First Report

Methods of Inducing Early Flowering in Oak

18th May 2017

Prepared for the
Future Trees Trust (FTT)

by

Rodrigo Olave & Colin Kelleher

AFBI & NBG

This report should be cited as:

Olave, R.J & Kelleher, C. (2017). *Methods of inducing early flowering in oak (Quercus robur* L*)*. Report prepared for Future Trees Trust.

Executive Summary

1. Relatively little is known about the effects of tree size and nursery culture on fructification of oak (*Quercus robur* L.) trees. Yet, accelerated growth is a primary determinant of changing from juvenile to mature trees and induction of early flowering. Early acorn production in oak trees could be an enormous benefit for genetic improvement.

2. The study used two experimental sites at Castlearchdale and Loughgall in Northern Ireland to evaluate the effects of two planting stock types (RPM® and CG) and crown release on acorn production of oak trees between 2001 and 2016.

3. Out of 150 and 200 RPM® oak trees at Castlearchdale and Loughgall, respectively, 12% of trees produced acorns at the age of 8 years old which was 6 times more than CG trees. RPM® oak trees began to fructify at the age of 5 (12%) at Loughgall, with 12% at 8 years old, increasing to 28% at 10 and decreasing to 17% the following year.

4. There was no evidence that by removing trees and opening tree plots at both sites in 2015, it could have influenced acorn production in 2016. However any effect might have been clouded by factors such as grey squirrel damage at Loughgall in 2015 and/or irregular natural fructification intervals of oak trees.

5. DNA analysis showed no evidence of grouping between populations. The ISSR are not located in coding regions and provided some reliable fingerprint profiles to be used to assess genetic diversity in the study population. A follow up work is in process to assess variation in gene responsible for flowering.

6. The results stress the importance of considering indirect factors and interactions when evaluating the effects of phenotypic and genotypes characteristics on fructification dynamic of oak trees. RPM® planting stock might have potential to increase acorn production, if used as rootstock to graft mature scions.

Contents

1. Introduction

Tree breeders in Britain and Ireland have expressed concern about difficulties to accelerate flowering of oak trees for their improvement programmes. Flowering of oak trees is a lengthy process that can take up to 30 years as natural seed production depends on maturation processes (Philipson, 1990). This is a serious problem for the Future Trees Trust (FTT) to accelerate progeny testing and speed the production of genetically improved seed.

Oak tree seedlings produced using the Root Production Method (RPM®) and established in 2001 at two experimental sites (Castlearchdale and Loughgall) in Northern Ireland have shown earlier flowering than conventionally raised Cell Grown (CG) tree seedlings. Reports from these experimental sites suggest that growing oak trees with the RPM® technique would induce flowering and acorn production in a shorter time by accelerated tree growth. The RPM®, developed by the Forrest Keeling Nursery in the US, and CG nursery techniques are a multistep system of producing containerised tree seedlings which places primary emphasis on the root system by using a series of containers on raised open benches (Walter et al., 2013).

Research (Meilan, 1997) has suggested that the most promising treatment for hastening the change from the juvenile to the mature phase is to grow plants as fast as possible to a certain minimum size, and then place them in flower inducing conditions. Thus from perceived advantages of RPM® planting stock to rapid growth and early flowering , a cut off tree seedling height of 76cm is recommended in the RPM® process.

The aims of this project were to examine the effect of tree seedling size and nursery stock type (RPM® and CG) on acorn production and their genetic predisposition to fructify from previous assessments at two field experiments in Northern Ireland. The influence of crown release by the removal of trees in 2015 to induce acorn production of RPM® and CG trees was also evaluated in 2016.

2. Methods

In January 2001, oak trials were established using RPM® and CG tree seedlings at Castlearchdale (lat 54.4^{o} N, long 7.7^{o} W) and Loughgall (lat 54.4 ^{o} N, long 6.6 ^{o} W) in Northern Ireland (Appendix 1). The sites were formerly grassland (mainly perennial ryegrass). The soils are predominantly freely drained, gleyed, overlaying glacial till at Castlearchdale and Brown Earth on Red Limestone Till at Loughgall. The mean annual temperature between 2001 and 2016 at Loughgall and Castlearchdale was 8.9^{o}C and 8.3^{o}C, respectively. Mean annual precipitation for Loughgall was 820mm and 1150mm for Castlearchdale during the same period. Soil pH was 5.51 and 7.41 for Castlearchdale and Loughgall, respectively.

2.1. Nursery Culture Process

RPM® seedling production: it is a patented procedure where acorns are germinated in flat trays (47cm x 37cm x 6cm) and after two months they are transferred into a bottomless band container (7.3cm x 7.3cm x 14cm) , which leaves the roots exposed to air, resulting in growth of many small lateral and fibrous roots. During May oak tree seedlings are transplanted into 7.5L containers for the remainder of the growing season.

CG seedling production: Acorns are germinated in trays and after two months they are placed in root-trainer containers which train the roots to grow down and become long and dense. Oak tree seedlings are transplanted into the field at the end of their growing season.

2.2. Experimental sites

Castlearchdale

RPM® and CG oak tree seedlings were planted at 2m x 2m in a randomised block design (Fig. 1). There were four treatments: RMP®1 and RPM®2 (trees under and over 76cm) and CG1 (cell grown 20-50cm) and CG2 (cell grown over 50cm); replicated in 3 blocks. Each plot (treatment) comprises 25 test trees with a single buffer row between plots and a double buffer row between blocks (Figure.1).

