

**REPRODUCTIVE BIOLOGY, PROPAGATION AND *EX SITU*
CONSERVATION STRATEGIES OF A CRITICALLY
ENDANGERED KENYAN HARDWOOD SPECIES: *IXORA*
SCHEFFLERI K. SCHUM. & K. KRAUSE SUBSP *KENIENSIS*.
BRIDSON**

Peter Kariuki Njenga

**A thesis submitted in fulfilment for the degree of doctor of philosophy in Botany in
the Jomo Kenyatta University of Agriculture and Technology**

2008

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature.....Date.....

Peter Kariuki Njenga

This thesis has been submitted for examination with our approval as university supervisors

1. Signature.....Date.....

Professor Victoria W. Ngumi.
JKUAT, Kenya

2. Signature.....Date.....

Dr David W. Odee
KEFRI, Kenya.

3. Signature.....Date.....

Dr Jane W. Kahia
CRF, Kenya

DEDICATION

This thesis is dedicated to my dear parents, the Late Mr Edward Njenga Kanori
and my mum Mrs Sarah Ruguru Njenga, my wife Gladys Karegi and my son
Prince Edward Njenga

GOD BLESS YOU

ACKNOWLEDGEMENTS

I would like to thank Professor Victoria W Ngumi, my first supervisor for her encouragement, patience and guidance in the preparation of this work. Without her co-operation, this work would not have been successfully accomplished.

I would also like to thank Dr David W Odee my second supervisor for all his criticisms and guidance in the writing up of this thesis.

I acknowledge the enormous support from my third supervisor, Dr Jane W Kahia for her continuous help and tireless efforts in monitoring my progress during all stages of this work.

I would like to greatly thank Deutscher Akademischer Austausch Dienst (DAAD) for giving me a scholarship to enable me pursue this research work. I sincerely thank JKUAT for giving me a partial study leave to carry out the research.

Sincere appreciation to Dr Robert Hoft for his help to locate the last refuge of the study species during the early stages of this work. I am grateful to Vincent Oeba for making useful comments on data analysis.

I am also indebted to the technologists, J.D. Kinyua of CRF, Josphat Muthanga and Josphat Mbogo of JKUAT and Mr John Gicheru of KEFRI for their technical assistance in my experiments. Thanks to Mr Joseph Machua of KEFRI for his advice on analysis of RAPD results.

Special thanks to John Kamau Muchuku for his tireless efforts in caring for the greenhouse plants and also for his help in collecting plant samples from the field.

I am also indebted to my wife Gladys and my son Njenga for their patience and tolerance as I prepared this work.

TABLE OF CONTENTS

DECLARATION.....	II
DEDICATION.....	III
ACKNOWLEDGEMENTS	IV
TABLE OF CONTENT	V
LIST OF TABLES	XII
LIST OF FIGURES.....	XIV
LIST OF PLATES.....	XVI
LIST OF APPENDICES	XIX
ACRONYMS AND ABBREVIATIONS.....	XX
ABSTRACT.....	XXII
CHAPTER 1	1
1 INTRODUCTION AND LITERATURE REVIEW.....	1
1.1 FAMILY RUBIACEAE AND GENUS IXORA.....	1
1.2 MORPHOLOGY AND ANATOMY	2
1.2.1 Habit	2
1.2.2 Leaves.....	3
1.2.3 Inflorescences	4
1.3 KARYOLOGY	4
1.4 REPRODUCTIVE BIOLOGY	5

1.4.1	Flower biology	5
1.4.2	Pollinators.....	5
1.4.3	Dispersal	6
1.4.4	Germination and seedlings	6
1.5	HABITAT	7
1.6	GENERIC DISTRIBUTION; AFRICAN CENTERS OF IXORA DIVERSITY	7
1.7	ECONOMIC IMPORTANCE OF PLANTS IN GENUS IXORA.....	8
1.8	IXORA SCHEFFLERI.....	8
1.9	RARE AND ENDANGERED SPECIES	12
1.10	CONSERVATION OF RARE TROPICAL PLANTS.....	13
1.11	OVERVIEW OF RECENT EXTINCTIONS	14
1.12	EX SITU CARE AND BIOTECHNOLOGY	16
1.13	STATEMENT OF THE PROBLEM	17
1.14	JUSTIFICATION OF THE STUDY.....	17
1.15	OBJECTIVES.....	20
CHAPTER 2		22
2.0 POLLINATION ECOLOGY AND BREEDING SYSTEMS OF IXORA		
SCHEFFLERI SUBSP KENIENSIS		22
2.1	INTRODUCTION	22
2.2	MATERIALS AND METHODS.....	24
2.2.1	The study area.....	24
2.2.2	Inflorescence Morphology, Flowering Phenology and Floral Biology	26
2.2.3	Floral Visitors	26

2.2.4	Breeding Systems.....	26
2.2.5	Pollen viability	27
2.2.6	Stigma receptivity	28
2.2.7	Seed germination trials.....	29
2.3	RESULTS.....	30
2.3.1	Flowering Phenology and Floral Biology	31
2.3.2	Inflorescence and Floral Morphology.....	31
2.3.3	Floral visitors	31
2.3.4	Breeding systems	32
2.3.5	Pollen viability.....	34
2.3.6	Stigma receptivity	34
2.3.7	Seed germination	37
2.4	DISCUSSION.....	41
CHAPTER 3		45
3.0 VEGETATIVE PROPAGATION OF <i>IXORA SCHEFFLERI</i> SUBSP		
<i>KENIENSIS</i>		45
3.1	INTRODUCTION	45
3.2	MATERIALS AND METHODS.....	48
3.2.1	Stock Plant Production	48
3.2.2	Collection and preparation of cuttings	48
3.2.3	Effect of Propagation Media on Rooting.	49
3.2.4	Effect of leaf area on rooting ability of leafy stem cuttings of <i>Ixora scheffleri</i> subsp <i>keniensis</i>	50

3.2.5	Effect of different concentrations of IBA and NAA on rooting of leafy stem cuttings of <i>Ixora scheffleri</i> subsp <i>keniensis</i> .	50
3.2.6	Effect of cutting Position on the mother plant on rooting	51
3.2.7	Seasonal periodicity of rooting	51
3.2.8	Effect of cutting length on rooting ability	51
3.2.9	Description of data and methods of analysis	52
3.2.10	Development of Logistic regression model	53
3.3	RESULTS	53
3.3.1	Effects of propagation media on rooting of cuttings	53
3.3.2	Effect of Indole butyric acid (IBA) and Napthalene acetic acid (NAA) on rooting of cuttings	59
3.3.3	Effect of Seasonal periodicity of rooting	64
3.3.4	Effect of Leaf Area on rooting	64
3.3.5	Effect of cutting position on mother plant	67
3.3.6	Effect of cutting length on rooting of <i>I.scheffleri</i> cuttings	68
3.4	DISCUSSION	71
CHAPTER 4		76
4.0 IN VITRO PROPAGATION/TISSUE CULTURE OF IXORA SCHEFFLERI SUBSP KENIENSIS		76
4.1	INTRODUCTION AND LITERATURE REVIEW	76
4.1.1	A Historical Background	76
4.1.2	Plants from meristems:	78
4.1.3	Plants from adventitious meristems:	79

4.1.4	Regeneration directly from explant tissue:	79
4.1.5	Regeneration of plants from callus:	80
4.1.6	Regeneration from cell suspensions:.....	80
4.1.7	Regeneration from protoplasts:.....	81
4.1.8	In vitro Propagation of Woody Perennials	81
4.2	MATERIALS AND METHODS.....	85
4.2.1	Introduction	85
4.2.2	Plant materials.....	85
4.2.3	Media Preparation.....	86
4.2.4	Plant growth substances	86
4.2.5	Sterilisation of thermolabile substances.....	87
4.2.6	Sterilization of explant material.....	87
4.2.7	Aseptic techniques	88
4.2.8	Dissecting tools.....	88
4.2.9	Incubation conditions	89
4.2.10	Culture methods for direct regeneration of shoots from explants	89
4.2.11	Elongation/multiplication of shoots	89
4.2.12	Rooting of shoots	91
4.2.13	Transplanting and Acclimatization	92
4.2.14	Induction and regeneration of somatic embryos	92
4.2.15	Washing up	93
4.2.16	Photography	93
4.2.17	Experimental Design and statistical analysis.....	93

