GENETIC VARIABILITY STUDIES IN A SELECTED POPULATION OF *AZADIRACHTA EXCELSA* (SENTANG) WITH AFLP MARKER TECHNOLOGY

CHIN CHIEW FOAN

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITI MALAYA

DISSERTATION PRESENTED FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY
UNIVERSITI MALAYA
KUALA LUMPUR

2002

ACKNOWLEDGEMENTS

I wish to thank the Ministry of Science and Environment for funding the research work under Malaysia Teaching Company Scheme (MTCS). I am grateful to the Executive Director of TropBio Research Sdn Bhd, Dato' Dr Salleh Mohd. Nor for granting the study opportunity.

I would like to express my heartiest gratitude to my supervisors, Prof Mak Chai and Associate Professor Dr Rofina Yasmin Othman for their valuable guidance, suggestions and criticisms throughout the course of this study.

The Forest Research Institute Malaysia (FRIM) is acknowledged for granting permission to access the forest reserves. My appreciation is also due to the staff in Forest Plantation Division in FRIM for their assistance in field sampling.

I would also like to thank Dr Low Fee Choon from Rubber Research Institute Malaysia (RRIM) and The Advanced Materials Characterization Allied Laboratories (AMCAL) of University Malaya for kindly allowing me to use the ABI 377 sequencer for my molecular work.

My special thanks go to all my friends and all my colleagues in TropBio Research Sdn Bhd especially Jamilah, Dr Jinil, Dr Jenni, Dr Tan Hong, Dr Francis Ng, Professor Mukherjee, Professor Mike Kearsey, Zaiton, Annie, Komala, Dr Kodiswaran, Balan and Mahendren who have given their help and support directly and indirectly.

And finally, I wish to express my greatest appreciation to all my family members for their unfailing support and encouragement to see me pull through my most difficult time. I would like to dedicate this work in memory of our beloved father and mother who had enriched our lives with their love and care.

ABSTRACT

GENETIC VARIABILITY STUDIES IN A SELECTED POPULATION OF *AZADIRACHTA EXCELSA* (SENTANG) WITH AFLP MARKER TECHNOLOGY

A maternal half-sib population of *Azadirachta excelsa* was established from seeds in a randomised complete block design with three replicates. 100 to 200 seeds were collected from each of the 13 selected mother trees. The mother trees were categorised into three groups *i.e.* large, medium and small according to their girth sizes. 50 progeny seedlings from each seedlot per replicate were planted in the experimental field. Genetic studies on phenotypic measurements and molecular marker analysis were carried out on the established *A. excelsa* population.

Measurements of quantitative traits including diameter breast height (Dbh) and total height were made. Mean Dbh of mother trees according to each category are highly correlated ($r^2 = 0.99$) to mean Dbh of progeny trees from each respective category. Since Dbh is highly correlated to tree volume ($r^2 = 0.96$), it can be taken as indicator for tree growth. The results based on phenotypic measurements of two and a half years old *A. excelsa* trees implicate that large trees (with high mean Dbh) tend to produce overall large size progeny trees. The estimated value of heritability for tree volume is 0.05 whilst for total height and Dbh, it is 0.06 and 0.09 respectively.

Amplified Fragment Length Polymorphism (AFLP) marker system was used to analyse DNA samples extracted from leaves of *A. excelsa* trees in the population. A total of 64 primer-pair combinations were tested. Twelve primer-pairs that produce the highest number of clear and reproducible polymorphic bands were selected. An average of 40 scorable amplified fragments can be obtained from each primer-pair combination. These primer-pairs were used for molecular marker analysis.

A dendogram that shows the relationship among various mother trees was constructed. The dendrogram provides a guide for selecting parental trees to carry out hybridization. For example, a cross between A1 and A2 mother trees is not advisable because they are closely related.

The mating system of the *A. excelsa* population was investigated using twenty- seven AFLP loci with the primer-pair combination EcoR I + AGG and Mse I + CAG. A multilocus mixed mating model was used to evaluate the mating system. The population was predominantly outcrossed ($tm = 0.810 \pm 0.086$) with no significant biparental mating ($tm - ts = 0.125$, $SE = 0.033$).