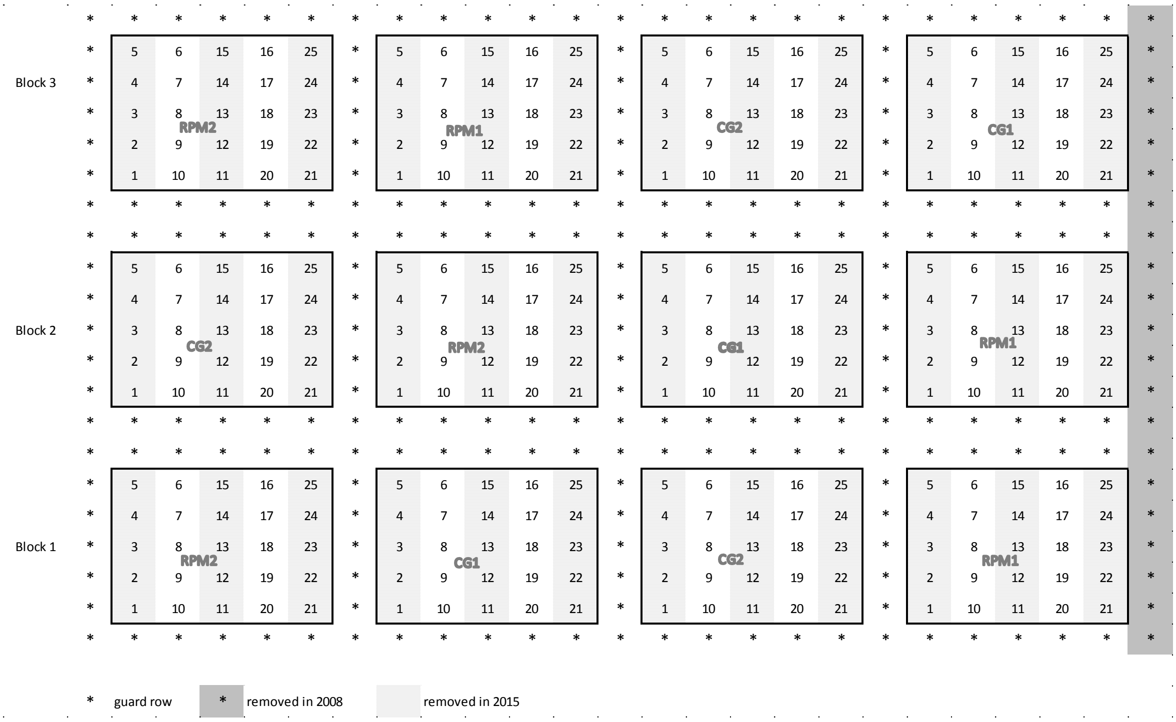

Figure. 1 Layout of oak trees, planted at Castlearchdale in Northern Ireland. Numbers and asterisks represent oak trees in each one of the field plot/treatments.

Loughgall

RPM® oak tree seedlings were planted at a density of 4m x 1.5m. The experimental design consisted of 4 randomised blocks containing 50 RPM® oak trees each (Figure 2) of varied sizes. The two treatments were the initial height before outplanting and whether or not they were greater than 76cm in height.

Site management

No fertiliser was applied to either site. At Loughgall, blocks were mechanically weeded when competition grew taller than approx. 5cm and the remaining vegetation around the trees was controlled using Gramoxone. At Castlearchdale, polypropylene mulch weed mats were placed round stem bases after planting and the area was only mechanically weeded in 2008. RPM® oak trees at Loughgall were sprayed with Topaz 50 WP (1,2kg/700 l water) to prevent mildew (*Microsphaera alphitoides*) in 2002.

Domestic and wild animals were excluded from the Castlearchdale experiment by a wired fence around the area and in Loughgall brown plastic tree shelters were used (72cm tall Gro-cones, Acorn Planting Products, UK) and removed in 2004.

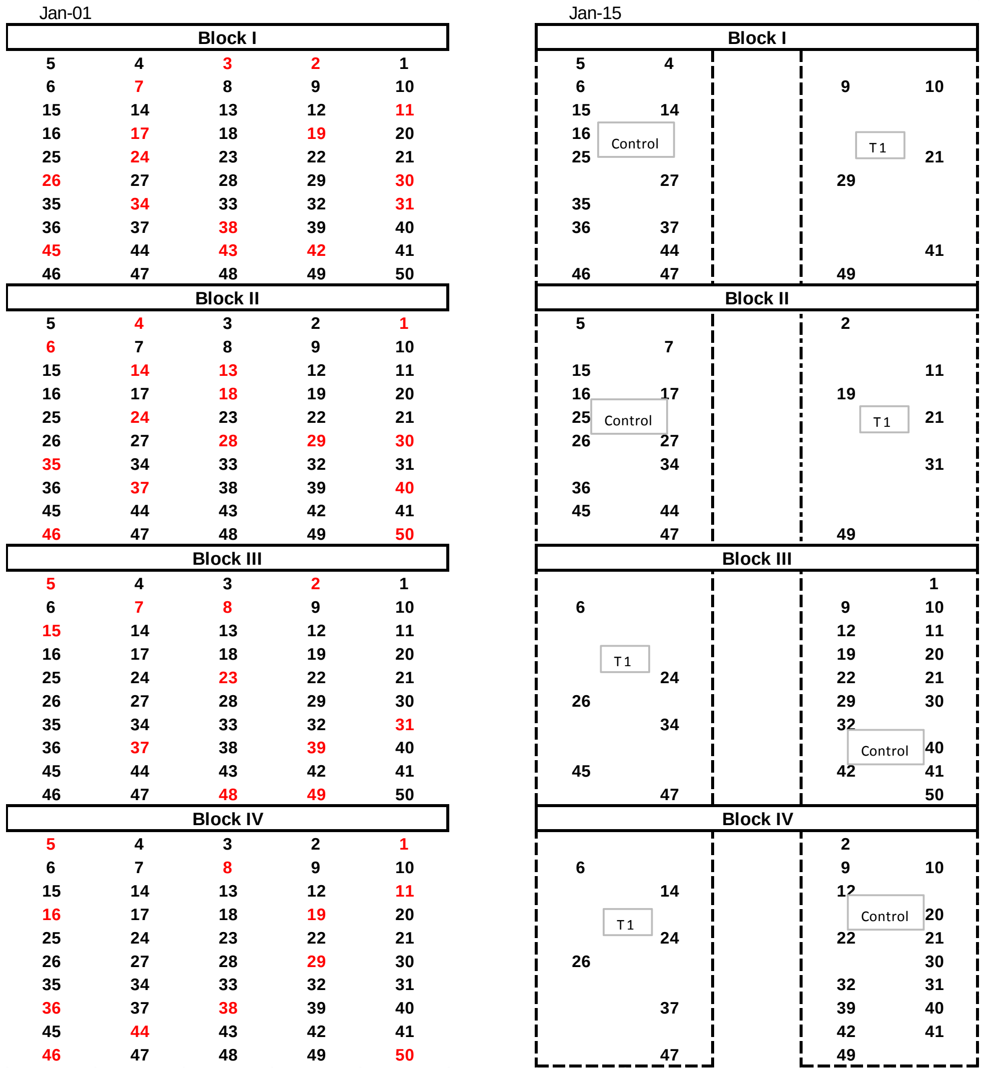

Figure 2. Layout of oak trees, planted at Loughgall in Northern Ireland. Numbers represent oak trees planted in 2001. Numbers in red represent trees removed in 2008. Blank spaces represent trees removed in 2015.

Crown release treatment applied in 2015

To assess the influence of crown release on acorn production of RPM® and CG trees, 180 and 76 trees were removed (Figure 1 and 2, Appendix 1) from Castlearchdale and Loughgall sites, respectively, during January and February 2015 (Table 1).

Table 1. Crown release treatment applied at Castlearchdale and Loughgall in 2015 to allow trees full light exposure to induce acorn production.

Site	Date	Treatment
Castlearchdale	January/February 2015	Three of the five rows of trees were felled in each plot leaving row 2 (trees 6-10), 4 (trees 16-20) and the buffer rows. Tree growing space: 4m x 2m.
Loughgall	January/February 2015	The third row of trees was completely removed and only two treatments retained (control and 6 standing trees per plot) and replicated 4 times. Approx. tree growing space: 4.5m x 4m.

2.3. Assessments

Field data (Initial height and basal diameter) were collected in 2001, after planting and again between 2002 and 2008 at Castlearchdale and Loughgall. Diameters at 70cm were measured in 2006 and 2008. Total height and DBH were again measured in 2015.

