3. METHODS AND MATERIALS

3.1 Setting up of half-sib progeny populations

3.1.1 Seed collection

Seeds were collected from 13 mature trees (approximately 40 years old) planted at the Forest Research Institute of Malaysia (FRIM). The mother trees were categorized into 3 groups based on their phenotypic characters. The 3 groups were : A-large; B-medium and C-small such that mother trees with diameter breast height (Dbh) greater than 0.6m were grouped as A; while mother trees with Dbh smaller than 0.6m and greater than 0.4m were classified into group B and mother trees with Dbh smaller than 0.4m were classified into group C (see Table 4.1). There were 4 trees in group A namely A1, A2, A3 and A4; 4 trees in group B namely B1, B2, B3 and B4; and 5 trees in group C namely C1, C2, C3, C4 and C5. Seeds were collected from selected individual mother trees by shaking the tree branches with a catapult. Freshly fallen seeds (identified by green stalk) were collected. Approximately 200 seeds were collected from each of the mother trees. Less seeds were collected from small trees (e.g. in C grade) (see Table 3.1). Fruit coat and pulp were removed from the seeds by rubbing them gently with wet sand. The seeds were washed in running water to remove all the sand. They were then air-dried on the bench. The dried seeds were stored in plastic bags until used.

Table 3.1 : Number of seeds collected from each selected mother tree

Characteristics of selected mother trees		Designation	Number of seeds
A group	Girth (m)		
	0.713	A1	200
	0.612	A2	200
	0.703	A3	200
	0.864	A4	200
B group			
	0.445	B1	200
	0.435	B2	200
	0.435	B3	200
	0.464	B4	197
C group			
	0.397	C1	200
	0.287	C2	98
	0.238	C3	200
	0.347	C4	200
	0.380	C5	119

3.1.2 Germination of seeds

Seeds were sown in trays (35 x 20 x 6 cm) at the nursery in the Genetics Garden of University Malaya (UM). Each tray consisted of potting mix containing a mixture of top soil and compost (N:P:K in the ratio of 15:15:15). Twenty seeds were planted in each tray. After one month, the germinated seedlings were transferred to 15 x 23 cm perforated polythene bags filled with the same potting mix. The seedlings were watered twice a day by mist irrigation.

3.1.3 Experimental design and layout

A randomised complete block design with three blocks was laid out in an experimental plot at Dangi, Negeri Sembilan. The experimental land was partitioned into 3 blocks due to the undulated nature of the experimental land. The block effect is therefore fixed. Each plot was planted with 50 trees. The layout of the planting design in the field is shown in Appendix 1. The planting distance was **2 m** between plants and 3 m between rows.

3.1.4 Agronomic practices

Planting holes of approximately 30 cm deep were made with a drill. The soil in each planting hole was mixed with approximately 500 g of potassium nitrate organic fertilizer (N: P_2O_5 : K_2O : MgO in the ratio of 6: 6: 6: 2). Approximately 500g of organic fertilizer was applied to each individual trees in every 3 months for a year after planting.

As there was no irrigation system installed at the experimental site, plants were watered using a long rubber tube connected to a nearby hose. Individual plants were watered every 2 weeks for a period of six months. Weeding was done manually around each individual plant at the initial stage of establishment.

3.1.5 Morphological data collection

Various phenotypic characters were measured from the 13 mother trees growing in FRIM. Observations were made on germination of seeds collected from each mother trees and measurements were made on the height and number of leaf nodes of each seedlings at 3 months interval. For the progeny trees (about 150 trees for each seedlot) planted at the experimental plot in Dangi, measurements were made at every 3 months intervals for a period of 3 years. The characters under assessment were :

Quantitative characters :–

a. Total height: Total height was taken as the distance between the shoot terminus and the base of the tree trunk. Mother trees were measured with optical clinometer (Model PM – 5/400PC, Suunto company, Healsimki, Finland) in meter. The use of the clinometer is based on the trigonometric principle as illustrated in Figure 3.1. One measurement was made at the tree tip, another at the foot of the stem. The two angles α_1 and α_2 were estimated from the clinometer and the distance of the observer from the tree, D, was also measured. The tree height AB was determined as follows :

$BC = D \tan \alpha_1$

$DC = D \tan \alpha_2$

As tree height is given by BC + CA, it follows that

$AB = D (\tan \alpha_1 + \tan \alpha_2)$

Progeny trees were measured with a calibrated 10 m pole in centimeter.