A strategy was developed to find markers linked to phenotypic traits under study. The strategy is a combination of Pseudotest cross and Bulk Segregant Analysis. It capitalised on the capability of AFLP marker system to produce a relatively large number of polymorphic bands per assay. By screening the mother trees population, the markers that occur very rarely in the population are assumed to be heterozygous. These markers were used to screen a small random sample of progeny trees from a selected seedlot. Rare occurrence of these markers indicates that the paternal parent could be homozygous null thus a test cross mating configuration is established. These selected segregating markers were then used to screen progeny trees with extreme phenotypic traits under study to increase the homozygous level of the trait. By applying this strategy, a putative marker at 75.5 bp was found to link to Dbh using E-AAC and M-CTC primer-pairs in B2 progeny trees. The marker was found to link to progeny trees with large Dbh. Using the same primer-pair combination, putative marker at 74 bp was found in trees with no forking characteristic. However, when a larger number of progeny samples were tested, the marker-trait linkage association disappeared. This could be caused by linkage equilibrium *i.e.* recombination occurs between the loci for the marker and gene coded for the trait because they are located far apart in the genome.

An *in vitro* micropropagation protocol for the production of *A. excelsa* plantlets has been established. Shoot tips cultured in MS medium supplemented with 1 mgL^{-1} BAP produced the highest number of shoots. Shoots were successfully induced from leaf cuttings. Leaf cuttings cultured on MS medium supplemented with 2 mgL^{-1} BAP, 1.2 mgL^{-1} kinetin and 6 mgL^{-1} adenine sulphate produced an average of 5 primordial shoots per leaf cutting. 25 % of these primordial shoots formed shoots with true leaves when cultured on MS medium supplemented with 1 mgL^{-1} BAP and 12.5 mgL^{-1} magnesium sulphate. 100 % of these shoots formed roots when they are transferred to medium supplemented with 10 mgL^{-1} NAA.

ABSTRAK

KAJIAN KEPELBAGAIAN GENETIK DALAM POPULASI TERPILIH *AZADIRACHTA EXCELSA* (SENTANG) MENGGUNAKAN TEKNOLOGI PENANDA AFLP

Sebuah populasi induk ibu separuh-sib *Azadirachta excelsa* telah ditubuhkan daripada biji benih dalam bentuk blok penuh rawak dengan tiga replikasi. 100 hingga 200 biji benih telah dipungut daripada setiap 13 pokok yang dipilih . Induk ibu dikategorikan kepada 3 kumpulan iaitu besar, sederhana dan kecil bergantung kepada saiz ukur lilit. 50 anak benih progeni daripada setiap lot biji benih bagi setiap replikasi telah ditanam dalam plot ujikaji. Kajian genetik bagi analisa fenotip dan penanda molekular telah dijalankan ke atas populasi *A. excelsa* yang ditubuhkan.

Pengukuran trait kuantitatif termasuk ukuran diameter aras dada (Dbh) dan jumlah ketinggian telah dilakukan. Min Dbh induk ibu berdasarkan kepada setiap kategori adalah berkait rapat (r^2=0.99) dengan min Dbh pokok progeni daripada setiap kategori masing -masing. Oleh kerana Dbh berkait rapat dengan isipadu pokok (r^2=0.96), ia boleh digunakan sebagai penunjuk tumbesaran pokok. Hasil kajian melalui pengiraan fenotip keatas pokok *A. excelsa* berusia dua setengah tahun menunjukkan bahawa pokok besar (dengan min Dbh yang tinggi) cenderung kepada penghasilan progeni yang mempunyai keseluruhan saiz yang besar. Nilai anggaran kebolehan mewaris untuk isipadu pokok ialah 0.05 sementara untuk jumlah ketinggian dan Dbh ialah masing-masing 0.06 dan 0.09.

Sistem penanda "Amplified fragment Length Polymorphism" (AFLP) telah digunakan untuk menganalisa sampel DNA yang diekstrak daripada daun pokok *A. excelsa* daripada populasi ini. Sejumlah 64 kombinasi primer telah diuji. Dua belas pasang primer yang menghasilkan jumlah tertinggi jalur polimorfik yang terang telah dipilih. Purata sebanyak 40 fragmen amplifikasi yang boleh diskor dan diamplfikasi-ulang boleh didapati daripada setiap kombinasi primer. Primer-primer ini telah digunakan untuk analisa penanda molekul.