Acorn production of individual trees was estimated by .observing the presence or absence of acorns in the interior of the treatment plots in a radius circle at each tree stem. Acorn assessment was carried out at Castlearchdale in 2008 and in 2005, 2008, 2010 and 2011 at Loughgall.

Due to a severe attack by grey squirrels (*Sciurus carolinensis*) in spring and summer of 2015 at the Loughgall site, a damage assessment was carried out in 2015 and an acorn assessment at both sites in September 2016.

2.4. DNA analysis

Leaves from 120 and 82 trees from Castlearchdale and Loughgall respectively were collected in September 2015 for DNA analysis. DNA samples were extracted and three testing ISSR loci (GTG5, ACTG5, CGA4) were employed to explore genetic diversity of the populations of trees producing and not producing acorns. The ISSR loci were chosen because of their proven ability to detect polymorphism. They are neutral genetic markers, i.e. they do not code for particular genes, and so they tend to accumulate more variation than conserved coding regions. DNA was extracted using a Maxwell 16 LEV Plant DNA Kit. All samples were first amplified with universal Chloroplast Primers CPE & CPF to ensure that all sample DNA was of amplifiable quality. Samples were amplified in a final volume of 25ul., 12.ul of 2x TopTaq (Qiagen, UK), 1ul of forward and reverse primers CP-E & CP-F @10um (Eurogentec, Belgium), 1ul template DNA and 9.5ul ddH2O. Subsequently reactions were amplified on Bioer thermal cyclers (LifePro, China) under the following conditions: 1 cycle of 95^{o}C for 5 mins, followed by 37 cycles of 95^{o}C for 30secs, 55^{o}C for 30 secs, 72^{o}C for 30 secs and finally followed by 1 cycle at 72^{o}C final extension for 10 mins. For the Inter Simple Sequence Repeats (ISSR), samples were amplified in a final volume of 25ul., 12.ul of 2x TopTaq (Qiagen, UK), 1ul primer (Eurogentec, Belgium), 1ul template DNA and 10.5ul ddH2O. After amplification all samples were analysed on a Capillary electrophoresis system - Qiaxcel Advanced (Qiagen, UK), to detect the presence of an ISSR fingerprint for each sample.

2.5. Statistics

Initial height, basal stem diameter and log relative growth data obtained from the Castlearchdale trial were subjected to analysis of variance and covariance, where both initial height and basal stem diameter at planting were used as the covariate.

The treatments were the RPM® and CG methods and the two size classes within each method.

Correlation coefficients were calculated for the height, basal diameter and their growth increments for the Loughgall experiment. These variables were also analysed for differences between the two treatments (initial height ≥76cm and initial height <76cm) using analysis of variance (ANOVA) to look for differences between both categories.

A logistic regression for both experiments was used to analyse a binomial response variable (1=yes, 0=no) to predict the acorn presence probability for RPM® and CG trees. In this the log "odds", i.e. log (p/(1-p)) where p=probability (acorn presence), were modelled on each of the plant-size-at-different-dates variables and on the initial plant height factor. Thus the estimated yearly acorn presence probability is expressed by a logistic function:

$$\log\left(\frac{p_1}{1-p_1}\right) = \text{Constant} + \text{Estimate} * \text{Predictor}(year)$$

ANOVA was also used to determine significant fructification percentage rate differences in relation to different crown release rates, planting stock type and tree seedling size at Castlearchdale and Loughgall after the removal of trees in 2015.

From the DNA fingerprint, the system created peak calling tables for each primer. The peak calling tables were then exported to Microsoft Excel where the peak calling results were converted to a presence/absence matrix for each primer by replacing 'presence' with 1 and 'absence' with 0. Subsequently the data from ISSR fragments were analysed using GenAlEx 6.5 to estimate diversity and to undertake a principal coordinate analysis (PCA) to investigate the data for clustering or grouping of individuals (Peakall and Smouse 2012).

3. Results

3.1. Growth effects

Figure 3 shows the overall treatment means for height and basal diameter and their changes over 15 years in the field. Initial height at planting time had a significant effect (p<0.001) on subsequent heights during the first 8 years for both RPM® and CG planting stock. However such differences in height disappeared over time.

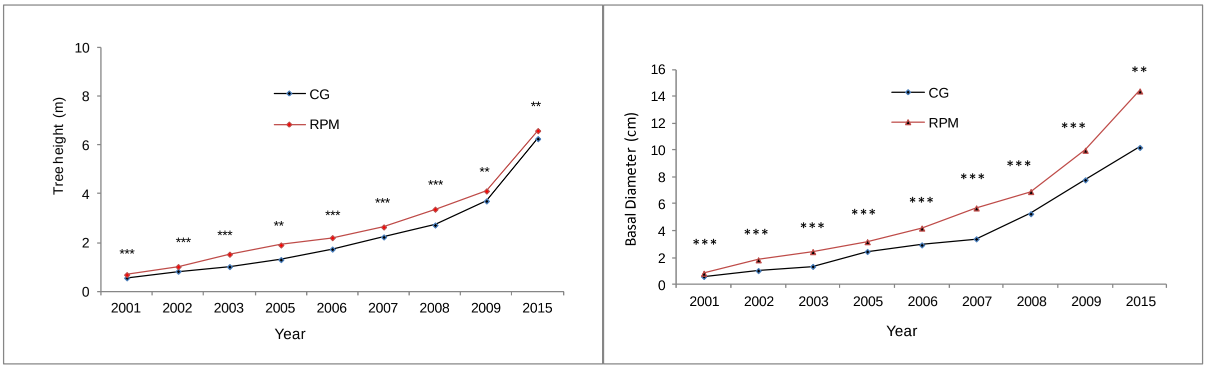

Figure 3. Mean variation of tree height and basal stem diameter of oak trees from two different planting stock types at planting (RPM® and CG) and over 15 years in the field at Castlearchdale. Significance: *** P<0.001; ** P<0.01.

RPM® trees had significantly (p<0.001) larger basal diameters than CG trees since planting (Figure 3). In 2008, when acorn production was assessed for the first time at Castlearchdale, RPM® trees were 1.1m and 1.7cm significantly (p<0.001) taller and thicker, respectively, than CG trees. Such difference was smaller for height and larger for basal diameter in 2015 (Figure 3).

However by analysing the variation of tree height and basal stem diameter of oak trees from the four different tree seedling sizes at Castlearchdale, Figure 4 shows that trees produced by the RPM® and CG techniques were consistently similar.

