evolution 24, 127-135.

Boyko A, Kovalchuk I. 2011. Genome instability and epigenetic modification - heritable responses to environmental stress? Current Opinion in Plant Biology 14, 260-266.

Caporaso JG, Kuczynski J, Stombaugh J, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336.

Chen SF, Wingfield MJ, Li GQ, Liu FF. 2016. *Corticimorbus sinomyrti* gen. et sp. nov. (Cryphonectriaceae) pathogenic to native *Rhodomyrtus tomentosa* (Myrtaceae) in South China. Plant Pathology 65, 1254–1266.

Dittrich-Schröder G, Wingfield MJ, Hurley BP, Slippers B. 2012. Diversity in *Eucalyptus* susceptibility to the gall-forming wasp *Leptocybe invasa*. Agricultural and Forest Entomology 14, 419-427.

Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, Dixon JE, Ecker JR. 2012. Widespread dynamic DNA methylation in response to biotic stress. Proceedings of the National Academy of Science 109, E2183-2191.

Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194-2200.

Elwell AL, Gronwall DS, Miller ND, Spalding EP, Brooks TLD. 2011. Separating parental environment from seed size effects on next generation growth and development in Arabidopsis. Plant Cell and Environment 34, 291-301.

Eusemann P, Schnittler M, Nilsson RH, Jumpponen A, Dahl MB, Wurth DG, Buras A, Wilmking M, Unterseher M. 2016. Habitat conditions and phenological tree traits overrule the influence of tree genotype in the needle mycobiome-*Picea glauca* system at an arctic treeline ecotone. New Phytologist 211, 1221-1231.

FAO. 2010. Global Forest Resources Assessment 2010 – Main report. *FAO Forestry Paper 163*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Galloway LF, Etterson JR. 2007. Transgenerational plasticity is adaptive in the wild. Science 318, 1134-1136.

Ganley RJ, Newcombe G. 2006. Fungal endophytes in seeds and needles of *Pinus monticola*. Mycological Research 110, 318-327.

García E, Alonso Á, Platas G, Sacristán S. 2013. The endophytic mycobiota of *Arabidopsis thaliana*. Fungal Diversity 60, 71-89.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. Molecular Ecology 2, 113-118.

Gourcilleau D, Bogeat-Triboulot M-B, Le Thiec D, Lafon-Placette C, Delaunay A, El-Soud WA, Brignolas F, Maury S. 2010. DNA methylation and histone acetylation: genotypic variations in hybrid poplars, impact of water deficit and relationships with productivity. Annals of Forest Science 67, 208-208.

Halpern SL. 2005. Sources and consequences of seed size variation in *Lupinus perennis* (Fabaceae): adaptive and non-adaptive hypotheses. American Journal of Botany 92, 205-213.

Hauser MT, Aufsatz W, Jonak C, Luschnig C. 2011. Transgenerational epigenetic inheritance in plants. Biochimica et Biophysica Acta 1809, 459-468.

Herman JJ, Sultan SE. 2011. Adaptive transgenerational plasticity in plants: case studies, mechanisms, and implications for natural populations. Forntiers in Plant Science 2, 1-10.

Holeski LM, Jander G, Agrawal AA. 2012. Transgenerational defense induction and epigenetic inheritance in plants. Trends in Ecology & Evolution 27, 618-626.

Holeski LM, Zinkgraf MS, Couture JJ, Whitham TG, Lindroth RL. 2013. Transgenerational effects of herbivory in a group of long-lived tree species: maternal damage reduces offspring allocation to resistance traits, but not growth. Journal of Ecology 101, 1062-1073.

Jablonka E, Raz G. 2009. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. Quarterly Review of Biology 84, 131-176.

Lafon-Placette C, Faivre-Rampant P, Delaunay A, Street N, Brignolas F, Maury S. 2013. Methylome of DNase I sensitive chromatin in *Populus trichocarpa* shoot apical meristematic cells: a simplified approach revealing characteristics of gene-body DNA methylation in open chromatin state. New Phytologist 197, 416-430.

Lamit LJ, Busby PE, Lau MK, et al. 2015. Tree genotype mediates covariance among communities from microbes to lichens and arthropods. Journal of Ecology 103, 840-850.

Linkies A, Graeber K, Knight C, Leubner-Metzger G. 2010. The evolution of seeds. New Phytologist 186, 817-831.

Lopez GA, Potts BM, Vaillancourt RE, Apiolaza LA. 2003. Maternal and carryover effects on early growth of *Eucalyptus globulus*. Canadian Journal of Forest Research 33, 2108-2115.

Luna E, Bruce TJ, Roberts MR, Flors V, Ton J. 2012. Next-generation systemic acquired resistance. Plant Physiology 158, 844-853.

Mendel Z, Protasov A, Fisher N, La Salle J. 2004. Taxonomy and biology of *Leptocybe invasa* gen. & sp. n. (Hymenoptera: Eulophidae), an invasive gall inducer on *Eucalyptus*. Australian Journal of Entomology 43, 101-113.