Satu dendogram yang menunjukkan hubungan antara pokok ibu telah dibina. Dendogram ini memberikan petunjuk bagi memilih pokok induk untuk menjalankan kajian hibridisasi. Sebagai contoh kacukan antara pokok ibu A1 dan pokok ibu A2 tidak digalakkan kerana kerapatan hubungan mereka.

Kajian ke atas sistem pengawanan populasi *A. excelsa* telah dilakukan menggunakan dua puluh tujuh loci AFLP dengan kombinasi pasangan primer. *Eco*RI + AGC dan *Mse*I + CAG. Model pengawanan bercampur multilokus telah digunakan untuk menilai sistem pengawanan. Kebanyakkan dari populasi telah berkacuk luar (tm = 0.810 + 0.086) tanpa percantuman dwi induk yang ketara (signifikan).

Satu strategi telah direka untuk mencari penanda yang berkait dengan trait fenotip yang dikaji. Strateginya adalah kombinasi kacukan "Pseudotest" dan "Analisa Bulk Segregant". Ia bergantung kepada kebolehan sistem penanda AFLP untuk menghasilkan jumlah jalur polimorfik per esei yang agak tinggi. Dengan menyaring populasi pokok ibu, penanda yang berlaku pada kadar yang rendah dalam populasi disifatkan sebagai heterozigos. Penanda ini telah digunakan untuk menyaring secara rawak sebahagian kecil sampel pokok progeni daripada lot biji benih terpilih. Kemunculan sekali sekala penanda ini menunjukkan kemungkinan induk bapa adalah 'homozygous null' dan dengan itu satu ujian konfigurasi kawanan silang (test cross mating configuration) telah dibuat. Penanda - penanda yang terpilih ini seterusnya digunakan untuk menyaring pokok progeni dengan trait fenotipik ekstrem dibawah kajian untuk meningkatkan kadar trait homozigos. Dengan strategi ini, penanda putatif bersaiz 75.5 bp telah didapati untuk menyambung kepada Dbh menggunakan primer- pasang E-AAC dan M-CTC dalam progeni B2. Penanda ini didapati berkait dengan pokok progeni yang mempunyai Dbh besar. Dengan menggunakan kombinasi primer-pasang, penanda putatif bersaiz 74 bp telah ditemui pada pokok dengan sifat bercabang. Bagaimanapun , apabila jumlah besar sampel progeny yang lebih besar di analisa, gabungan perkaitan penanda-trait di dapati hilang. Ini mungkin disebabkan oleh keseimbangan (equilibrium) perkaitan seperti rekombinasi telah berlaku diantara loci untuk penanda dan gen yang mengkodkan trait, oleh kerana kedudukan antara mereka yang jauh didalam genom.

Kaedah micropropagasi *in vitro* untuk menghasilkan plantlet *A. excelsa* telah ditentukan. Hujung pucuk yang dikulturkan didalam media MS yang ditambah dengan 1mg/L BAP, menghaslikan jumlah tertinggi pucuk. Pucuk juga berjaya diaruh daripada eksplan daun. Keratan daun di kulturkan atas media MS yang di tambah 2 mg/L BAP ,1.2 mg/L knetin dan 6mg/L adenin sulfat telah menghaslikan purata 5 pucuk primodia bagi setiap keratan. 25% daripada pucuk primodia ini membentuk pucuk dengan daun sebenar apabila di kulturkan di atas medium MS dengan 1mg/L BAP dan 12.5 mg/L magnesium sulfat 100% pucuk ini membentuk akar apabila dipindahkan ke media mengandungi10 mg/L NAA.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	**ii**
ABSTRACT	**iii**
ABSTRAK	**v**
TABLE OF CONTENTS	**vii**
LIST OF TABLES	**xi**
LIST OF FIGURES	**xiv**
LIST OF PLATES	**xvii**
LIST OF ABBREVIATIONS	**xviii**
1. INTRODUCTION	
1.1 Status of the forestry industry	2
1.2 Status of the forestry industry in Malaysia	3
1.3 Status of forest tree improvement	4
1.4 Status of research in *Azadirachta excelsa*	6
1.5 Aims and objectives of the study	7
2. LITERATURE REVIEW	
2.1 Background of *A. excelsa*	
2.1.1 Origin and Geographical Distribution	9
2.1.2 Taxonomy	10
2.1.3 Botanical characteristics	10
2.2 Background of tree improvement	
2.2.1 Strategy of provenance trials	13
2.2.2 Strategy of progeny trials	14
2.2.3 Strategy of clonal trials	14
2.2.4 Limitations to forest tree improvement	15
2.3 Genetic marker technology	
2.3.1 Morphological markers	17
2.3.2 Protein markers	20
2.3.3 Molecular markers	21
2.3.3.1 RFLP (Restriction fragment length polymorphism)	22
2.3.3.2 RAPD (Random Amplified Polymorphic DNA)	25