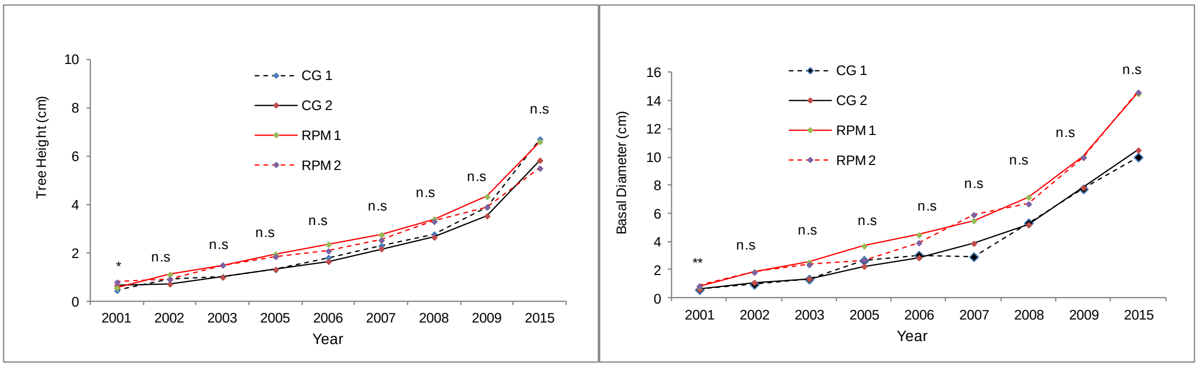

Figure 4. Mean variation of tree height and basal stem diameter of oak trees from four different sizes at planting and over 15 years in the field at Castlearchdale. Significance: *P<0.05; n.s; not significant.

By 2015, mean height of RPM®1 and RPM®2 trees was 6.6m and 5.5m, respectively. In contrast, height for CG1 and CG2 was lower but they were statistically similar.

Similarly, trees produced by the RPM® technique consistently had the greatest basal stem diameter. The average basal diameter of both RPM® types of trees increased 13.5cm in 15 years, whereas CG trees increased only 9.6 cm, respectively. The basal diameter of RPM®2 trees (>76cm in height) increased the most over the 15 years in the field (Figure 4).

Figure 5 shows the treatment means for initial height and basal stem diameter of oak trees over 15 years in the field at Loughgall. Initial planting stock sizes (76cm) did have a small effect on subsequent heights. RPM® planting stock below 76cm tall grew proportionally more than those above 76cm. These differences disappeared over time. Although height was good for both planting stocks (≥76cm and <76cm) over 15 years, clearly trees below 76cm grew faster than those above 76cm, averaging 8.1m and 8.2m, respectively, at 15 years old.

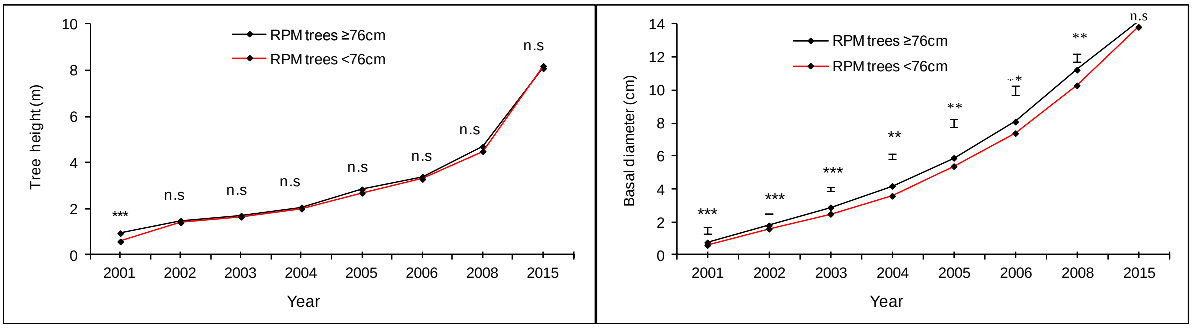

Figure 5. Mean variation of tree height and basal stem diameter of oak trees from two different sizes at planting (Initial height ≥76cm and <76cm) and over 15 years in the field at Loughgall. Vertical lines represent least significant difference at 5% level. Significance: *** P<0.001; ** P<0.01; *P<0.05; n.s; not significant.

Basal stem diameter (Figure 5) of RPM® trees above 76cm remained significantly (P<0.001) larger than those below 76cm for the eight first growing seasons. However in subsequent years their basal stem diameters were similar, averaging 14.2cm and 13.8c respectively.

Initial height and basal diameter between RPM® trees at Loughgall were weakly correlated (Appendix 1) with subsequent height and basal diameter. However these correlations were not very strong, for example the correlation between initial basal diameter and height decreased over the years. The strongest correlation was that between initial basal diameter and height in 2008, though this also decreased over time (Appendix 1).

Correlations (Appendix 1) between mean initial heights and diameters and subsequent heights and basal diameters in the field declined over time, demonstrating that below 76cm, planting stock can still grow faster and eventually catch up with those initially planted out above 76cm.

3.2. Fructification

The acorn assessment of 2008 at Castlearchdale showed that RPM® plots had 10% more trees producing acorns than CG plots at the age of 8 years. However, overall only 12% and 2% of all RPM and CG trees, respectively, produced acorns at the age of 8 years old. Nevertheless, in 2016, one year after applying the crown release treatment when RPM® and CG trees were 16 years old, only 9% of RPM® and CG trees had produced acorns.

Table 2. Observed number and percentage of RPM® and CG trees with acorn within each treatment at Castlearchdale.

Castlearchdale	Presence of acorns							
	2008				2016			
	RPM1	RPM2	CG1	CG2	RPM1	RPM2	CG1	CG2
Age	8	8	8	8	16	16	16	16
No Trees	7	11	1	2	6	3	5	4
Height (m)	3.6	3.4	3.3	2.6	6.3	6.4	5.6	5.6
Basal Diameter (cm)	7.6	7.6	7.2	5.1	14.2	14.4	13.5	13.8
% Trees with acorns	9.3	14.6	1.3	2.6	20	10	23.3	13.3

Table 3 shows the probability of RPM® and CG trees fructifying based on growth parameters and a fructification event in 2008. Although there is a significant relationship between growth parameters and fructification ($p<0.001$) the probability of producing acorns by either planting stock is low (12.7% and 2.2%) but significantly different.

Table 3. Logistic regression for estimating the probability of acorn production based on height, basal diameter and DBH of RPM® and CG trees at Castlearchdale.

Model	chi pr.	t pr.	Predictions from regression model	
			Prediction	SE
Regression	<0.001			
Constant		<0.001		
Covariate (H, BD, DBH)		<0.05		
Treatment	0.004			
CG1			0.014^{a}	0.143
CG2			0.031^{a}	0.021
RPM1			0.097^{b}	0.034
RPM2			0.157^{b}	0.042

Prediction values in the same column for a given planting stock type followed by different superscripts are significantly different. P<0.05. SE: Standard Error.

12% of all RPM® trees began to fructify at 5 years old at Loughgall which were on average just above 3m, with a basal diameter of 5.9cm (Table 4). This 12% was maintained after three years and RPM® trees produced acorns in each of almost every year from 2008 onwards. From the same year, the percentage of RPM® trees with acorns increased from 12% to 28% in 2010 and decreased to 17% in 2011. In contrast in 2016, one year since the crown release treatment was applied, 52% of trees produced acorns. However it could have been a stress effect in response to grey squirrel attack in 2015.