4.3	RESULTS.....	94
4.3.1	Shoot elongation	94
4.3.2	Effect of auxins on in vitro rooting of <i>I. scheffleri</i> subsp <i>keniensis</i> second generation microcuttings:.....	98
4.3.3	Effect of auxins (NAA and IBA) on <i>ex vitro</i> rooting of <i>Ixora scheffleri</i> subsp <i>keniensis</i> second generation microcuttings	103
4.3.4	Induction and regeneration of somatic embryos	105
4.4	DISCUSSION.....	107
CHAPTER 5		110
5.0 DETECTION AND ANALYSIS OF GENETIC VARIATION IN <i>IXORA SCHEFFLERI</i> SUBSP <i>KENIENSIS</i> USING RAPD MARKERS		110
5.1	INTRODUCTION	110
5.2	MATERIALS AND METHODS.....	113
5.2.1	Plant material:	113
5.2.2	DNA isolation	113
5.2.3	RAPD reaction and Gel electrophoresis.....	114
5.2.4	Data analysis	115
5.3	RESULTS.....	116
5.4	DISCUSSION.....	118
CHAPTER 6		123
6.0 OVERALL CONCLUSIONS AND RECOMMENDATIONS		123
6.1	VALUE FOR CONSERVATION EFFORTS.....	126

6.2	<i>IXORA SCHEFFLERI</i> SUBSP <i>KENIENSIS</i> CONSERVATIONS OPTIONS AND MANAGEMENT PLANS	127
6.2.1	<i>Ex situ</i> conservation	127
6.3	CONCLUSION.....	129
6.4	RECOMMEDATIONS AND WAY FORWARD	130
	REFERENCES.....	134
	APPENDICES	165

LIST OF TABLES

Table 2-1	Percentage fruit set emanating from various breeding experiment treatments	33
Table 2-2	Comparison of the various treatments in the logistic model.....	34
Table 2-3	Summary of average number of fruits set	35
Table 2-4	Comparison of fruits set on the various days in the logistic model.....	36
Table 3-1	Regression analysis results. Reference levels: week 5 and FRS	57
Table 3-2	Percentages of the cuttings that rooted under different hormonal concentration	59
Table 3-3	Comparisons of various terms in the logistic model. References; Week5, IBA and 0 μ g	63
Table 3-4	Comparisons of various terms in the logistic model. Reference week 5 and 0 cm ²	66
Table 3-5	Summary of % rooted	67
Table 3-6	Summary of % rooted under different cutting length of <i>I.scheffleri</i> across the weeks	68
Table 3-7	Comparisons of various terms in the logistic model. Rerences: 100 mm and week 5.....	69
Table 4-1	Solvents used to solubilise plant growth substances	87
Table 4-2	Summary of the effect of combinations of cytokinins on shoot length and number of micro-shoots	95
Table 4-3	Mean comparisons of shoot length and no. of microshoots.....	96

Table 4-4	Percentage of <i>in vitro</i> shoots that rooted under different hormonal concentrations.....	100
Table 4-5	<i>Ex vitro</i> rooting results.....	103
Table 4-6	Summary of number of roots under different concentrations	104
Table 5-1	List of primers screened for the RAPD reaction	115
Table 5-2	Means of Nei's gene diversity and Shannon's information index and standard deviations for the single population of <i>Ixora scheffleri</i> subsp <i>keniensis</i>	116

LIST OF FIGURES

Figure 2-1	Percentage number of fruits set emanating from different days of stigma receptivity. Vertical bars represent standard error of means, n = 100.	36
Figure 3-1	Overall percentage rooted at end of 8 weeks. (Vertical bars represent standard error of means, n = 100).	54
Figure 3-2	Trend of rooting under various growing media (Vertical bars represent standard error of means, n = 100)	55
Figure 3-3	Overall percentage rooting across the weeks.....	58
Figure 3-4.	Percentage rooting trend for IBA and NAA under different concentrations during the seventh week	60
Figure 3-5.	Accumulated average rooted cuttings under various concentrations of IBA and NAA. Vertical bars represent the standard error of means, n = 100. .	61
Figure 3-6.	Cumulative rooting % under rainy and dry seasons. Vertical bars represent the standard error of means, n = 100.....	64
Figure 3-7.	Rooting progression across the weeks. Vertical bars represent the standard error of means, n = 100.....	65
Figure 3-8	Overall percentage of cuttings that rooted under different leaf areas. Vertical bars represent the standard error of means, n = 20.	66
Figure 3-9.	Cumulative percentage of cuttings that rooted based on position of cutting on mother plant. Vertical bars represent the standard error of means, n =10	67
Figure 3-10	Overall cumulative rooting % under different cutting lengths. Vertical bars represent the standard error of means, n = 100.....	68

Figure 4-1	Percentage rooting under different IBA concentrations. Vertical bars represent the standard error of means, n = 20.....	101
Figure 4-2	Percentage rooting under different NAA concentrations. Vertical bars represent the standard error of means, n = 20.....	102

LIST OF PLATES

Plate 1.1	The tree habit of <i>Ixora scheffleri</i> subsp <i>keniensis</i>	3
Plate 1.2	An inflorescence of <i>Ixora scheffleri</i> subsp <i>keniensis</i>	4
Plate 1.3	A sprouting (S) stump of <i>Ixora scheffleri</i> subsp <i>keniensis</i>	10
Plate 1.4	A dead stump of <i>Ixora scheffleri</i> subsp <i>keniensis</i>	11
Plate 1.5	Area of endemism of <i>Ixora scheffleri</i> subsp <i>keniensis</i> . See the introduced exotic <i>Cupressus lusitanica</i> after cutting down the <i>Ixora</i> . Only one individual is left in this location	11
Plate 1.6	A well laid out canopy of <i>Ixora scheffleri</i> subsp <i>keniensis</i>	20
Plate 2.1	Map of the study area showing the location of Ragati forest	25
Plate 2.2	Map of specific location of 29 individuals located during the study at Ragati forest (GPS coordinates given in appendix 8).	30
Plate 2-3	<i>Ixora scheffleri</i> subsp <i>keniensis</i> dried drupes and seeds near proximity of mother plants where they have been dropped by frutivorous birds and mammals.....	38
Plate 2-4	A faecal lump of <i>Ixora scheffleri</i> subsp <i>keniensis</i> seeds defecated by a monkey	38
Plate 2-5	<i>I. scheffleri</i> subsp <i>keniensis</i> drupes harvested after one month	39
Plate 2-6	<i>I. scheffleri</i> subsp <i>keniensis</i> drupes harvested after one and a half.....	39
Plate 2.7	<i>I. scheffleri</i> subsp <i>keniensis</i> drupes harvested after 2 months	40
Plate 2-8	<i>I. scheffleri</i> subsp <i>keniensis</i> drupes harvested after 3 months	40
Plate 2-9	Embryos extracted from 3-month-old seeds of <i>I. scheffleri</i> subsp <i>keniensis</i>	

Plate 3-1	Uprooted <i>Ixora scheffleri</i> subsp <i>keniensis</i> rooted cuttings after 4 Months ...	55
Plate 3-2	Potted <i>Ixora scheffleri</i> subsp <i>keniensis</i> seedlings. The one on the extreme right is newly potted while the other two are six months old since the commencement of rooting process.	70
Plate 3-3	Out planted vegetatively propagated <i>Ixora scheffleri</i> subsp <i>keniensis</i> planted at the <i>Ixora</i> conservation plot at JKUAT.	71
Plate 4-1	A freshly inoculated shoot tip explant	96
Plate 4.2	An <i>in vitro</i> shoot after 2 weeks. Note the drying off of the original leaves and the development of new leaves.	96
Plate 4-3	An <i>in vitro</i> shoot with the original leaves completely fallen off showing the emergence of new leaves.	97
Plate 4-4a	An actively growing <i>in vitro</i> shoot after 5 weeks.	97
Plate 4-5	A rooted microplant cultured in MS medium supplemented with 50 μ M IBA after 9 weeks	99
Plate 4-6	A transplanted seedling that flowered after four months.	99
Plate 4-7	Rooted microplants under green house conditions after one month.	100
Plate 4-8	Callus induction in <i>Ixora scheffleri</i> subsp <i>keniensis</i> leaf explant cultured in 1 μ M TDZ: 1 μ M IBA after 4 weeks.	105
Plate 4-9	Callus induction in <i>Ixora scheffleri</i> subsp <i>keniensis</i> leaf explant cultured in 1 μ M TDZ: 1 μ M IBA after 7 weeks. Note the browning of the callus at the edges.	105
Plate 4-10	Fresh Leaf explants cultured in MS medium supplemented with 2,4-D ...	106
Plate 4-11	Callus formed in MS medium supplemented with 2,4-D	106