2.3.3.3 AFLP (Amplified Fragment Length Polymorphism) 27
2.3.3.4 Microsatellites 28
2.3.4 Marker system selection 30
2.3.5 Application of genetic marker system to forest trees 32
2.3.5.1 Tree Genome 32
2.3.5.2 Applications to the field of forestry 34

2.4 Micropropagation 36
2.4.1 Principles of the technology 37
2.4.2 Organogenesis 37
2.4.3 Embryogenesis 38
2.4.4 Application in forest tree improvement 39

3. METHODS AND MATERIALS

3.1 Setting up of half-sib progeny populations 41
3.1.1 Seed collection 41
3.1.2 Germination of seeds 43
3.1.3 Experimental design and layout 43
3.1.4 Agronomic practices 43
3.1.5 Morphological data collection 44
3.1.6 Statistical analysis 46

3.2 Molecular analysis
3.2.1 Counting the chromosome number of *A. excelsa* 48
3.2.2 Amplified Fragment Length Polymorphism (AFLP) 48
3.2.2.1 DNA extraction 48
3.2.2.2 Restriction digestion of genomic DNA 49
3.2.2.3 Ligation of adapters 50
3.2.2.4 Preamplification reactions (PA) 50
3.2.2.5 Selective AFLP amplification (SA) 51
3.2.3 Analysis of SA samples in a 377 ABI sequencer 51
3.2.3.1 Preparation of sequencing gel 51
3.2.3.2 Preparation of samples 52
3.2.3.3 Electrophoresis parameters 52
3.2.3.4 Data collection and analysis 52
3.2.4 Estimation of genetic variability 53
3.2.4.1 Sampling 53
3.2.4.2 Genetic analysis 53
3.2.5 Estimation of outcrossing rate 54
3.2.5.1 Sampling 54
3.2.5.2 Genetic analysis 54

3.3 *In vitro* micropropagation
3.3.1 Chemicals 55
3.3.2 Preparation of stock solutions and culture media 56

3.3.3 Preparation of axenic plant materials 57
3.3.4 *In vitro* shoot culture of *A. excelsa* 57
3.3.4.1 Induction of shoot buds from shoot tip explant 57
3.3.4.2 Induction of shoots from leaf explants 58
3.3.5 Roots induction 58
3.3.6 Incubation of cultures 59
3.3.7 Weaning of plantlets 60
3.3.8 Statistical analysis 60

4. RESULTS

4.1 Morphological characters assessment of mother trees and seeds germination 61

4.2 Morphological character assessment of seedling plants 64

4.3 Morphological character assessment of plants in the field 71
4.3.1 Dbh 71
4.3.2 Total height 81
4.3.3 Number of nodes and canopy diameter 87

4.4 Analysis of variance (ANOVA) on quantitative characters 91
4.4.1 Diameter at breast height (Dbh) 91
4.4.2 Total height 94
4.4.3 Tree volume 98
4.4.4 Number of nodes 100
4.4.5 Canopy diameter 100

4.5 Assessment on qualitative characters 103
4.5.1 Bending or straightness of tree trunk 103
4.5.2 Early or late branching habits 103
4.5.3 Forking 108