Table 4. Observed number and percentage of RPM® trees with acorn within each treatment at Loughgall.

	Presence of acorns									
Loughgall	2005		2008		2010		2011		2016	
	≥76cm	<76cm	≥76cm	<76cm	≥76cm	<76cm	≥76cm	<76cm	≥76cm	<76cm
Age	4	4	8	8	10	10	11	11	16	16
No Trees	11	13	6	18	17	42	9	16	13	30
Height (m)	3.2	2.9	5.4	4.9					9.1	9.2
Basal Diameter (cm)	6.0	5.8	13.6	11.9	*Not measured*				17.5	16.3
% Trees with acorns	17	9.5	9.3	13.2	34	43	18	16.5	48	54

Logistic regressions between growth variables and fructification events (Tables 5 and 6) at Loughgall also show that although height is significantly ($p<0.001$) correlated with acorn production, the fructification probability is very low (15%) despite acorn production being more likely to occur in taller trees than trees with larger basal diameter.

Despite the probability of acorn production increasing with height from 2005 to 2008, and this explanatory variable being found to be significant, the probability of trees to produce acorns was very low (approx 15%) for the majority of cases (Tables 5 and 6).

Table. 5. Logistic regression parameters for estimating the probability of acorn production based on height, basal diameter and DBH of RPM® trees at Loughgall.

Effect	2005			2008			2010			2011		
	chi pr.	Estimate	t pr.	chi pr.	Estimate	t pr.	chi pr.	Estimate	t pr.	chi pr.	Estimate	t pr.
Regression	0.015			0.006								
Constant		*-5.38*	*<0.001*		*-5.8*	*<0.001*						
Height 2002		*0.0228*	*0.016*		*2558*	*0.008*						
Regression	0.005			<0.001			0.031			0.033		
Constant		*-6.99*	*<0.001*		*-7.9*	*<0.001*		*-3.53*	*0.006*		*-5.58*	*0.002*
Height 2003		*0.0289*	*0.006*		*0.034*	*0.002*		*0.0157*	*0.033*		*0.0212*	*0.035*
Regression	0.005			0.001			0.004			0.032		
Constant		*-5.53*	*<0.001*		*-6.13*	*<0.001*		*-3.492*	*<0.001*		*-4.58*	*<0.001*
Height 2004		*0.01672*	*0.007*		*0.01944*	*0.002*		*0.0127*	*0.005*		*0.01258*	*0.034*
Regression	0.008			0.005			0.001			0.032		
Constant		*-5.92*	*<0.001*		*-6.2*	*<0.001*		*-4.13*	*<0.001*		*-4.95*	*0.001*
Height 2005		*0.01352*	*0.011*		*0.0144*	*0.007*		*0.01144*	*0.002*		*0.01043*	*0.038*
Regression	0.028			<0.001			0.014			0.02		
Constant		*-5.56*	*0.002*		*-11.41*	*<0.001*		*-3.59*	*0.002*		*-7.21*	*<0.001*
Height 2006		*0.01046*	*0.036*		*0.0265*	*<0.001*		*0.00812*	*0.018*		*0.01528*	*0.004*
Regression	0.029			<0.001								
Constant		*-5.23*	*0.001*		*-7.83*	*<0.001*						
Height 2008		*0.00685*	*0.037*		*0.01203*	*0.001*						
Regression				0.010								
Constant					*-6.95*	*0.002*						
Height 2015					*0.00632*	*0.017*						
Regression				0.007								
Constant					*-6.74*	*0.001*						
Overall Height					*0.0066*	*0.013*						

Table 6. Logistic regression parameters for estimating the probability of acorn production based on height, basal diameter and DBH of RPM® and CG trees at Loughgall.

Effect	2005			2008			2010			2011		
	chi pr.	Estimate	t pr.	chi pr.	Estimate	t pr.	chi pr.	Estimate	t pr.	chi pr.	Estimate	t pr.
Regression	0.017											
Constant		*-4.67*	*<0.001*									
RCD 2001		*0.359*	*0.019*									
Regression	0.008											
Constant		*-4.83*	*<0.001*									
BD at P		*4.09*	*0.001*									
Regression				0.021								
Constant					*-5.49*	*<0.001*						
Diamater 70					*0.0291*	*0.03*						
Regression				0.009								
Constant					*-5.2*	*<0.001*						
BD Increment 70					*0.519*	*0.015*						
Regression							0.007					
Constant								*-3.76*	*<0.001*			
Diamater 2005								*0.511*	*0.009*			
Regression							0.018					
Constant								*-3.69*	*0.003*			
Diamater 2006								*0.367*	*0.022*			
Regression							0.011					
Constant								*-3.86*	*0.002*			
Overall Diamater 2006								*0.424*	*0.014*			
Regression										0.002		
Constant											*-4.91*	*<0.001*
DBH 2015											*0.2118*	*0.004*

In relation to the crown release treatment applied in 2015, the assessment of all visible bark stripping damage caused by grey squirrels was quantified as moderate, as all trees at Loughgall had approximately between 10 and 20% of their bark removed. Oak tree plots at Castlearchdale didn't have any damage or signs of presence of grey squirrels in the area. Despite the outbreak of grey squirrel bark stripping at Loughgall, the acorn assessment carried out in 2016 showed (Figure 6) that the treatment did not have a significant effect on the number of trees producing acorns at either site in 2016. On average the percentage of trees with acorns between RPM® and CG trees was statistically similar. However there were more trees with acorns in those trees that were smaller at planting time.

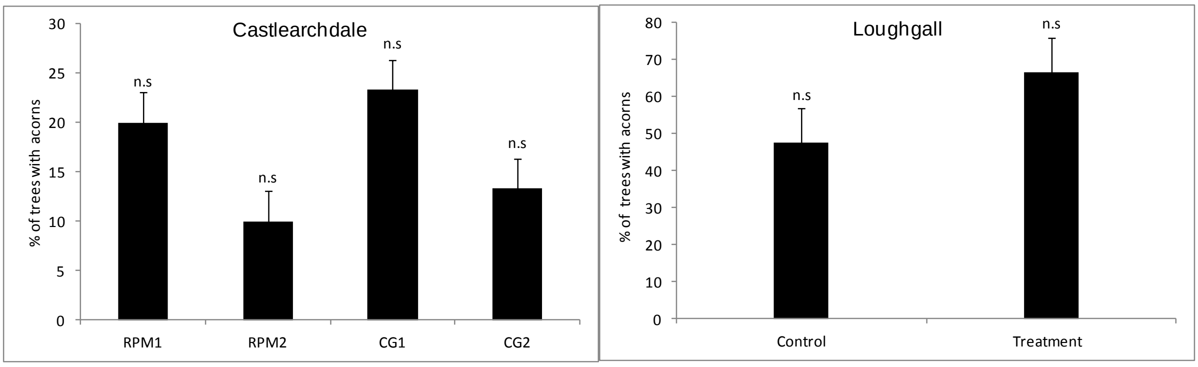

Figure 6. Effect of crown release treatment on mean percentage of trees with acorns, following the removal of trees in 2015 at Castlearchdale and Loughgall. Error bars are one standard error; n.s; Not significant.