- Plate 5.1 An example of an *Ixora scheffleri* subsp *keniensis* population RAPD profile generated using primer KF01. (Profile for 18 individuals sampled). Arrows indicate some of the polymorphic bands scored. 117
- Plate 6.1 A *coffea Arabica* (Ruiru 11) scion grafted on *I. scheffleri* subsp *keniensis* rootstock after 2 weeks. Note the buds sprouting from the scion. 131

LIST OF APPENDICES

Appendix 1. Development of logistic regression model	165
Appendix 2. Summary ANOVA table for Mean rooting percentage in the different rooting media across the weeks.....	167
Appendix 3. Summary ANOVA table for orthogonal contrasts between the weeks in various rooting media	168
Appendix 4. Accumulated ANODE on number of cuttings that rooted across the weeks under IBA and NAA.....	169
Appendix 5. Accumulated ANODE on effect of leaf area on rooting	169
Appendix 6. Accumulated analysis of deviance (ANODE)	170
Appendix 7. Mechanical and physical properties of <i>Ixora scheffleri</i> subsp <i>keniensis</i> compared to <i>Cupressus lusitanica</i> and <i>Croton megalocarpus</i> (Measurements were done at KEFRI wood center, Karura forest, Kenya).	170
Appendix 8. The GPS coordinates for the 29 individuals located in study area.	172

ACRONYMS AND ABBREVIATIONS

CGIAR	Consultative Group on International Agricultural Research
CIFOR	Centre for International Forestry Research
COFO	FAO's Committee on Forestry
DDW	Double distilled water
ICRAF	International Centre for Research in Agroforestry
IPGRI	International Plant Genetic Resources Institute
KEFRI	Kenya Forestry Research Institute
FAO	Food and Agricultural Organization of the United Nations
KFMP	Kenya Forestry Master Plan
IBA	Indole-3-butyric Acid
NAA	∞ Napthaleneacetic acid
BAP	Benzylaminopurine
KIN	Kinetin
TDZ	Thidiazuron (N-phenyl-N'1, 2,3-thidiazol-5-ylurea)
MS	Murashige and Skoog medium (1962)
°C	Degree celcius (Centigrade)
df	Degrees of freedom
2,4-D	2,4-dichlorophenoxyacetic acid
<i>et al</i>	And others
ed.(s)	Editor (s)
e.g	For example
Fig	Figure

FGR	Forest Genetic Resources
H	Hour
μM	Micromolar
mg/l	Milligrams per litre
min	Minute
mm	Millimeter
NRC	National Research Council
n.s	Non significant
no.	Number
pp	page (s)
%	Percentage
pH	$-\log \text{H}^+$ concentration
p	Probability
RAPD	Randomly Amplified Polymorphic DNA
S	Second
s.e	Standard error
v/v	Volume per volume

ABSTRACT

Ixora scheffleri K.Schum.& K.Krause subsp. *keniensis* Bridson is a critically endangered Kenyan hardwood species on the brink of extinction in its area of endemism. The pollination ecology and breeding system of *I. scheffleri* subsp *keniensis* was investigated at the singular natural population at the Mount Kenya region of Kenya in an attempt to unravel the causes of its decimation. *I. scheffleri* subsp *keniensis* was in flower from November to March (Fruits April to July) with a peak flowering in February. All the 8 insect species regularly visiting the flowers frequently made contacts with the stigmas and they carried copious amounts of pollen. Three Lepidoptera, two Diptera, one Coleopteran one Hymenoptera and one Thysanoptera were the visitors that frequently visited the flowers. *I. scheffleri* subsp *keniensis* produced fruits through both self and cross-pollination. The tree species showed high fruit production under natural, open pollination conditions. The control, wind and/or insect pollination treatment resulted in 79.5% fruit set. The spontaneous autogamy treatment resulted in 36.5% fruit set. No germination of *I. scheffleri* subsp *keniensis* seeds was observed either in the field or in the laboratory experiments.

The effect of Indole butyric acid (IBA), Napthalene acetic acid (NAA), Propagation media, season, leaf area, cutting position on mother plant and cutting length on rooting was evaluated. The Forest soil: Sand (FRS: S) rooting medium recorded the highest percentage of rooting after 8 weeks (69%). This was significantly different ($p < 0.01$, ANOVA) between FRS: S and the rest of rooting media tested. Both IBA and NAA influenced maximum rooting at a concentration of 50-55 μg ($p < 0.05$). The rooting of the

cuttings did not differ significantly ($p=0.779$) across the seasons (rainy and dry season). Percentage rooting of the cuttings from the various leaf areas increased with increase in leaf area from 0-80 cm² but reduced as the leaf area increased to 100 cm². Cuttings obtained from the upper third of the mother plant significantly rooted better ($p<0.01$, cumulated ANOVA) than the ones obtained from the lower third.

An *in vitro* propagation method of *I. scheffleri* subsp *keniensis* by means of axillary buds proliferation was also developed. The nodal explants were cultured on full strength Murashige and Skoog's salt medium supplemented with different concentrations of cytokinins. The concentration 25 μ M BAP: 0.5 μ M TDZ had the highest effect on the elongation of the *in vitro* shoots and on the mean number of microshoots. There was a significant difference ($p=0.039$, log linear modeling procedures for count data) in the number of microshoots that emerged under a concentration of 25: 0.5 and the rest but no significant difference ($p>0.05$) between 20:1.0, 20:1.5 and 25: 0.1 BAP: TDZ μ M combination. Based on these results, one nodal explant can give rise to 12 plantlets in one year. Results showed that both IBA and NAA achieved their optimum *in vitro* rooting at a concentration of 50 μ M. There was a significant difference in the mean percentage of the microshoots that rooted ($p<0.001$) between the two hormones where IBA performed better than NAA. The *ex vitro* rooting results showed that there was significant difference ($p=0.030$, ANOVA based on mean rooting percentage, s.e.d=2.98) between the two hormones in inducing rooting where IBA performed better than NAA. Somatic embryogenesis was also attempted using leaf discs as explants. The discs were cultured in MS medium supplemented with various concentrations of 2,4-D and TDZ. Callus initiation was evident in all cases but there was no embryogenesis recorded

RAPD analysis was carried out for 20 individuals in the single population of *I scheffleri* subsp *keniensis*. From a set of 40 primers screened, eight produced clear RAPD patterns consisting of a total of 213 scorable markers, 177 of which (80.3%) were polymorphic. The mean level of genetic diversity within the population based on Nei's (1973) gene diversity measure was 0.2438. The mean based on Shannon's information index was 0.3776.

The scientific findings recorded herein clearly indicate the possibility of mass propagation of rare and critically endangered plant species using micropropagation and macropropagation protocols when factors affecting these processes are clearly optimized.

CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Family Rubiaceae and genus *Ixora*

The Rubiaceae are the fourth largest angiosperm family after the compositae, the orchidaceae and the Fabaceae and consist of more than 10,000 species and 640 genera. The family has a cosmopolitan distribution but is predominantly tropical. The current subfamilial classification (Robbrecht, 1988, 1994) recognizes four subfamilies: the Cinchoideae, the Ixoroideae, the antirheoideae and Rubioideae. The Ixoroideae consist of five tribes, one of which is the tribe pavetteae, comprising 20 genera and about 1200 species. Among the pavetteae, the genus *Ixora* is the largest and most widespread. Bridson and Robbrecht (1985) divided the genera of the pavetteae into two groups: those with affinities to *Ixora* and those with affinities to *Tarenna*. The other tribes are Gardenieae, Octotropideae, Aulacocalyceae and coffeae. Two of the more well known Rubiaceae genera are members of Ixoroideae: the economically important *coffea arabica* and the often-cultivated *Gardenia* (Katarina and Birgitta, 2000).

Ixora is a large rain forest genus of shrubs and small trees. Estimates of species number range between 300 and 400 (Mabberley, 1987, Bridson, 1988). No modern monographs of the genus exist. Recent flora treatments include Flora of Tropical East Africa (Bridson, 1988), Flora of Tropical West Africa (Keay, 1963) and Flora *Vitensis nova* (Smith and Darwin, 1988).

Ixoras are easily recognizable at genus level among other things by the articulate petioles that results in clear-cut genus delimitation. At species level however, identification is

much more difficult. The overall similarity between the species renders distinction difficult since it is based on minor and often quantitative characters.