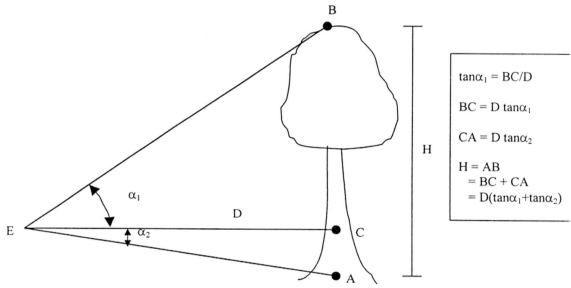

Figure 3.1 : Height measurements - trigonometrical principle (adapted from Köhl, 1993)

Increment of height was calculated over one year period (annual growth)

b. Diameter at breast height (dbh): Mother (m) – circumference ($2\pi r$) of the tree at a height of 1.3 m from the ground was measured with a diameter tape, dbh is estimated as $2\pi r/\pi$; Progeny (cm) - measured with a pair of Vernier calipers at a height of 1.3 m from the ground. Increment of dbh was calculated over one year period (annual growth)

c. Canopy diameter (d): Mother (m) – radii of the tree canopy at four sides were measured with a calibrated 10 m pole ; Progeny (m) - radii of the tree canopy at four

sides were measured with a calibrated 1m pole. An average radius (r) is obtained and canopy diameter (d) is calculated as d = 2r.

d. Number of leaf nodes – seedling - counting on the number of leaf nodes; Progeny – counting on the number of leaf nodes after the trees were one year old in the field

Qualitative characters :– scored by ratings of the following

a. forking of stem – fork or no fork

b. straightness of tree trunk – straight or bent

c. early branching habit – with or without the appearance of first whorl of branches

3.1.6 Statistical analysis

Statistical computation packages used for analyzing the data were Statistical Analysis Software (SAS) and Statistica. Analysis of variance was carried out using PROC ANOVA in SAS. Least significant difference (LSD) was used to compare treatment means by applying the following formula:

$LSD_{0.05} = 1.96\sqrt{(S^2(1/N_1+1/N_2))}$

Where S^2 = Total mean square error

N_1 = value of mean 1

N_2 = value of mean 2

Tree volume (V) is calculated using the formula $V = \pi r^2 htx0.7 = \pi(dbh/2)^2 htx0.7$

Where ht = total height ; dbh = diameter breast height and 0.7 is the reduction factor for taper (Ahmad Zuhaidi Y and Weinland G, 1995). Increment of tree volume was

calculated based on the increment obtained from total height and diameter breast height over 1 year period.

Mean, variance and standard deviation for each seedlot were obtained. Correlation and regression between mother and progeny trees were also established to investigate segregation of characters from the mother to the progenies.

An analysis of variance was performed on the data using the following model:

$$Y_{ijk(l)mn} = \mu + bi + Pa_j + Pa(k)_{j(l)} + Pr_m + bPa(k)_{ij(l)} + bPa_{ij} + bPr_{im} + \varepsilon_{ijklmn}$$

Where,

$Y_{ijk(l)mn}$ = effect of n^{th} data i^{th} block, j^{th} parent, $j(l)^{th}$ parent(category) and m^{th} progeny

μ = common mean

P_{aj} = effect of j^{th} parent

Pr_m = effect of m^{th} progeny

Another analysis of variance was done using the following sources of variances (extracted from the above method) :