Smaller RPM®1 and CG1 planting stocks had more trees with acorns than taller planting stocks (RPM®2 and CG2). RPM trees with acorns at Loughgall were highest at the crown release treatment plot with a much wider tree growing space than the control.

3.3. Genetic variation

Out of three ISSR primers screened, all of them were found to give satisfactory amplification in the population studied. The genetic distance analysis shown in Figure 7 did not indicate any clear grouping of the individuals. Axis 1 accounts for 39% of the variation and Axis 2 accounts for 19% of the variation, so much of the variation is still unaccounted for. There is a wide spread of the individuals across the axes and no obvious grouping, which would indicate genetic groupings. Neither is there a grouping based on acorn production.

The analysis of the sample populations using three primers shows that although there is some degree of variation between populations (i.e. Non acorn trees and acorn trees) the genetic diversity is moderate.

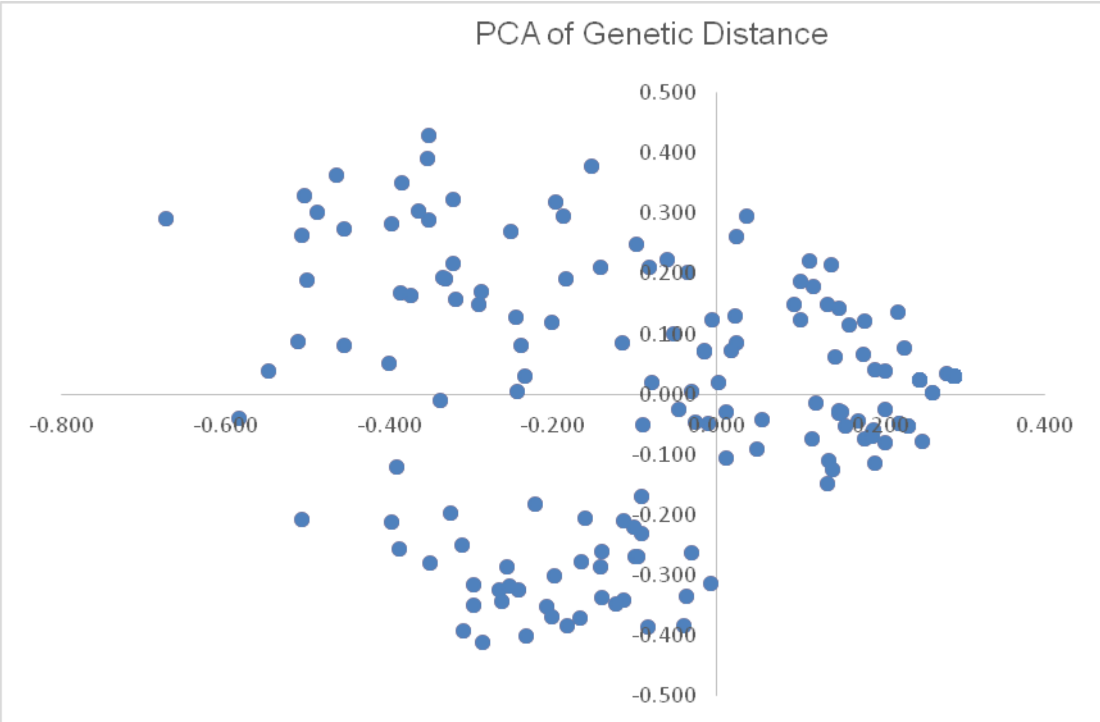

Figure 7. Principal Coordinate Analysis of the genetic distance data of RPM® and CG planting stock from Castlearchdale and Loughgall.

Locus GTG5 resulted in a total of 26 different alleles (bands), CGA4 had 21 alleles and ACTG5 had 14 alleles. Table 7 shows the numbers of alleles and the percentage of variable or polymorphic loci for each grouping or treatment in the different sites. The average number of alleles per population was 21 but the standard deviation was large due to the fact that some populations had only one individual.

Table 7. The number of alleles per population/treatment and the percentage of polymorphic loci per population (NB. Some populations only contain 1 individual).

Population	Site	No. Alleles	% Polymorphism
RPM	Loughgall	23	37.70%
CG1	Castlearchdale	28	45.90%
CG2	Castlearchdale	37	60.66%
RPM2	Castlearchdale	12	0.00%
RPM1	Castlearchdale	36	59.02%
RPM2	Castlearchdale	35	57.38%
CG1	Castlearchdale	10	0.00%
RPM	Loughgall	22	36.07%
RPM	Loughgall	40	65.57%
CG1	Castlearchdale	35	57.38%
CG2	Castlearchdale	27	42.62%
RPM2	Castlearchdale	13	19.67%
RPM1	Castlearchdale	29	47.54%
RPM2	Castlearchdale	19	31.15%
RPM	Loughgall	12	19.67%
RPM	Loughgall	0	0.00%
RPM	Loughgall	17	27.87%
RPM	Loughgall	12	19.67%
RPM	Loughgall	37	60.66%
RPM	Loughgall	19	31.15%
CG1	Castlearchdale	15	0.00%
CG2	Castlearchdale	7	0.00%
CG1	Castlearchdale	23	37.70%
CG2	Castlearchdale	28	45.90%
CG1	Castlearchdale	17	21.31%
RPM1	Castlearchdale	19	22.95%
RPM2	Castlearchdale	22	36.07%
RPM1	Castlearchdale	0	0.00%
	Mean	21.21	31.56%
	SE	11.03	4.06%

Table 8 shows average gene diversity and expected heterozygosity for the populations included in the study together with the total average. The expected heterozygosity is very low, indicating that the diversity overall is low. In addition the number of alleles per individual (Na) and the effective number of alleles (Ne) is very low.

Table 8. Diversity statistics estimated using the ISSR data from 28 populations.

	Mean	SE
N (mean sample no. Per pop)	7.143	0.107
Na (no. Different alleles)	0.663	0.022
Ne (no. Effective alleles)	1.101	0.005
He (Expected heterozygosity)	0.069	0.003

SE: Standard Error.

This shows (Table 8) that there is limited genetic diversity in the samples tested and thus high family relatedness. This is probably due to the fact that the individuals were all from a single seed batch and from one or a limited set of mothers and so are more genetically uniform than a wild population.

4. Discussion

The RPM® technique is based on root pruning, fertilisation and extending the growing season in the greenhouse (Dey et al., 2004; Dey et al., 2010) which aids the stimulation of flowering (Meilan, 1997). According to research in the US (Grossman et al 2003; Walter et al., 2013), large initial height RPM® tree seedlings are more likely to become firmly established and flower in a shorter time. Therefore these nursery practices that favour rapid accelerated vegetative growth, might have reduced the time taken for some RPM® trees to reach flowering time. Research (Meilan, 1997) has suggested that if pollination is not a limiting factor, seed production is roughly a function of plant size in most species. Oak trees grown by the RPM® process at both sites grew faster than CG trees as well as those produced conventionally.