1.2 Morphology and anatomy

1.2.1 Habit

A shrubby habit is common in the African *Ixoras*. Many species are described as small to medium sized or even large shrubs, with a height varying between 0.5 and 6m. In fourteen species however tree habit occurs (De Block, 1998). These fourteen include the four montane species (*Ixora albersii*; 3 – 12m, *I. burudiensis*; 3 – 16m, *I. foliosa*; 5 – 27m and *Ixora scheffleri*; 2 – 20m) in which the tree habit is predominant (plate 1.1) and which possess the largest trees among the African representatives. Three African *Ixora* species are monocaulous dwarf plants. According to Robbrecht (1988), monocaulous dwarfs are low unbranched woody understorey plants of which one single stem frequently bears the usually large leaves aggregated towards the tip. Examples are the Congolian *Ixora nana*, *I. minutiflora* and *I. synactica*.



Plate 1.1 The tree habit of *Ixora scheffleri* subsp *keniensis*

1.2.2 Leaves

All African species are petiolate, with the exception of *I. synactica*, which possesses large sessile, cordate stem clasping leaves. In this species, the stem clasping leaves form large cups in which water and debris accumulate (De Block, 1998). This allows the plant to realize additional water and nutrients uptake by adventitious roots that grows into the debris collecting cups.

The stipules are interpetiolar and consist of a truncate to triangular or ovate sheath, topped by a narrow awn. The size of the stipules is very variable according to their position and strongly depends on the size of the branch they envelop. In *Ixora scheffleri*, the length of the awn is one of the distinguishing characters between the two subspecies recognized. (1.5 – 5mm long in subsp *scheffleri* and 0.5 – 1mm long in subsp *keniensis*).

1.2.3 Inflorescences

The inflorescences are always terminal. They may vary greatly in size. In Africa the largest ones are found in *I. aneimenodesma*, upto 20cm and 30cm wide. The inflorescences may be sessile or pedunculate. The length of the peduncle is very variable and may reach 25 – 30cm (e.g. *I. hippoperifera*). Sessile and pedunculate inflorescences with relatively short peduncles upto 10cm in length are erect (e.g. *I. brachypoda*, *I. foliosa* and *I. scheffleri*).



Plate 1.2 An inflorescence of *Ixora scheffleri* subsp *keniensis*

1.3 Karyology

The first report of a chromosome number of the genus *Ixora* dates from 1937 (Fagerlind, 1937). The basic chromosome number in *Ixora* = 11. Only one species, *Ixora duffi* is reported to possess a different chromosome number, namely $n=10$ (Sharma and Chartterjee, 1960). Polyploidy seems to be relatively unimportant in *Ixora*. Almost all

species are diploid. Exceptions are *I. rosea* (triploid: Sharma and Chartterjee, 1960) and *Ixora notoniana* (tetraploid: Bir, Singh and Gill, 1984).

1.4 Reproductive biology

1.4.1 Flower biology

Flowering Pattern: No data are available for African species (De Block, 1998). The Madagascan *I. platythyrsa* has a flowering period of several months. Nillsson *et al* (1990) reported that flowers at anthesis at any one day never exceeded 4% of the total number in an inflorescence. At a rate of more than 200 flowers, this results in very long-lived inflorescences (up to 1 month).

Floral organization: Flowers in *Ixora* are hermaphroditic as is generally the case in Rubiaceae. The Australian *I. baileyana* was reported to be androecious (Adams, Bridson and Robbrecht, 1987). All flowers on one herbarium specimen out of six studied were male.

Secondary Pollen Presentation: *Ixora* exhibits secondary pollen presentation, a complex reproductive strategy promoting out breeding (Yeo, 1993, Robbrecht, 1988, Ladd, 1994), which in the Rubiaceae family is sometimes termed “Ixoroid pollen mechanism”. This strategy involves proterandry and relocation of pollen from the anthers onto a receptaculum pollinis.

1.4.2 Pollinators

Detailed information on pollinators is relatively rare. In the Madagascan *I. platythyrsa*, (Nillson *et al* 1990) pollination is effected by small moths. Vogel (1954) listed *I.narcissodora* and *I. scheffleri* as examples of moth pollinated *Ixoras*. Outside Africa,

Ixoras with bright red and orange red flowers occur. The flowers are odourless and rich in nectar. These are visited by butterflies e.g. *I. coccinea* and *I. congesta* (as *I. grifithii*, Kato, 1996).

1.4.3 Dispersal

Dispersal is endozoochorous. Initially, the dispersal unit is the entire fruit but after passing through the digestive system of the animal, the pyrene becomes the diaspore (De Block, 1998). The small fleshy fruits are eaten and dispersed by frugivorous birds and small mammals. Seed dispersal by animals and especially by birds, which have large home ranges, is generally considered highly efficient (Erickson and Brenner, 1991). Using species number per genus as a measure of success, Erickson and Bremer (1991) showed that in the Rubiaceae, the possession of drupes, which invariably means dispersal by animals, combined with the possession of a shrub habit was an extremely successful strategy. This agrees well with the statement of Snow (1981) who described Rubiaceae fruits as an important food source for frugivorous birds in the tropics especially for smaller unspecialized frugivores that forage in the lower levels of the vegetation. Bremekamp (1937) suggested distribution by sea currents for some *Ixora* species that grow on the seashore e.g. Malaysian *I. Timorensis*.

1.4.4 Germination and seedlings

Germination data on *Ixora* species are incomplete and no data are available for African species (De Block, 1998.). Data exists for *I. ferrea* (Duke, 1965) which is a non-African species. Halle (1966) mentioned *Ixora* seeds exceptionally germinating on the mother

plant. Von Teichman and Van Wyk (1994) reported *Ixora* as a genus having recalcitrant species.

1.5 Habitat

In Africa, 27 out of 37 species occur in lowland forests, four more in montane evergreen forests up to an altitude of 2500m (*I. albersii*, *I. burundiensis*, *I. foliosa* and *I. scheffleri*). Some species prefer wet habitat e.g. *I. longipedunculata* inhabits riparian and swamp forests (De Block, 1998).

1.6 Generic distribution; African centers of *Ixora* diversity

The genus is exclusively tropical and penetrates only slightly if at all into subtropical regions. It has its center of diversity in Asia where there are over 300 species. The highest species numbers are found in Southeast Asia and Malaysia culminating in Borneo (Bremekamp, 1937). In Africa, it is concentrated in the Guineo – Congolian region and the northern part of the Indian coastal Belt (Zanzibar – Inhambane Region). Most African *Ixoras* are endemic species and show a distribution restricted to one phytochorion within the Guineo – Congolian region to one of its three domains. In Eastern Africa, *I. tanzaniensis* is endemic to the Zanzibar – Inhambane Region, while the widely distributed *I. narcissodora* is a transgressor occupying the Zanzibar – Inhambane region. Four species are endemic to the Afromontane Region: *I. foliosa* is restricted to the West African Domain. In Eastern Africa, *I. Scheffleri subsp scheffleri* is endemic to Uluguru – Mulanje Domain and subsp *keniensis* and *I. Albersii* to Imatongs Usambara domain. A fourth montane species occurs in Kivu – Ruwenzori Domain (De Block, 1998)

1.7 Economic importance of plants in genus *Ixora*

African *Ixoras* whether trees or shrubs are always relatively small. There are no species, which produce valuable timber. Nevertheless some uses for wood and bark have been reported. The hardwood of *I. brachypoda* is used to construct paddles, bows arrows and proas (De wildeman, 1920) while the fine bark supplies roofing tiles. The hardwood of *I. Scheffleri* furnishes building poles. The wood of *I. narcissodora* is described as durable and probably useful (Williamson, 1955). The wood of *I. laxiflora* is said to be trimmed into poles or cut to small planks in Liberia (Burkhill, 1998). The stems of *I. Seretii* are used to construct traps to catch game. *I. Brachypoda* has multiple uses: Its leaves are burned to mask the human smell during hunting (De Wildeman, 1920). Its fruits serve as bait during fishing. Root bark juice from this species forms one of the many ingredients of the arrow poison of the mbuti pygmies from the Ituri forest in Congo, Kinshasha (Tanno, 1981). The fruits of *I. brachypoda* are reported as edible (De Wildemann, 1914, Abbiw, 1990). Bark of the same species is put in sugarcane beer to facilitate fermentation (De Wildemann, 1920). Fruits of *Ixora longependunculata* are used to perfume the body and to make fragrant necklaces (Cooper and Record, 1931). Burkhill (1988) reported that the leaves of *I. aggregata* were used as juju to magically invoke strong winds during the burning of freshly cleared forest.

1.8 *Ixora scheffleri*

Two sub species have been identified: *Ixora scheffleri* subsp *keniensis* and *Ixora scheffleri* subsp *scheffleri*. Beentje (1988) lists *Ixora scheffleri* subsp *keniensis* as rare, vulnerable and endangered. The reason for this status is that logging has seriously depleted the camphor forests where it grows in its area of endemism (Plate 1.3 -.1.4)

Bridson (1988) and Beentje (1994) report that this subspecies is possibly extinct since the most recent collection was from 1967.