$Y_{ijk} = \mu + B_i + Pr_j + (BPr)_{ij} + \varepsilon_{ijk}$

Y_{ijk} = k^{th} observation of i^{th} block and j^{th} progeny

μ = common mean

B_i = effect of i^{th} block, i = 1,2

Pr_j = effect of j^{th} progeny mean, where j = 1, ……..,13

$(BPr)_{ij}$ = interaction effect of i^{th} block and j^{th} progeny mean

ε_{ijk} = random error with $N(0, \sigma^2)$

3.2 Molecular analysis

3.2.1 Counting the chromosome number of *A. excelsa*

Freshly grown root tips of approximately 1 cm length were excised from a one year old seedling grown in a potting container at about 10 à.m. in the morning. They were washed in running tap water to remove traces of soil. The root tips were then submerged in 0.05% colchicine for 4 hours to cause the living chromosomes to contract and separate from each other. The samples were then soaked in distilled water for half an hour. The root tips were further fixed in 6:3:1 methanol : acetic acid : chloroform mixture for 1 hour at room temperature to preserve the chromosomes. They were then hydrolysed in 1N hydrochloric acid for 20 minutes at 60 °C. A root tip was placed on a microscope slide covered with acetocarmine and macerated thoroughly with a sharp lancet. A cover glass was then placed over it and pressed heavily with the thumb to flatten the cells. The slide was then be placed on a microscope and viewed under 100x magnification.

3.2.2 Amplified Fragment Length Polymorphism (AFLP)

DNA samples of each selected tree were analyzed by AFLP reactions. The protocol for the AFLP reactions was modified from Vos *et al.* (1995).

3.2.2.1 DNA extraction

Leaf samples were collected from all the mother trees and selected progeny trees. Each individual leaf was washed with 1% SDS and rinsed with water to remove dust. Genomic DNA was extracted from leaf samples using the procedure modified from

Doyle and Doyle (1987). The protocol for the modified DNA extraction procedure is outlined in Appendix 2. DNA concentrations of each extracted DNA sample were estimated and standardised against known concentrations of lambda DNA (Promega) on 1.2 % agarose gels. This is done by mixing 10 uL of DNA sample with 2 uL of loading dye (See Appendix 2) and electrophoresed in 1.2 % agarose gel at 80 voltage for 2 hours. The gel was soaked in 10 % ethidium bromide solution for 30 minutes before viewing under UV light in a Gel Doc 1000 apparatus (Bio-Rad Laboratories, Hercules, California).

3.2.2.2 Restriction digestion of genomic DNA

Approximately 200 ng/μL of each DNA sample was digested with 2 μL of restriction digestion mixtures consisting of 1.25 units of *Eco*RI and 1.25 units of *Mse*I restriction endonucleases (Life Technologies, Inc., Gaithersburg, MD, USA)(see Appendix 3). The mixture was incubated for 2 hours at 37 °C. After complete digestion, the restriction endonucleases were inactivated by heating the digested sample mixtures at 70 °C for 15 minutes. A duplicate set of reactions was carried out. One set of DNA samples was used for checking complete digestion of the reaction samples. This is done by loading 20 μL of the reaction sample (added 4 μL loading dye) parallel to 5 μL of DNA sample (added 1 μL loading dye) on 1.2 % agarose gel. The gel was run at 100V for 90 minutes.

3.2.2.3 Ligation of adapters

Ligation was performed in a 25 μL reaction, which contains 24 μL of adapter ligation solution (see Appendix 3) and 1 μL of T4 DNA ligase (Life Technologies, Inc.). The mixture was centrifuged briefly and incubated at 20 °C ± 2 °C for 2 hours. The ligation mixture of each sample was diluted 10-fold with TE buffer (see Appendix 3). The unused portion of the reaction mixture was stored at –20 °C.

3.2.2.4 Preamplification reactions (PA)

In a 0.2 mL thin-walled microcentrifuge tube, 40 μL of pre-amplification primer mixture, which consists of 15 ng/μL *Eco*RI-PA, 15 ng/μL *Mse*I-PA (see Appendix 3) and 10 mM dNTP (Promega, Madison, USA), was added to 5 μL of the ligation mixture from section 3.2.2.3 together with 5 μL of 10 x PCR buffer and 1 unit of Taq DNA polymerase (Promega). A negative control was set up with all the reaction mixture added except the DNA template (*i.e.* the ligation mixture). The content was mixed gently and centrifuged briefly to collect reaction mixture. Each tube of reaction mixture underwent 20 cycles of PCR in a Perkin-Elmer 9600 thermal cycler (Perkin Elmer, Norwalk, CT, USA) *i.e.* 95 °C for 30 seconds to denature the DNA; 56 °C for 60 seconds for primers annealing and 72 °C for primers extension for 60 seconds. The soak temperature is 4 °C. The success of the preamplification reaction was checked by running 10 μL of the reaction sample with 2 μL loading dye in 1.2% agarose gel. The preamplified samples were diluted 20-fold with TE buffer. Both unused diluted and undiluted preamplified samples were stored at –20 °C.