Oak trees at both sites which were small initially grew more in the subsequent periods than the larger planting stock and in some cases, outgrew them. However while the use of the RPM® technique has had an obvious effect on the early growth of oak trees, the influence on fructification was not reliable. Nevertheless, grafting, for example, of mature scions onto RPM® trees, which causes phase reversion in woody plants, if used as rootstock, might help even more to shorten the juvenile period and induce acorn production.

Although RPM® oak trees were established as separated experiments, the response of oak trees to growth and acorn production were better at Loughgall than those in Castlearchdale, as a consequence of more suitable environmental conditions in the former site. Oak tree size and flowering increased with the application of the RPM® process (Table 4). Although oak trees raised by the RPM® process in Castlearchdale increased in height, they did not respond as well as those from Loughgall to acorn production (Table 3).

A few studies (Groosman et al., 2003; Dey et al., 2004; Dey et al., 2010) have addressed the relationship between initial height, basal diameter and acorn production in RPM® trees. There was a significant relationship between initial height, basal diameter and acorn production for RPM® trees at both sites over the first few years but this declined over time (Tables 3, 5 and 6).

Correlation coefficients were determined to see if initial height and basal diameters were good parameters on which to discard trees before outplanting. This could also give some indications about fructification of RPM® trees, as was found by Grossman *et al*., 2003 and Day et al., 2004 in the US. However, the correlation from both sites do not clearly discriminate well between initial tree seedling sizes, to allow deciding if a particular tree would be able to grow faster and produce acorns quicker.

While the factors responsible for an unusual ability of large tree seedlings to fructify using the RPM® process are not fully understood, the RPM® process has been successfully used to induce acorn production of different oak tree species in the US. Dey et al*.,* (2004) found that 18 to 24 months old Swamp white oak (*Quercus bicolor*) RPM® tree seedlings planted in Missouri, produced acorns in each of the first 4 years following outplanting. The same author as well as Grossman *et al.,* (2003) state that acorn production was more likely to occur in the first year for large basal stem diameter (>1.3cm) and tall (>1.5m) RPM® tree seedlings. In Castlearchdale and Loughgall, the majority of tree seedlings tested had less basal diameter and height than 1.3cm and 1.5m, respectively, after one year in the field, though after three growing seasons in the field, all oak trees reached more than 1.3cm and 1.5m in basal stem diameter and height, respectively, which would increase the probability of producing acorns at these two sites.

Although logistic regressions were significant (Tables 3, 5 and 6), acorn production did appear to be more related to height than basal diameter or DBH. Research on RPM® trees (Grossman et al., 2003 and Dey et al., 2004) in the US suggests that the probability of producing acorns is significantly related to initial basal diameter and height of Swamp white RPM® oak trees at >12.7 mm and 1.5 m, respectively, according. This would suggest that with the initial height of RPM® planting stock planted at Castlearchdale and Loughgall, fructification could have occurred much quicker than after 5 years. However, other environmental factors could have been affected the flowering process, thus requiring additional years for the majority of trees to produce acorns at these two sites.

Releasing crowns to allow trees full light exposure, increases light intensity for more carbohydrate synthesis in the trees, and this might help to reduce the length of the juvenile (non flowering) phase of oak trees. Although the treatment applied in 2015 could have helped advance the transition to the fructification phase, this could have also been a year outside the good mast production intervals which occur between 5 and 15 years with infrequent flowering in the intervening years (Phillipson, 1990) or a stress effect from grey squirrel damage at Loughgall. Therefore these abiotic and biotic factors during the two consecutive years in 2015 and 2016, could have contributed to variation in acorn production at both sites, independently of planting stock type/size and/or release crown.

In relation to the DNA analysis used for estimating genetic diversity of the oak population, no clear grouping or genetic element could be determined from the analysis. The markers used didn't group populations according to fructification patterns. Thus the relationship between populations shown in Figure 7 suggests little in terms of acorn presence/absence patterning and indicates there is no genetic component to the results. It is worth mentioning that ISSRs are considered dominant genetic markers, i.e. the respective parental alleles cannot be inferred from the data and there is no homology between bands. The DNA analysis showed that the predisposition to produce acorns in RPM® oaks appear to have generally a poor genetic link. So, despite the ability to flower of oak trees being subject to pronounced genetic and environmental influences which may be affected by tree size (Phillipson, 1990), clustering analysis did not indicate any genetic relatedness for trees that showed early fructification. The ISSR markers are neutral (i.e. non-coding) and so for a more accurate assessment of the genetic component involved estimation of variation in flowering/fruiting genes would be a useful addition. This analysis is ongoing and will be presented in a final report.

The observed heterozygosity was much lower than values from other oak diversity studies (Cottrell et al., 2003), which have found values ranging from 0.5 to 0.8 with microsatellites. Although this does indicate uniformity in the study population, it is difficult to compare levels of heterozygosity between different markers. The ISSRs were useful in identifying variation and could be complemented further by microsatellite analysis or indeed by analysis of genetic variation in flowering genes.

5. Conclusions

The relationship between planting stock (RPM® and CG) and acorn production across two sites in Northern Ireland, following flowering events in 2005, 2008, 2010 and 2011, suggest that there is a significant possibility for young and taller RPM® trees to produce acorns but the probability is low (12% to 15%) but significantly higher than CG planting stock. Growth increments were not good predictors for fructification probability.

Despite significant relationships and some positive correlations between initial height and fructification when population data were combined, the relationship between planting stock type/size and acorn production was complex in that acorn production occurred across a wide range of tree sizes, with almost complete overlap in size between acorn and non-acorn trees. The repeated crop assessment revealed a varied predisposition of individual trees to fructification.

Although RPM® planting stock studied at Castlearchdale grew faster and taller than CG planting stock, on average only 12% of trees had acorns at the age of 8 years old, increasing to 20% after removing trees in 2015. In contrast fructification at Loughgall was higher but probably altered by diverse external factors (e.g. grey squirrel damage).

Results suggest that initial tree size and basal diameter may play a role in triggering flowering events. However, it is unclear why there is such a low size range of flowering trees, i.e. whether this is just a consequence of environmental factors, or whether the optimal size for flowering does indeed vary among individuals.

The DNA analysis using ISSRs failed to group populations according to acorn presence or absence, suggesting there is very little structuring in the population. It also indicates that there is not a significant genetic component in the results and that variation in fructification is primarily determined by treatment. To be confident of this additional study on variation in genes responsible for flowering and fruiting is ongoing. The ISSR fingerprint profiles will be useful in combination with future analysis in distinguishing individuals and populations.

6. Acknowledgements

This project was funded by Future Trees Trust. The authors gratefully acknowledge the field assistance of Mrs Chris McCann, James Gilmore from AFBI and Forest Service NI. Special thanks to Dr Peter Savill, Mr John Fennessey, Mr Bede Howell, Dr Jo Clark and Mr Greg Forbes for their valuable comments and Dr Sally Watson (AFBI) for assistance with statistical analyses.