Ixora scheffleri subsp keniensis is listed in the 2004 IUCN Red list of threatened species as critically endangered. Its designation in the Red list category and criteria is CR B1 + 2.

- CR signifies critically endangered. A taxon is critically endangered when it is facing an extremely high risk of extinction in the wild in the immediate future
- B implies that the extent of occurrence is estimated to be less than 100 Km² or area of occupancy estimated to be less than 10 Km²
- Extent of occurrence is defined as the area contained within the shortest continuous imaginary boundary which can be drawn to encompass all the known, inferred or projected sites of present occurrence of a taxon
- Area of occupancy is defined as the area within its extent of occurrence that is occupied by a taxon. The measure reflects the fact that a taxon will not usually occur throughout the area of its extent of occurrence that may, for example contain unsuitable habitats.
- 1 implies severely fragmented or known to exist at only a single location
- 2 implies continuing decline, observed, inferred or projected in any of the following:
 - (a) Extent of occurrence
 - (b) Area of occupancy
 - (c) Area, extent and or quality of habitat
 - (d) Number of locations or subpopulations
 - (e) Number of mature individuals.

Habitat destruction and fragmentation have restricted an increasing number of plant species to small and isolated populations. Even in intact habitat remnants, these populations face an increased risk of extinction because of environmental, demographic and genetic stochasticity (Fisher and Mathies, 1998).

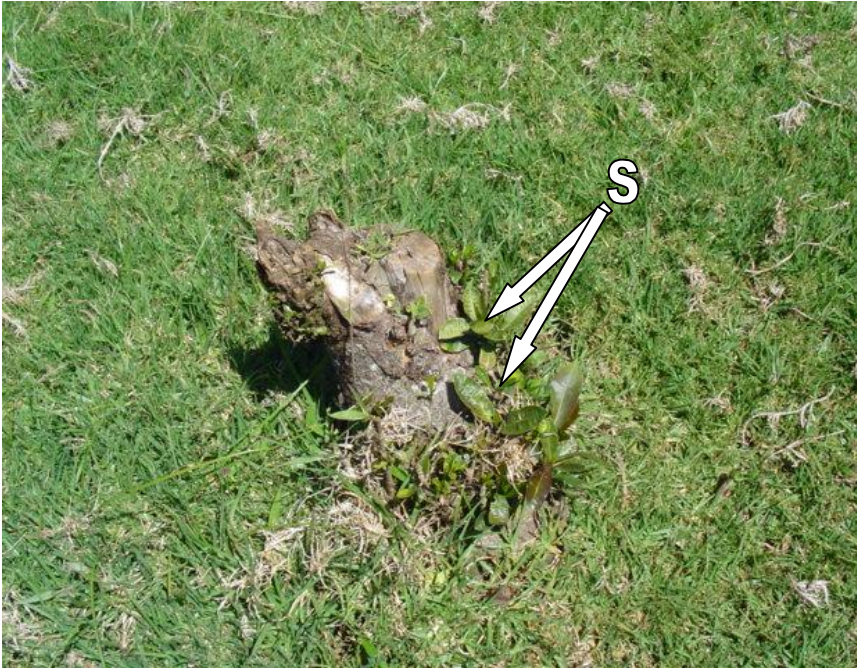


Plate 1.3 A sprouting (S) stump of *Ixora scheffleri* subsp *keniensis*



Plate 1.4 A dead stump (S) of *Ixora scheffleri* subsp *keniensis*



Plate 1.5 Area of endemism of *Ixora scheffleri* subsp *keniensis*. See the introduced exotic *Cupressus lusitanica* after cutting down the *Ixora*. Only one individual is left in this location (I).

1.9 Rare and endangered species

Rarity and endangerment are not synonymous. Rarity is an expression of the pattern of distribution and abundance of a species at a specified time. Endangerment (also known as threat) refers to factors (generally anthropogenic) that may make a species more susceptible to decline or extinction. Various historical, ecological and genetic hypotheses have been presented as to the causes and consequences of rarity (Stebbins, 1980, Baskauf *et al*, 1994, Morse, 1996, Crins, 1997). Since it has become clear that no single explanation can be applied to all cases, field studies are required for the identification of these factors (Henifin, *et al*, 1981). Falk and Holsinger (1991) distinguish four types of rarity for tropical forest trees.

- (1) The first category includes species that are uniformly rare. Such species typically occur in low population densities with an average of one reproductively mature individual or less per hectare. Species with low population densities usually have mechanisms that allow long distance dispersal of pollen and seed.
- (2) The second category contains species that are common in some places but rare in between. Areas in which a species is common may serve as “sources” for propagules that establish in “sink” habitats where the species is not abundant and where in the long run reproductive output is low and local mortality exceeds the recruitment of new individuals (Pulliam, 1988)
- (3) In the third category are species that are local endemics. Gentry and Dodson (1987) have suggested that many species in such groups may be ephemeral arising through chance hybridization of incompletely

genetically isolated forms that lead to distinctive new forms in tiny numbers that are easily eliminated before they have a chance to multiply and spread.

- (4) In the fourth category are species that are clumped even when overall population density is very low. Here small differences in nutrient status may have a major influence on the ecological distribution of species such that population become fragmented into soil determined ecological islands of various sizes.

1.10 Conservation of rare tropical plants

Conservation Biology, an emerging discipline promises to help us decide what facets of nature to preserve, how to avoid extinctions and how to restore ecological damage (Western, 1992). Conservation Biology is partly a response to a Biological crisis (Soule, 1986). Broadly the aims of conservation Biology are as follows:

- (a) Provide scientific conservation principles
- (b) Identify conservation problems
- (c) Establish corrective procedures
- (d) Bridge science and management by making scientists responsive to the conservation problems and managers responsive to Biological issues.

Conservation of rare tropical forest plants, particularly trees may require large protected areas of hundreds of square Kilometers (Bawa and Ashton, 1991). Three principle arguments can be advanced to justify the conclusion

- (i) Many rare species in tropical forests at a given site probably represent occasional emigrants from population centers occurring elsewhere (Hubbell and Foster, 1983)
- (ii) Most tropical forest trees engage in mutualistic interactions with fungi (mycorrhizae) pollen and seed vectors for their survival. This emphasizes the importance of maintaining diverse habitats and communities for the conservation of tropical biodiversity.
- (iii) Population genetic studies of some canopy species show that these trees are widely outcrossed (O'Malley and Bawa, 1987). For canopy trees that occur at densities of one adult or less per hectare, a large area would be needed to maintain minimum viable populations

Conservation of rare tropical trees *ex situ* is also difficult for a number of reasons. First, only a limited number of large woody plants can be cultivated in a botanical garden or an arboretum. Second, the complex sexual systems and pollination mechanisms found in many species may not always ensure seed set under *ex situ* conditions. Third, the seeds of most species are fleshy and techniques for their cryogenic preservation are not well developed (Eberhart *et al*, 1991).

1.11 Overview of recent extinctions

Extinctions are the events that the conservation movement aims to prevent (Western, 1992). The arsenal of extermination has involved at least four sets of mechanisms: - Overkill, habitat destruction and fragmentation, impact of introduced species and secondary extinctions (Diamond, 1984).

- (1) Overkill: Before the advent of agriculture, overkill – the hunting of animals at rates faster than they could reproduce themselves was probably the main mechanism by which humans exterminated animals. Overkill has also befallen plant species, such as the wine palm and various sandalwoods which are logged for their sap or wood. Compared to other mechanisms of extermination, overkill is now probably less important than it was formerly, simply because most of the preferred or susceptible victims have already been eliminated
- (2) Habitat destruction and fragmentation: Since the advent of agriculture, humans have been destroying natural habitats by clearing them for agriculture as well as for timber or domestic livestock. Other modes of habitat destruction have involved fire, draining of wetlands and destruction of wetlands by introduced grazing and browsing animals. This has contributed for most plant extinctions
- (3) Impact of introduced species: Almost all examples involve naive victims: that is native species with no prior experience of the introduced species in question. For example introduced goats, rabbits and other herbivores have brought a lot of destruction on island plants. This has occurred because most island plants evolved in the absence of mammalian grazers and browsers and lack the adaptations such as thorns, spines and unpalatable chemicals evolved by continental plants long exposed to mammalian herbivores as anti herbivore protective devices.