4.6 Correlations 111

4.7 Variance component analysis 113

4.8 Heritability 114

4.9 Selection of plus trees 115

4.10 Molecular markers analysis 118
4.10.1 Estimation of chromosome number and DNA content 118
4.10.2 AFLP marker technology 118
4.10.2.1 Selection of primer pairs 118
4.10.3 Genetic variability 130

4.10.4 Estimation of outcrossing rate 137
4.10.5 Assessment of molecular markers linked to morphological traits 142
4.10.5.1 Implementation of the proposed strategy 142

4.11 Micropropagation
4.11.1 Introduction of axenic shoot tips 155
4.11.2 Induction of buds and shoots from shoot explants 155
4.11.3 *In vitro* culture of *A. excelsa* from leaf explants 159
4.11.3.1 Induction of shoots from leaf explants 159
4.11.3.2 Shoot development 162
4.11.4 Effect of NAA and IBA on root induction 162
4.11.5 Weaning of plantlets 167

5. DISCUSSION

5.1 Genetic information derived from morphological trait measurement 169
5.1.1 Direct growth related traits 170
5.1.2 Indirect growth related traits 172

5.2 Molecular marker analysis 173
5.2.1 Genetic variability study 173
5.2.2 Study on outcrossing rate with dominant AFLP markers 176
5.2.3 Searching for molecular markers linked to morphological traits 179
5.2.3.1 Strategy for finding the molecular markers 180
5.2.3.2 Linkage disequilibrium 185
5.2.4 Application of markers linked to desirable traits 185
5.2.5 Implications of the strategy to forest tree breeding programs 186
5.2.6 Limitations of applying AFLP marker system in half-sib population 187
5.2.7 Morphological data versus AFLP markers for genetic analysis 188

5.3 The role of *in vitro* propagation in the improvement program of *A. excelsa* 189
5.3.1 *In vitro* shoot culture of *A. excelsa* 190
5.3.2 Somatic embryogenesis of *A. excelsa* 190
5.3.3 Clonal fidelity 191
5.3.4 Field performance of propagules 192

5.4 "Pyramiding" of QTLs for next generation of breeding 192

5.5 Recommendations for future studies 194

6. REFERENCES 197

APPENDIX

1	217
2	218
3	219
4	222
5	223
6	224

LIST OF TABLES

Table No.		Page
2.1	Comparisons of molecular marker systems	33
3.1	Number of seeds collected from each selected mother tree	42
3.2	Component of media for shoot buds induction	58
3.3	Component of media for root induction	59
4.1	Morphological characteristics of selected mother trees	62
4.2	Percent germination of seeds from each category of mother trees	63
4.3	Percent of seedlings at extreme upper end of height and number of leaf node distribution in each tree group over 3 months and 6 months growth period	66
4.4	Number of trees at the upper range of Dbh distribution curve after 1, 1.5 or 2.5 years of growth in percentage	80
4.5	Number of trees at the upper range of tree height distribution curve after 1, 1.5 or 2.5 years of growth in percentage	86
4.6	ANOVA on mean Dbh	93
4.7	Diameter breast height (Dbh)	93
4.8	ANOVA on mean total height	95
4.9	Total height based on Dbh sizes	95
4.10	Total height	97
4.11	ANOVA on mean tree volume	99
4.12	Tree volume	99
4.13	ANOVA on mean number of nodes	101
4.14	Mean number of nodes	101
4.15	ANOVA on mean canopy diameter	102