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Appendix 1. Study sites at Castlearchdale and Loughgall, Northern Ireland.

Planting in 2001 at Castlearchdale.

Planting in 2001 at Loughgall.

Crown release treatment, 2015, Castlearchdale.

Crown release treatment, 2015, Loughgall.

Oak tree plots in 2016 at Castlearchdale.

Oak tree plots in 2016 at Loughgall.

Appendix 2. Correlation coefficients and their significances for the relationship between initial and subsequent growth parameters of RPM® oaks planted at Loughgall.

Field Data	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Initial Height																																
Height 2002	0.20*																															
Height 2003	0.14	0.67																														
Height 2004	0.13	0.34	0.68																													
Height 2005	0.10	0.35	0.56	0.58																												
Height 2006	0.15	0.38	0.57	0.58	0.78																											
Height 2008	0.11	0.31***	0.45	0.49	0.63	0.74																										
Height 2015	-0.02	0.23**	0.38	0.42	0.58	0.69	0.70																									
Initial Basal diameter	0.67	0.33	0.15	0.07	0.12	0.11	0.05	-0.06																								
Basal diameter 2001	0.68	0.32***	0.14	0.04	0.11	0.10	0.04	-0.08	0.99																							
Basal Diameter 2002	0.61	0.34	0.13	0.01	0.06	0.08	0.06	-0.09	0.73	0.73																						
Basal Diameter 2003	0.53	0.35	0.20*	0.01	0.16	0.19*	0.15	0.07	0.63	0.62	0.79																					
Basal Diamter 2004	0.38	0.27***	0.23**	0.17*	0.20*	0.26**	0.22**	0.14	0.43	0.42	0.53	0.68																				
Basal Diamter 2005	0.47	0.36	0.36	0.23**	0.37	0.43	0.40	0.30***	0.47	0.47	0.60	0.70	0.72																			
Basal Diameter 2006	0.42	0.39	0.39	0.28***	0.44	0.51	0.41	0.40	0.48	0.48	0.55	0.66	0.68	0.87																		
Diameter_at_70cm 2006	0.41	0.28***	0.34	0.27***	0.46	0.49	0.41	0.36	0.45	0.45	0.54	0.63	0.61	0.73	0.79																	
DBH 2015	0.13	0.28***	0.30***	0.18*	0.36	0.54	0.44	0.46	0.14	0.17*	0.17*	0.21**	0.29***	0.41	0.54	0.50	0.68															
height_increment 2002	-0.60	0.67	0.44	0.18*	0.21**	0.20*	0.17*	0.20*	-0.24**	-0.25**	-0.18*	-0.11	-0.06	-0.06	0.005	-0.07	-0.03	0.14														
height_increment 2003	-0.09	-0.54	0.27***	0.33	0.18*	0.15	0.11	0.13	-0.26**	-0.26**	-0.29***	-0.23**	-0.08	-0.05	-0.06	0.02	0.06	-0.03	-0.36													
height_increment 2004	0.06	-0.08	0.12	0.81	0.34	0.33	0.31***	0.27**	-0.03	-0.06	-0.10	-0.09	0.04	0.03	0.06	0.09	0.11	0.01	-0.11	0.23**												
height_increment 2005	-0.01	0.08	0.001	-0.29***	0.61	0.35	0.26**	0.27***	0.08	0.09	0.07	0.13	0.07	0.21**	0.25**	0.28***	0.25**	0.24**	0.08	-0.12	-0.39											
height_increment 2006	0.09	0.07	0.05	0.04	-0.26**	0.40	0.22**	0.22**	-0.01	-0.01	0.03	0.05	0.10	0.12	0.14	0.09	0.14	0.32***	-0.01	-0.03	0.02	-0.35										
height_increment 2008	0.01	0.09	0.11	0.16*	0.17*	0.12	0.76	0.37	-0.04	-0.04	0.01	0.04	0.08	0.18*	0.12	0.13	0.13	0.12	0.06	0.01	0.13	0.05	-0.06									
height_increment 08-15	-0.11	0.06	0.15	0.17*	0.28***	0.34	0.14	0.80	-0.12	-0.15	-0.18*	-0.02	0.01	0.08	0.21*	0.16	0.23**	0.27***	0.13	0.10	0.11	0.16	0.12	-0.13								
Overall_height_increment 01-15	-0.21*	0.19*	0.34	0.39	0.55	0.65	0.67	0.98	-0.19*	-0.21*	-0.20*	-0.0352	0.06	0.20*	0.31***	0.28	0.35	0.43	0.31***	0.15	0.25**	0.27***	0.19*	0.36	0.81							
basal_diameter_increment 2002	0.37	0.25**	0.08	-0.02	0.01	0.05	0.06	-0.07	0.33***	0.32***	0.88	0.66	0.45	0.51	0.43	0.44	0.32***	0.12	-0.08	-0.23**	-0.10	0.04	0.06	0.04	-0.14	-0.14						
basal_diameter_increment 2003	0.18*	0.19*	0.18*	0.08	0.18*	0.20*	0.17*	0.22**	0.22**	0.20*	0.17*	0.74	0.51	0.46	0.45	0.41	0.38	0.16	0.02	-0.04	-0.03	0.13	0.05	0.06	0.16	0.18*	0.09					
basal_diameter_increment 2004	0.05	0.06	0.14	0.18*	0.14	0.19*	0.17*	0.12	0.03	0.02	0.03	0.05	0.76	0.37	0.35	0.28***	0.39	0.21*	0.01	0.09	0.14	-0.02	0.09	0.07	0.03	0.11	0.03	0.04				
basal_diameter_increment 2005	0.26**	0.21**	0.26**	0.15	0.31***	0.34	0.34	0.28***	0.20*	0.22**	0.28***	0.25**	-0.07	0.64	0.49	0.38	0.34	0.26**	-0.02	0.02	-0.01	0.23**	0.06	0.17*	0.11	0.22**	0.24**	0.10	-0.32***			
basal_diameter_increment 2006	0.04	0.18*	0.16*	0.16	0.24**	0.28***	0.13	0.28***	0.14	0.15	0.07	0.12	0.13	0.02	0.52	0.33	0.46	0.39	0.12	-0.04	0.08	0.13	0.08	-0.07	0.28***	0.27***	-0.004	0.11	0.07	-0.12		
basal_diameter_increment 70 06	0.17*	0.17*	0.26**	0.22**	0.30***	0.37	0.30***	0.323***	0.15	0.17*	0.16	0.25**	0.40	0.44	0.57	0.25**	0.85	0.57	0.01	0.08	0.08	0.14	0.12	0.09	0.20*	0.29***	0.11	0.22**	0.33***	0.19*	0.39	
Overall_basal_diameter_increment	0.34	0.37	0.39	0.29	0.45	0.53	0.43	0.44	0.36	0.35	0.48	0.61	0.66	0.85	0.99	0.77	0.84	0.55	0.04	-0.03	0.08	0.25**	0.15	0.13	0.24**	0.36	0.41	0.45	0.37	0.49	0.53	0.58