(4) Chains of extinctions: Species interact with each other as predators, mutualists, competitors, and herbivores. Thus a change in abundance of one species is likely to lead to changes in abundance of other species (Pimm, 1979, Diamond and Case, 1986). Whenever one species is exterminated, its disappearance may lead to increases in abundance of other species (e.g. of its direct competitors and prey) and to decreases in abundance of other species (e.g. its predators, mutualists and competitors of its competitors). For example extinctions of Hawaiian plants of genus *Hibiscaedelphus*, which depended on the honey creepers for pollination, while the decline and reproductive failure of the tree *Calvaria major* on Mauritius has been attributed to extinction of dodo which may have been its main seed dispersal agent.

1.12 *Ex situ* care and biotechnology

In biological conservation, *ex situ* care and biotechnology are crisis responses to the threat of extinction (Conway, 1991). *Ex situ* care is the management of populations of wild plants or animals away from their natural distribution. Its techniques include propagation, storage and replacement in case of plant species. Biotechnology in the context of biological conservation seeks to modify or utilize some aspects of the biology of a taxon using the paraphnelia of genetics and reproductive physiology (Conway, 1991). The lessons of conservation biology (MacArthur and Wilson, 1967) suggest that small isolated populations will rarely survive without artificial help.

According to Myers (1991) “We may learn how to manipulate habitats to enhance survival prospects. We may learn how to propagate threatened species in captivity. We

may be able to apply other emergent conservation techniques, all of which could help to relieve the adverse repercussions of broad scale deforestation. But in the main, the damage will have been done”.

1.13 Statement of the problem

Ixora scheffleri subsp *keniensis* is classified as critically endangered and therefore the single natural population is a candidate for conservation and improvement programmes. However, there is lack of information on its reproductive biology, propagation and the diversity of the existing genepool. Therefore a study was carried out on the reproductive biology, propagation and genetic diversity of the species for the purpose of initiating appropriate conservation measures.

1.14 Justification of the study

The rate of extinction during the past 600 million years has been estimated to be roughly one species per year (Raup and Sepkowski, 1982). Today the rate is surely hundreds of times higher, possibly thousands of times higher (Ehrlich and Ehrlich, 1981). Moreover, whereas past extinctions have occurred by virtue of natural processes, today, the virtually exclusive cause is man.

The main mechanisms of extinctions namely, loss of habitat, pollution, introduction of predators/competitors/diseases and overexploitation tend to be studied in isolation from one another. Very little is studied about the dynamic interplay between the discrete mechanisms. When we consider the probable outcome of several mechanisms operating at once, we can reasonably surmise that many of their effects will amplify one another through synergistic interactions (Myers, 1991). This is mainly because an organism's

tolerance of one stress tends to be lower when other stresses are in operation. With respect to tropical forests, what are the synergistic factors at issue? The majority of species in tropical forests tend to be characterized by traits that leave them singularly susceptible to endangerment and extinction. Most exist at low densities and many are endemic (Janzen, 1983). Many species feature narrow ecological specialization. *Ixora scheffleri subsp keniensis* satisfies all these attributes.

“The world Charter for Nature” passed by the United Nations general assembly in 1984 pointed out that all living species should be respected no matter whether they are useful or not to mankind. We should save as many species of the planet as possible and argue later about whether saving species by artificial means was in their best interest.”

To conserve biological diversity, conservation programmes must be guided by the Biology of the species or systems that they seek to conserve (Falk & Holsinger, 1991). The practice of conservation however takes place in a context of limited resources. Consequently, practitioners must often extrapolate and improvise making pragmatic decisions based on incomplete understanding of the Biology of the species or systems they wish to conserve.

Information on reproductive biology and life history is important for the conservation of endangered species. A population’s reproductive strategy affects its effective population size, the distribution of genetic variation and the designing of sampling and management strategies for conservation (Olfeit *et al*, 1998). In this study the flowering pattern, pollination, fruiting, dispersal and seed germination were considered.

The study also aimed at establishing appropriate *in vitro* propagation and macropropagation protocols to raise plantlets for *ex situ*, *circa situ* and *in situ*

conservation of this species. If used in conjunction with conventional methods, *in vitro* culture techniques can contribute markedly in the breeding of *Ixora scheffleri* subsp *keniensis* by increasing the number of plantlets produced per parent genotype.

An understanding of the intraspecific distribution of genetic variation within the single population of *Ixora scheffleri* is essential for producing appropriate conservation and sustainable utilization strategies. In this study, Random Amplified Polymorphic DNA (RAPD) analysis was carried out. RAPD analysis requires only small amounts of DNA, does not require prior DNA sequence information and is simple and quick to perform.

It is hoped that the information gotten in this study will provide a useful impetus for further study or conservation management of this critically endangered species.



Plate 1.6 A well laid out canopy of *Ixora scheffleri* subsp *keniensis*

1.15 Objectives

The study aimed at developing effective strategies and sound methods to mitigate the extirpation of the critically endangered endemic Kenyan hardwood species: *Ixora scheffleri* subsp *keniensis*. The specific objectives were:

- (1) To develop vegetative propagation methods and determine the optimal factors influencing the processes that determine rooting of cuttings of this species.
- (2) To develop *in vitro* propagation methods of this tree and determine the optimal cytokinin and auxin levels.
- (3) To determine genetic variation using Random Amplified Polymorphic DNA (RAPDS) in the single population of this species
- (4) To determine seed germinability of this species
- (5) To determine the rates of successful offspring recruitment through seed germination in natural population and formulate an updated diagnostic of this species in Kenya
- (6) To identify preliminary steps that are urgently required for laying a foundation for a long-term conservation strategy for *Ixora scheffleri* subsp *keniensis*
- (7) To determine the breeding systems of the species, to test for self-pollination, out crossing and autogamy and determine the probable limiting factors in reproductive success.
- (8) To carry out a survey and distribution of the species in its area of endemism.

1.15: Research Hypotheses

1. The breeding systems of *Ixora scheffleri* subsp *keniensis* pose a major bottleneck in the reproductive success of this species.
2. *Ixora scheffleri* subsp *keniensis* can be successfully propagated through vegetative propagation, tissue culture and seeds.
3. There exists genetic variation among individuals of the single *Ixora scheffleri* subsp *keniensis* population.

CHAPTER 2

2.0 POLLINATION ECOLOGY AND BREEDING SYSTEMS OF *IXORA SCHEFFLERI* SUBSP *KENIENSIS*

2.1 Introduction

The study of reproductive biology is fundamental for systematic and evolutionary studies as well as conservation (Ornduff, 1969; Anderson, 1995). Conservation or restoration programmes cannot be effective without an understanding of breeding systems and pollination. Breeding systems in plants are one of the major aspects that influence their population structures, and hence their genetic diversity (Hamrick, 1992). The ability of any organism to produce genetic variation for any trait, including resistance to pathogens, is partially determined by its breeding system. Some species are intolerant of self-fertilization and do counter it by developing either pre- or post-zygotic rejection of selfing (Charlesworth & Charlesworth, 1987). The breeding system of a species (such as selfing rates) may vary along geographical or ecological gradients, especially under stress (Dafni, 1992). Hence the result from one population cannot always be applied to the whole species range. Knowledge of the breeding systems provides an essential background for evaluating the dependence of seed production on pollination rate and type in the effort to understand the mechanisms of gene flow within and between populations. This information is crucial in breeding programmes, where effective pollination of seed orchards is necessary and in developing seed collection strategies. Breeding systems have a direct influence on genetic structure of a population and therefore are essential in

sampling parental trees for breeding and gene conservation, designing multiplication populations such as seed orchards and to achieve the intended packaging of required genetic traits in progeny (Adams, 1992).

Generally, knowledge of the biology of tropical forests and in particular the reproductive biology of woody species is very limited (National Research Council [NRC], 1991). This is a drawback in their conservation and improvement programmes. Different approaches have been used to gain an understanding of breeding systems in plants and estimate parameters describing breeding systems. The most conventional method used is controlled pollination to assess self compatibility (Perez-Nasser, *et al*, 1993).

Flowers play an important role in controlling pollen flow within and between flowers and within and between plants. The temporal and spatial separation of the male and female functions of flowers in plants has traditionally been regarded as a means to avoid self pollination (Faegri and Van der Piljl, 1979). Knowledge of floral morphology and on its temporal pattern of development is essential and can be used in determining a species mode of pollination. These have implications on pollination and on the subsequent gene flow in a given population. Development of stigma and pollen grains can be checked *in vivo* or *in vitro*.

Observations of features, including floral morphology and phenology as well as pollination studies can provide inferences into plant breeding systems. No information is available on the identity of the pollinators in *Ixora scheffleri* subsp *keniensis* or on their relationship with the plant. In this study we investigated the reproductive biology of *Ixora scheffleri*, including the pollination ecology, breeding systems, stigma receptivity, seed set and pollen viability with the objective of contributing to the knowledge of the biology

of this rare plant. It is hoped the information in this chapter will provide a useful impetus for further study or conservation management of this critically endangered species.