4.16	Mean canopy diameter	102
4.17	Number of bent trees in different progeny tree groups	106
4.18	Number of progeny trees with early branching habits	107
4.19	Number of trees with forking habits in various progeny tree groups	110
4.20	Correlations of growth related traits among different progeny groups (below diagonal) and categories (above diagonal).The values in diagonal are correlations between mother and progeny group, and categories (and in parentheses)	111
4.21	Correlation between tree volume and indirect growth related traits among different progeny tree groups	112
4.22	Expected mean square (EMS) and mean square (MS) values of various sources of variance	113
4.23	Variance components of selected growth related traits. Estimated variance components are in parentheses	114
4.24	Frequency of plus tree occurrence in various progeny tree group	117
4.25	Number of bands obtained for each primer-pair combination	127
4.26	Number of polymorphic bands scored with various selected primer-pair combinations	128
4.27	Primer names and sequence for six selective amplified fragment length polymorphism primer combinations	130
4.28	Similarity matrix among mother trees based on Nei's estimate of similarity coefficient	132
4.29	Similarity matrix among progeny trees based on Nei's estimate of similarity coefficient	135
4.30	Estimates of AFLP marker allele frequencies (+), their respective standard deviations (σ) and χ^2 statistics for agreement with the mixed-mating model	139
4.31	Maternal genotype for the 6 families determined by AFLP assay (in parentheses) or inferred by MLDT. Genotype 1 : homozygous for "band presence" allele (+/+); genotype 2 : heterozygous (+/-); genotype 3 : homozygous (-/-); genotype 0 : either genotype 1 or 2	140

4.32	Estimates of multilocus outcrossing rates (t_m) single-locus outcrossing rate (t_s) and fixation index (F)	141
4.33	Variance of various progeny tree groups	143
4.34	Number of segregating markers for B2 obtained by each primer-pair combination	145
4.35	Number of segregating markers for C3 obtained by each primer-pair combination	154
4.36	Shoots and buds development from shoots cultured on various concentrations of BAP and kinetin	158
4.37	Callus and shoot formation from leaf explants cultured on various concentrations of BAP, kinetin and adenine sulphate (after 12 weeks of culture)	161
4.38	Shoots developed from primordial shoots cultured on various concentrations of BAP and magnesium sulphate	163
4.39	Mean number of roots produced from in vitro shoots cultured on various concentrations of NAA or IBA	166

LIST OF FIGURES

Figure No		Page
2.1	Comparisons of various molecular marker technologies	24
3.1	Height measurements – trigonometrical principle	45
4.1	Mean distribution of 13 mother plant seedlings for height after 3 months of growth	67
4.2	Mean Distribution of 13 mother plant seedlings for height after 6 months of growth	68
4.3	Mean Distribution of 13 mother plant seedlings for number of leaf nodes after 3 months of growth	69
4.4	Mean Distribution of 13 mother plant seedlings for number of leaf nodes after 6 months of growth	70
4.5	Mean Dbh distribution for each progeny tree group A in different replicates over 2.5 years	75
4.6	Mean Dbh distribution for each progeny tree group B in different replicates over 2.5 years	76
4.7	Mean Dbh distribution for each progeny tree group C in different replicates over 2.5 years	77
4.8	Mean tree height distribution for each progeny tree group A in different replicates over 2.5 years	82
4.9	Mean tree height distribution for each progeny tree group B in different replicates over 2.5 years	83
4.10	Mean tree height distribution for each progeny tree group C in different replicates over 2.5 years	84
4.11	Mean canopy diameter (m) distribution for each progeny tree group in different replicates	88
4.12	Mean number of nodes distribution for each progeny tree group in different replicates	89

4.13 Genomic DNA extracted from *A. excelsa* leaves. Lane 1: 25 ng λ DNA; Lane 2: 50 ng λ DNA; Lane 3: 100 ng λ DNA; Lane 4: 250 ng λ DNA; Lanes 5 to 14: DNA samples 120

4.14 Genomic DNA digested with *Eco*RI and *Mse*I. Lane 1: 25 ng λDNA; Lane 2: 50 ng λDNA; Lane 3: 100 ng λDNA; Lane 4: 250 ng λDNA; Lane 5,7,9,11 and 13: DNA samples from *A. excelsa* leaves; Lanes 6,8,10,12 and 14: respective fully digested DNA samples 122

4.15 Preamplification reactions. Lane 1: 100 bp DNA ladder; Lanes 2-9: preamplification reaction samples; Lane 10: a negative control that contained the primer but no template DNA 123

4.16 Selective amplification reaction. Lane 1: 100 bp ladder; Lane 2 to 17 : selective amplification reaction samples. Lane 18 : a negative control with no DNA template added 124