3.2.2.5 Selective AFLP amplification (SA)

The following components were added to the diluted PA samples from section 3.2.2.4 : 1 μL of 1μM fluorescent-dye labeled E-primer (FAM-blue, JOE-green, TAMRA or NED-yellow) (Perkin-Elmer); 1 μL of 5 μM unlabeled M-primer; 2 μL 10 x PCR buffer; 0.2 μL of 5 U/μL Taq Polymerase (Promega); 0.4 μL of 10mM dNTPs (Promega); 1.2 μL of 25 mM $MgCl_2$ and 9.2 μL sterilized distilled water in a 0.2 mL thin-walled microcentrifuge tubes. The content of the tubes was mixed gently and centrifuged briefly to collect reaction mixtures. PCR reaction was performed in a PE 9600 thermal cycler as follows:

a. One cycle at 94 °C for 30 seconds; 65 °C for 30 seconds and 72 °C for 60 seconds.
b. The annealing temperature was lowered 0.7 °C each cycle during 12 cycles. This gives a touch down phase of 13 cycles.
c. 23 cycles were performed at 94 °C for 30 seconds; 56 °C for 30 seconds and 72 °C for 60 seconds.

The SA reactions were checked by running 5 μL of each SA samples with 1 μL of loading dye on 1.2% agarose gel. The gel was run at 100V for 45 minutes.

3.2.3 Analysis of SA samples in a 377 ABI sequencer

3.2.3.1 Preparation of sequencing gel

A 4 % acrylamide gel was prepared by adding 18 g urea (Ultra pure grade; Amresco, Solon, Ohio) and 0.5 g mixed-bed resin (MB150 Resin Amberlite; Amresco) to 5 mL of Long Ranger gel and 28 mL autoclaved distilled water (sd H_2O). The mixture was

stirred to dissolve. It was then filtered and degassed using a vacuum pump. 5 mL of 10 x TBE buffer was added and the solution was brought to 50 mL with sd H_2O. 250 µL of 10% ammonium persulfate (0.1 g ammonium persulfate in 1 mL sd H_2O) and 35 µL TEMED were added to the solution. The solution was mixed gently and injected quickly to the gel casting mould. The gel was left for 2 hours to set.

3.2.3.2 Preparation of samples

1 µL of ROX-500 internal standard (see Appendix 3) was added to 1 µL of multiplex SA sample consisting of a combined mixture of 0.3 µL FAM-SA, 0.4 µL JOE-SA and 0.3 µL TAMRA/NED-SA samples. The sample mixtures were mixed briefly by vortexing. The samples were then denatured by heating to 94 °C for 3 minutes and quickly placed in ice before loading onto the polyacrylamide gel.

3.2.3.3 Electrophoresis parameters

Samples were loaded onto the 4% polyacrylamide gel in the ABI PRISM 377 automated sequencer. The gel was run in 1x TBE running buffer (Appendix 3) at 100 V for 2.5 hours. The temperature was maintained at 55 °C during the gel run.

3.2.3.4 Data collection and analysis

All data were analyzed according to the procedure described in ABI PRISM GeneScan analysis software user's manual (1997) and Genotyper DNA fragment analysis software user's manual (1997). As samples migrated beyond the fluorescent detector, the ABI GeneScan analysis software version 2.0, running on a Macintosh

computer, detected the fluorescent signal and assigned a peak size for each fragment. The GeneScan data were exported to the ABI Genotyper software for automated band scoring. For all the gel run, a minimum peak level of 50 was set to eliminate bands that were too faint or background noise. The electropherogram of each gel lane was subsequently examined to pick up discrete peaks for analysis. These selected peaks can then be converted into binary digits and scored as (1) presence or (0) absence, which were output as ASCII files. The ASCII files were further exported to the worksheet file Microsoft Excel for arranging and organizing the data.

3.2.4 Estimation of genetic variability

3.2.4.1 Sampling

All the thirteen mother trees and five randomly selected progeny trees from each progeny tree group were used for this study. The leaves were taken from the progeny trees when they were one year old.