The aim of the study reported in this chapter was to investigate the pollination ecology and breeding system of *Ixora scheffleri* subsp *keniensis* single population for the purpose of generating relevant information for its sustainable management through scientifically based conservation, improvement and propagation strategies.

2.2 Materials and Methods

2.2.1 The study area

Ragati Forest is situated in the South-Western part of Mount Kenya (plate 2.1 & 2.2). The area is situated between latitude 0° 9' and 0°26' South and longitudes 37°3' and 37°15' East. It lies east of Nyeri town. The bulk of the area lies within Nyeri and a part of Kirinyaga Districts of central province. There are five forest stations namely Ragati, Kabaru, Chehe, Hombe and Naru Moro. Ragati Forest covers an area of 8,773 hectares. The area ranges in altitude between 1768m in the Southwest near Nyeri Town and 3200m at the boundary of Mt Kenya National Park, the 3,200m contour being the boundary between forest reserve and the National Park. There are normally two rainy seasons during the year, the first extending from about mid March to end of May and the second from about mid October to mid December. The wettest month is usually April. The mean annual rainfall is 855 – 1682 mm and the mean annual temperature is 24.2 ° C. The driest period of the year is June to September and a shorter dry period between January and mid March (Ragati Forest Station Annual Report, 1998). The East African Herbarium information, the personal collections of Robert Hoft and information from people in the

neighborhood of the forest area were consulted in search of localities and phenological information of the study species. Since 2002 visits to the area have been done to try and locate the tree species at least five times in a year. The last visit was in April 2007.

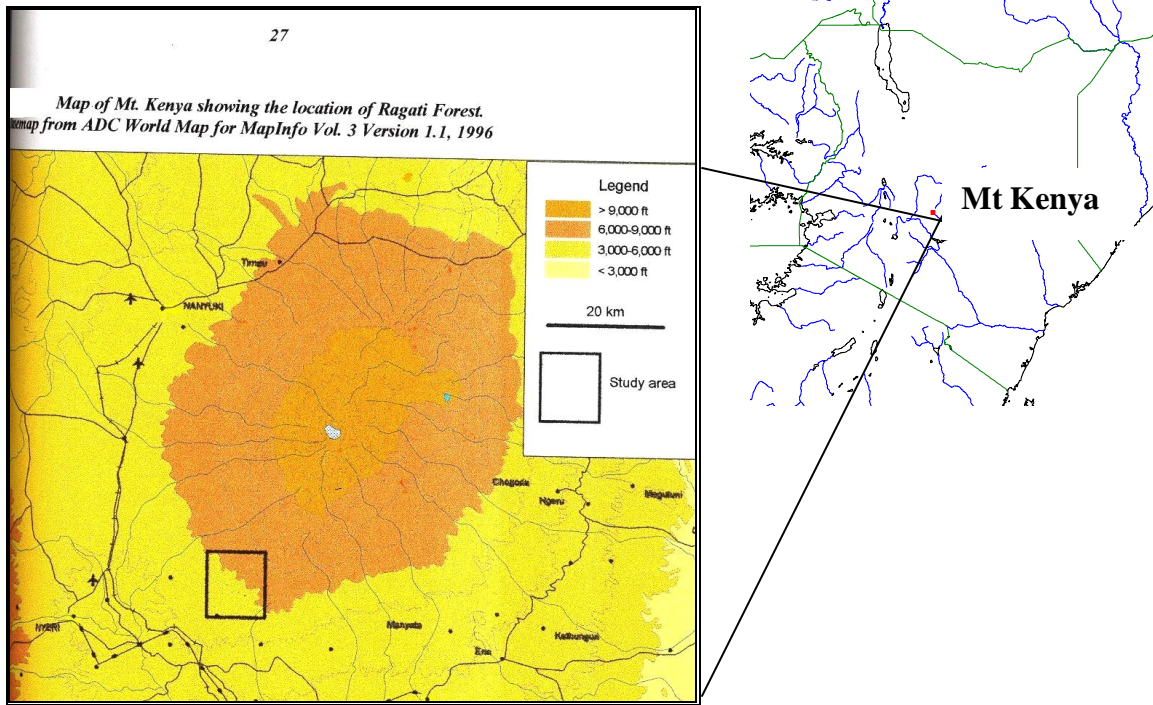


Plate 2.1 Map of the study area showing the location of Ragati forest

2.2.2 Inflorescence Morphology, Flowering Phenology and Floral Biology

Ten individuals were chosen at random and tagged during the first day of flowering. From this day on, daily counts of open flowers were made and opening sequences were determined for each inflorescence. Data were obtained for anthesis period, flower longevity, availability of pollen and fragrance production.

2.2.3 Floral Visitors

The behavior and movements of the insect visitors to the flowers as well as the number of visits to the flowers were recorded during a 3-hour period in February of 2004 and 2005. The studies were recorded during the peak of flowering between 10.00 am and 1.00 pm. Four trees selected on the basis of easy proximity to the flowers for observation of the visitors were used in this study. Observations were made on a total of 20 inflorescences on each tree. 30 minutes were utilized to observe the behavior of the visitors as they approached the flowers. Twenty-minute intervals between the census periods were utilized for capturing the specimens of the insect visitors using a hand held net. Pollen loads of the captured floral visitors were dispersed in a phosphate buffer 0.1 M and counted under a light microscope. Identification of the pollen grains was made by comparison with a reference collection at JKUAT, GK, Botany laboratory. Identification of the insect visitors was carried out at the Kenya Forestry Research Institute (KEFRI), entomology laboratory.

2.2.4 Breeding Systems

An experiment was conducted to distinguish between wind and insect pollination by exclusion of various potential pollen vectors. Over a four-day period, seven

inflorescences (with ten newly opened flowers each) on each of 20 plants were randomly assigned to one of seven treatments:

1. Open pollination (control, wind and/or insect pollination)
2. Emasculation (wind and/or insect pollination but not autogamy)
3. Coarse mesh bagging (wind but not insect pollination)
4. Fine mesh bagging (spontaneous autogamy)
5. Emasculation with fine mesh bagging (agamospermy)
6. Xenogamy was tested by artificial pollination with pollen from flowers of other individuals in the population.
7. Artificial pollination with pollen from other flowers of the same individual was conducted to test geitonogamy

Anthers in the emasculated treatments were removed using fine forceps to prevent autogamy. They were emasculated before flower opening at least two days before anthesis. The holes of the coarse mesh used in this study was $1 \times 1 \text{ mm}^2$, slightly larger than the mesh size known to allow flow of willow pollen and pollen of *Espelatia* (Berry & Calvo, 1989). The difference in seed production was evaluated using ANOVA and binary logistic modeling procedures.

2.2.5 Pollen viability

Pollen viability was tested by staining with 1% methylene blue mixed with 1% neutral red and 1% aniline blue following Dafni (1992). Pollen staining capacity depends not only on the viability but also on the content of pollen grains, which may differ considerably from the true value of pollen viability. Therefore an additional examination was done to elucidate the germinability of the pollen grains. Pollen was extracted and

germinated in Petri dishes with sucrose concentrations of 10%. The Petri dishes were left at room temperature for a maximum of 24 h. Pollen grains were considered to have germinated when the pollen tube length was greater than or equal to pollen diameter. Pollen was observed using a light microscope at x 100 magnification.

2.2.6 Stigma receptivity

In vivo and *in vitro* assessment of stigma receptivity of the species was carried out. In both methods, artificial pollination of flowers at different stages of development was carried out. In the *in vivo* experiment, flowers of sampled trees were emasculated and hand pollinated at different stages of development (starting just before anthesis and ending at the wilting stage) and bagged to isolate them from foreign pollen grains. Ten trees were randomly sampled. Ten inflorescences were marked and tagged on each sampled tree. The flowers were then emasculated and hand pollinated at an interval of one day (starting from the onset of anthesis). On each day 10 emasculated flowers per tree were hand pollinated with outcross pollen giving a total of 100. After the hand pollination, the inflorescences were recovered until the following day's treatment to exclude visitors. The number of fruits resulting from each day's treatment was considered the estimate of stigma receptivity.