Mousseau TA, Fox CW. 1998. The adaptive significance of maternal effects. Trends in Ecology & Evolution 13, 403-407.

Nakabonge G, Roux J, Gryzenhout M, Wingfield MJ. 2006. Distribution of chrysoporthe canker pathogens on *Eucalyptus* and *Syzygium* spp. in Eastern and Southern Africa. Plant Disease 90, 734-740.

Oksanen J, Kindt R, O'Hara RB. 2005. vegan: Community Ecology Package version 1.6-9.

Peñuelas J, Terradas J. 2014. The foliar microbiome. Trends in Plant Science 19, 278-280.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945-959.

Raj S, Bräutigam K, Hamanishi ET, Wilkins O, Thomas BR, Schroeder W, Mansfield SD, Plant AL, Campbell MM. 2011. Clone history shapes *Populus* drought responses. Proceedings of the National Academy of Sciences 108, 12521-12526.

Rajala T, Velmala SM, Tuomivirta T, Haapanen M, Müller M, Pennanen T. 2013. Endophyte communities vary in the needles of Norway spruce clones. Fungal Biology 117, 182-190.

Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, Agrawal AA, Felton GW, Jander G. 2012. Herbivory in the previous generation primes plants for enhanced insect resistance. Plant Physiology 158, 854-863.

Rastogi G, Coaker GL, Leveau JHJ. 2013. New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS Microbiology Letters 348, 1-10.

RCoreTeam. 2014. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Rix K, Gracie A, Potts B, Brown P, Spurr C, Gore P. 2012. Paternal and maternal effects on the response of seed germination to high temperatures in *Eucalyptus globulus*. Annals of Forest Science 69, 673-679.

Roach DA, Wulff RD. 1987. Maternal effects in plants. Annual Review of Ecology and Systematics 18, 209-235.

Saunders M, Kohn LM. 2009. Evidence for alteration of fungal endophyte community assembly by host defense compounds. New Phytologist 182, 229-238.

Vannier N, Mony C, Bittebière A-K, Vandenkoornhuyse P. 2015. Epigenetic mechanisms and microbiota as a toolbox for plant phenotypic adjustment to environment. Frontiers in Plant Science 6, 1159.

Vining KJ, Pomraning KR, Wilhelm LJ, Priest HD, Pellegrini M, Mockler TC, Freitag M, Strauss SH. 2012. Dynamic DNA cytosine methylation in the *Populus trichocarpa* genome: tissue-level variation and relationship to gene expression. BMC Genomics 13, 1-19.

Violle CH, Castro H, Richarte J, Navas ML. 2009. Intraspecific seed trait variations and competition: passive or adaptive response? Functional Ecology 23, 612-620.

Vivas M, Kemler M, Slippers B. 2015. Maternal effects on tree phenotypes: considering the microbiome. Trends in Plant Science 20, 541-544.

Vivas M, Zas R, Sampedro L, Solla A. 2013. Environmental maternal effects mediate the resistance of Maritime pine to biotic stress. *PLoS ONE* doi:10.1371/journal.pone.0070148

Vorholt JA. 2012. Microbial life in the phyllosphere. Nature Reviews Microbiology 10, 828-840.

White T, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Shinsky J, White T, eds. *PCR Protocols: A Guide to Methods and Applications.* San Diego: Academic Press, 315-322.

Wilson D. 1996. Manipulation of infection levels of horizontally transmitted fungal endophytes in the field. Mycological Research 100, 827-830.

Wingfield MJ, Brockerhoff EG, Wingfield BD, Slippers B. 2015. Planted forest health: The need for a global strategy. Science 349, 832-836.

Wingfield MJ, Roux J, Slippers B, Hurley BP, Garnas J, Myburg AA, Wingfield BD. 2013. Established and new technologies reduce increasing pest and pathogen threats to Eucalypt plantations. Forest Ecology and Management 301, 35-42.

Yakovlev I, Lee Y, Rotter B, Olsen J, Skrøppa T, Johnsen Ø, Fossdal C. 2014. Temperature-dependent differential transcriptomes during formation of an epigenetic memory in Norway spruce embryogenesis. Tree Genetics & Genomes 10, 355-366.

Yakovlev IA, Asante DK, Fossdal CG, Junttila O, Johnsen O. 2011. Differential gene expression related to an epigenetic memory affecting climatic adaptation in Norway spruce. Plant Science 180, 132-139.

Yakovlev IA, Fossdal CG, Johnsen Ø. 2010. MicroRNAs, the epigenetic memory and climatic adaptation in Norway spruce. New Phytologist 187, 1154-1169.

Zhang J, Kobert K, Flouri T, Stamatakis A. 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614-620.

Zhu QH, Shan WX, Ayliffe MA, Wang MB. 2016. Epigenetic mechanisms: an emerging player in plant-microbe interactions. Molecular Plant Microbiome Interaction 29, 187-196.

Zimmerman NB, Vitousek PM. 2012. Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. Proceedings of the National Academy of Science 109, 13022-13027.