3.2.4.2 Genetic analysis

A population genetic freeware analysis package known as PopGene (Yeh, 1996) was used. The data were analyzed using Nei's similarity index: ($S_{AB} = 2N_{AB}/(N_A+N_B)$, where N_{AB} is the number of shared fragments; N_A the number of fragments from accession A; and N_B the number of fragments from accession B (Nei and Li, 1979). Average linkage cluster analysis was performed based on Nei's similarity index.

3.2.5 Estimation of out-crossing rate

3.2.5.1 Sampling

Six progeny tree groups were selected for this study. They were A1, A2, B1, B2, C1 and C2. Twenty trees were randomly selected from each group for analysis. Leaves were collected from the trees when they were one year old.

3.2.5.2 Genetic analysis

Mating systems were estimated using MLDT computer program of Ritland (1992) for a mixed mating model. The model assumes that progenies result from a mixture of random out-crossing and self-fertilization. From progeny array data and through maximum likelihood procedures, the program simultaneously estimated: (1) multilocus population out-crossing rate (t_m) by the Newton Raphson method; (2) the average single locus population out-crossing rate (t_s); (3) the average single locus inbreeding coefficient of maternal parents (F); (4) the pollen and ovule gene population frequencies, either separately or average; and (5) variances of the above quantities using bootstrap method where the progeny array (within families) is the unit of resampling (100 bootstraps were used). Out-crossing rates t_m for single families and t_s for each locus together with their variances (based on 100 bootstraps) were also calculated. The significant difference between t_m and t_s was also tested using Student t-test as $t = (D - 0)/S_D$, where D is the mean difference between t_m and t_s, and S_D as the mean standard error.

Assumptions of the mix mating model according to Ritland and Jain (1981) and Brown *et al.* (1989) are as follows :

1. Mating events are due to random out-crossing or self-fertilization.
2. The pollen pool is assumed to be homogeneous over all the maternal trees.
3. Segregation within locus assumed not to be linked to other loci.
4. The loci employed are not affected by selection, mutation or other genetic changes between fertilization and time of assay.
5. Segregation in the heterozygous maternal trees is assumed to be strictly Mendelian in 1:1 ratio for both pollen and ovule production.

Differences between pollen and ovule frequencies were tested by computing a chi-square distribution statistics as $\chi^2 = 2NFst\ (a-1)$, with (a-1) degrees of freedom, where N is the number of samples examined, a is the number of alleles for the locus and Fst is the genetic differences between populations, treating pollen and ovules as separate populations (Murawski and Hamrick, 1992; Hall *et al.*, 1994).

3.3 *In vitro* micropropagation

3.3.1 Chemicals

The chemicals used in all experiments were of Analar grade and purchased from Merck, Darmstadt, Germany and Sigma Chemical Company, Saint Loius, Missourri, USA. The agar used for solidifying culture media was Agar Bacteriological Technical grade (Difco Laboratories, Detroit, Michigan, USA).

3.3.2 Preparation of stock solutions and culture media

The basal medium used in the experiment was Murashige and Skoog (1962) (Sigma M5519). The inorganic salts and vitamins were prepared as four main stock solutions, namely macro elements (10x concentrated), micro elements (1000x concentrated), FeEDTA (10x concentrated) and vitamins (1000x concentrated). Sucrose (30 gL^{-1}), myo-inositol (100 mgL^{-1}) and agar (8 gL^{-1}) were added individually to each litre of basal medium prepared. For subculturing to maintain the plant in tissue culture, basal medium in the form of prepared powder obtained from Sigma Chemical Company was used.

The auxins, NAA (Sigma N0640), IBA (Sigma I5386) and BAP (Sigma B9395), kinetin (Sigma K3378) and GA_3 were prepared as 1 $mgmL^{-1}$ stocks.

The pH of all media were adjusted to 5.7 prior to addition of sucrose. The volume of media dispensed were 40 mL for Magenta vessels and 25 mL for petri dishes. Autoclaving was carried out at 121 °C and 1.1 $kgcm^{-2}$ for 15 minutes. The media were then cooled and stored in a cold room (4 °C) until required. The media were allowed to warm to room temperature before they were used in culture work. Where petri dishes were used, the media were autoclaved in reagent bottles before being dispensed in a laminar flow cabinet.