4.17 An AFLP gel profile showing testing of 64 primer-pair combinations using A4 mother plant as DNA template 126

4.18 An AFLP gel profile showing band pattern generated by amplification of 4 mother tree DNA template using various primer-pairs. The templates are Lanes 1, 5, 9, 13 and 17 : A1 ; Lanes 2, 6, 10, 14 and 18 : A2 ; Lanes 3, 7, 11, 15 and 19 : B3; Lanes 4, 8,12,16 and 20 : B4. Some of the polymorphic bands are shown by the white arrows. 129

4.19 Dendrogram of mother tree populations generated by Unweighted Pair Group Method using Arithmetic Average (UPGMA) 133

4.20 A dendrogram showing relationship among progeny tree populations generated by UPGMA 136

4.21 Candidate molecular marker linked to Dbh using B2 DNA samples. Fragment size ~ 75.5 bp (see arrows and green boxes) occurs only in trees with large Dbh *i.e.* B2(53), B2(57), B2(80), B2(93), B2(111), B2(130) and B2(133); but not in trees with small Dbh *i.e.* B2(8), B2(28), B2(55), B2(95) and B2(137) 147

4.22	Candidate molecular marker linked to forking characteristics using B2 DNA samples. Fragment size ~ 74 bp (see arrows and green boxes) occurs only in unforked trees *i.e.* B2(53), B2(55), B2(130) and B2(137) but not in forked trees *i.e.* B2(8), B2(28) and B2(95)	149
4.23	Screening of 30 B2 DNA samples for fragment size ~ 74 bp (see arrows and green boxes). B2(8), B2(28), B2(72), B2(82), B2(89) and B2(95) – forked trees. The rest – unforked trees	150
4.24	Candidate molecular marker (fragment size ~ 86.5 bp – see arrows and green boxes) linked to early branching using C3 DNA samples. Early branching trees are C3(12), C3(49), C3(60), C3(115), C3(126) and C3(156) Non-early branching trees are C3(83), C3(117), C3(139) and C3(164)	152
4.25	Screening of other C3 DNA samples. Early branching trees are C3(2), C3(13), C3(51),C3(61), C3(68), C3(143) and C3(154) Non-early branching trees are C3(26), C3(52), C3(78) and C3(111)	153
4.26	Roots formation with the application of NAA or IBA	165

LIST OF PLATES

Plate No.		Page
1	Seeds of *A. excelsa* after removal of fruit coat and pulp	12
2	Six months old *A. excelsa* seedlings ready to be transplanted to the experimental field. Note that the length of the ruler is 1 m.	72
3	Progression growth of *A. excelsa* trees in the field over the period of 2.5 years	73
4	Variation of sizes in the population. Arrows indicate small trees	74
5	Arrow indicates bending of tree trunk	104
6	(A) Arrows indicate growth of first whorl of branches (B) A close up view of first whorl of branches	105
7	(A) Arrow indicates forking of the tree at tree trunk (B) A close up view of the forked tree	109
8	Chromosomes in an *A. excelsa* cell of squash root tip. Pictures A & B, magnification : 10 x 100. Picture C, magnification : 100 x 100 (2N = 28)	119
9	(A) Shoots have been removed to expose the growth of buds at the base. (B) Arrows indicate some of the buds developed into shoots	156
10	Induction of shoots from leaf cuttings - (A) Development of callus on the edges of leaf cutting. (B) Some protruding structures start to form (C) Formation of primordial shoots (D) Formation of true leaves (E) Development of primordial shoots into shoots. Time from leaf culture is shown in parenthesis.	160
11	Comparison of different concentrations of IBA and NAA on root induction after 6 weeks of growth	164
12	Formation of *in vitro* plantlet ready for weaning	168

LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAP	Benzylaminopurine
bp	Base pair
BSA	Bulk segregant analysis
CTAB	Cetyltrimethyl ammonium bromide
2,4-D	2,4-dichlorophenoxyacetic acid
Dbh	Diameter at breast height
DNA	Deoxyribonucleic acid
EDTA	Diaminoethanetetra-acetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	α - Naphthalene acetic acid
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
SG	Selective genotyping
MLDT	Multilocus estimation of outcrossing with dominant markers