3.3.3 Preparation of axenic plant materials

Twenty shoot tips were excised from six months old Sentang seedlings grown in UM genetic garden. They were washed briefly with FlowLab non-toxic detergent and stirred in 0.125 mgL^{-1} Benlate for approximately 1 hour. The explants were submerged in 70% alcohol for 1 minute. They were then sterilised for 15 minutes in 20% commercial bleach (Clorox, Colgate-Clorox (M) Ind., Petaling Jaya, Malaysia) with a drop of Tween 20 (Polyoxyethylene-sorbitan monolaurate, Sigma P1379) added. The cultures were rinsed one time with autoclaved distilled water. The explants were further submerged in 30% Clorox with Tween 20 added for 15 minutes. They were then rinsed three times with autoclaved distilled water. The entire process was conducted in the laminar flow cabinet.

3.3.4 *In vitro* shoot culture of *A. excelsa*

Induction of shoot growth from axenic *A. excelsa* explants was investigated by using MS media supplemented with various concentrations of BAP or kinetin. An *in vitro* plantlet was obtained when roots were induced from an axenic shoot with various concentrations of NAA or IBA.

3.3.4.1 Induction of shoot buds from shoot tip explant

The experiment followed a completely randomized factorial design. The treatments were full-strength MS medium supplemented with the following concentrations of BAP or kinetin (Table 3.2):

Table 3.2 : Component of media for shoot buds induction

Plant growth hormones	Concentrations (mgL^{-1})
BAP	0.0
	0.1
	0.2
	0.5
	1.0
	2.0
Kinetin	0.0
	0.1
	0.2
	0.5
	1.0
	2.0

The source of explants was shoot tips excised from *in vitro* shoot culture. Four replicates of five shoot tips each were used for the experiment. The experimental cultures were checked regularly for the number of shoot buds and shoots growth.

3.3.4.2 Induction of shoots from leaf explants

Leaves were excised from *A.exclesa* shoots cultured *in vitro* in a tissue culture vessel. Individual leaves were cut into 1 cm by 1cm square before being cultured onto petri dish with MS medium supplemented by various concentrations of BAP, kinetin and adenine sulphate. Each petri dish consisted of 10 leaf cuttings and there were 2 petri dishes per treatment.

3.3.5 Roots induction

In vitro shoots growing more than 4 cm in height from 3.3.4.1 and 3.3.4.2 were used in the root induction experiment. The treatments were full-strength MS medium supplemented with the following concentrations of NAA or IBA (Table 3.3) :

Table 3.3 : Component of media for root induction

Plant growth hormones	Concentrations (mgL^{-1})
NAA	0
	1
	2
	5
	10
IBA	0
	1
	2
	5
	10

Ten replicates with two shoots per tube were set up for each treatment. The experimental cultures were checked regularly to record the number of shoots rooted and the number of roots produced.

3.3.6 Incubation of cultures

All cultures were maintained at 25 ± 1 °C in a temperature-controlled room with a 12 hour photoperiod provided by Philips TLD 36W/54 fluorescent lights. The PAR (Photosynthetic active radiation) ranged from 12 to 15 $\mu Em^{-2}s^{-1}$ and the relative humidity from 67 to 75 %. Cultures that were incubated in the dark were placed in a cabinet in the same environment. The distribution of culture vessels on racks was completely randomized.

3.3.7 Weaning of plantlets

Rooted plantlets were removed from the cultured vessels and rinsed under running water to wash off the agar. They were planted in plastic trays measuring 5x5x12 cm filled with mashed Jiffy (AS Jiffy Products Ltd., Norway). The plantlets were transferred to a 26 ± 2 °C growth incubator and watered daily. After one month, each plantlet was scored for survival. All the rooted shoots from the rooting experiment (see section 3.3.5) were used. The weaning experiment was laid out in a completely randomized design.

3.3.8 Statistical analysis

Data were analyzed using analysis of variance and student t-test for unplanned comparison among treatment means. Percentages were transformed into arc-sine values while mean numbers were transformed into square-root value before ANOVA was performed. Only the results for significantly different treatments were back-transformed. Back-transformation was done according to the transformation used (Sokal and Rohlf, 1981).