In the *in vitro* experiment, hand pollinations were done and pollen tubes counted to establish the time of peak pollen germination. Forty five flowers on three individuals were used for this experiment. They were emasculated before anther dehiscence and covered with fine bridal veil to exclude visitors. Starting at 10.00 am, every 15 min, five flowers were uncovered, artificially pollinated with outcross pollen, and then recovered. The flowers were left on the plants for 24 h after which they were collected and fixed in

Carnoy's solution. The pistils were excised, cleared in 0.8 N NaOH at 60 C, washed in deionized water, squashed on a microscope slide in a drop of 0.1% aniline blue in 0.1 N K₃PO₄, and observed for pollen tubes under a microscope using UV light following the methods outlined by Preston (1991).

2.2.7 Seed germination trials

Various seed dormancy-breaking treatments were carried out following the methods of International Board for Plant Genetic Resources (1985). Seeds from fruits at different developmental stages were tested for their ability to germinate. After the treatments, the seeds were cultured between filter papers in petri dishes. Another set of seeds was planted in sterilized sand. The treatments were:

1. Presoaking the seeds in 2,4-D followed by warm stratification
2. Removal of seed covering structures and then presoaking for 24 h in kinetin at 10⁻⁴ M
3. Thiamine: reapplied, 48 h, 0.1%
4. Folic acid: pre-applied, 48 h, 0.1%
5. Ferrous sulphate: pre-applied, 48 h at 10ppm.

Another set of experiments was carried out based on the methods of Milthorpe and Milthorpe (1962). The procedure is referred to as seed advancement treatment or drought hardening. The experiments were performed as follows:

1. Weighed *Ixora* seeds were subjected to seed advancement treatments. The treatments were allowing the seeds to imbibe a volume of distilled water, 10ppm Indole acetic acid (IAA) in distilled water, 10ppm IAA in acetone and 0.2% zinc sulphate for 48 hours. The seeds were then dried over silica gel to their initial

weight. Both the treated and the untreated control seeds were surface sterilized with 0.2% mercuric chloride solution before plating them out on two layers of filter papers in petri dishes. The filter papers were kept moist with distilled water at room temperature.

2. Weighed *Ixora* seeds were subjected to advancement treatment with anhydrous acetone to find out the effect of acetone on its own. The procedure was as described in (1) above. In each experiment, 200 seeds were used.

2.3 Results

Using GPS coordinates, a distribution map of 29 individuals located during the study was generated using the ARC GIS programme (Plate 2.2).

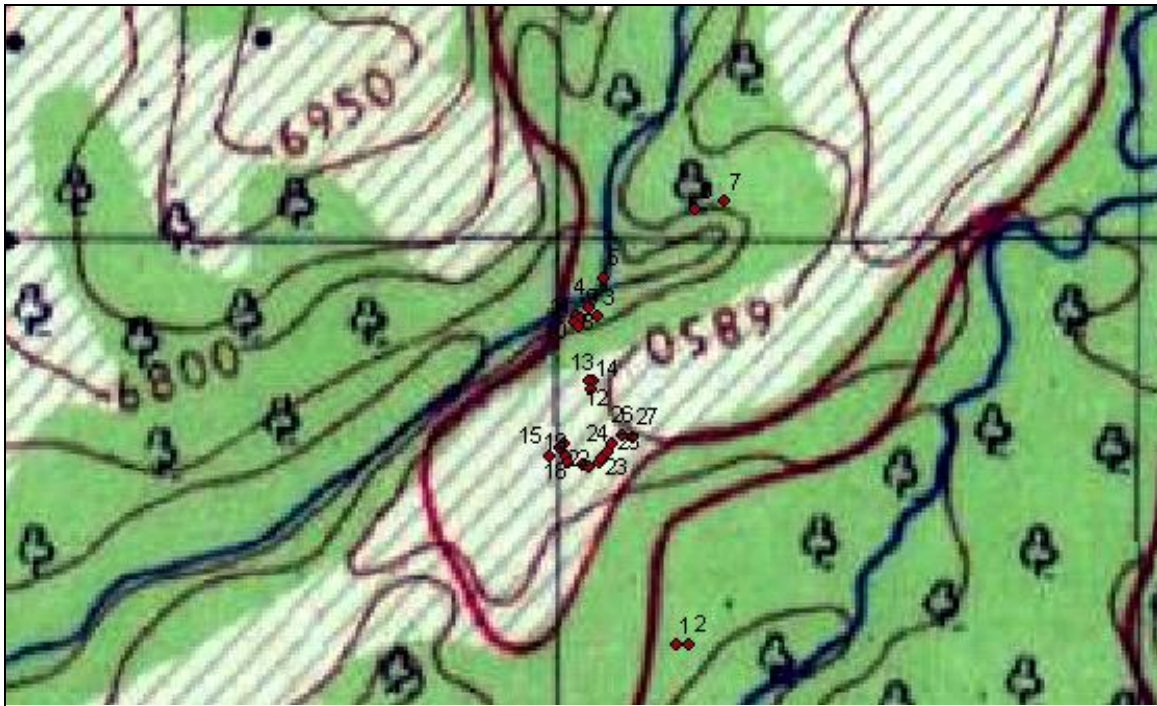


Plate 2.2 Map of specific location of 29 individuals located during the study at Ragati forest (GPS coordinates given in appendix 8).

2.3.1 Flowering Phenology and Floral Biology

Ixora scheffleri subsp *keniensis* was in flower from November to March (Fruits April to July) with a peak of flowering in February. The flowers at anthesis at any one day never exceeded 10% of the total number in an inflorescence. At a rate of more than 200 flowers, this results in a very long-lived inflorescence (up to 1 month). Furthermore, inflorescences in an individual plant were in various stages of development. Flowers in this plant are hermaphroditic as is generally the case in the Rubiaceae. The flowers are sweetly fragrant.

2.3.2 Inflorescence and Floral Morphology

The inflorescence in *Ixora scheffleri* subsp *keniensis* is terminal and pendunculate. The inflorescences are trichotomously branched (4 – 10 cm wide) and 6 – 15 cm long) (see plate 1.2). The inflorescence is erect or rarely drooping. The flowers are actinomorphic and 4 – merous. The flowers have a short calyx and long narrow cylindrical corolla tube with relatively short corolla lobes. The corolla has a white to pink colour. The calyx lobes are persistent (0.4 – 0.7 cm long). The lobes have rounded or obtuse tips. Stamens with filaments 1.2 – 2.3 mm long, anthers 3.4 – 6 mm long. Style exerted 4 – 6 mm, including the long recurving stigmatic arms.

2.3.3 Floral visitors

All the 8 insect species regularly visiting the flowers of *I. scheffleri* subsp *keniensis* frequently made contacts with the stigmas. Examination of the captured insects revealed copious amounts of *I. scheffleri* subsp *keniensis* pollen attached to their bodies. These visitors were consequently deemed to be effective pollinators. Three Lepidoptera (*Pieris*

brassicae: Family; Pieridae, *Aporia crataegi*: Family; Pieridae and *Galleria mellonella*: Family; Pterophoridae), two diptera (*Syrphus ribesii*: Family; Syrphidae and *Episyrphus balteaus*: Family; Syrphidae), one coleoptera (*Chalcophora mariana*: Family; Buprestidae), one hymenoptera (*Apis mellifera*: Family; Apidae) and one thysanoptera (*Kakoyhrips pisivorus*: Family; Thripidae) were the visitors that frequently visited the flowers. Another insect visitor noted in large numbers belong to order Hemiptera, Family Pentatomidae; genus, *Chlochroa*; species, *ligata*. They laid eggs on lower surfaces of *Ixora* leaves. These are locally known as stink bugs. They were found to be predaceous on other insect visitors.

2.3.4 Breeding systems

Table 2.1 summarises the results of the breeding experiments. The control, wind and/or insect pollination treatment resulted in 79.5 % fruit set. However this percent fruit set was not significantly different from that emanating from geitonogamy treatment (74.5 %). The spontaneous autogamy treatment resulted in 36.5 % fruit set. This observation indicates that the species is self compatible and capable of autogamy (Table 2-1). Agamospermy (0 %) was not detected in this experiment. Open pollination and geitonogamy treatment resulted in substantially higher fruit set than the pollinator – exclusion treatment (79.5, 74.4 and 36.5 % respectively).

Table 2-1 Percentage fruit set emanating from various breeding experiment treatments

Treatment	T	M %	M/t
A	114	57c	11.4
B	88	44b	8.8
C	149	74.5a	14.9
D	159	79.5a	15.9
E	73	36.5d	7.3
F	0	0	0
G	130	65e	13
s.e.d		4.77	0.954

Key

A = Wind and/or insect pollination but not autogamy

B = Wind but not insect pollination

C = Geitonogamy

D = Wind and/or insect pollination

E = Spontaneous autogamy

F = Agamospermy

G = Xenogamy

s.e.d. = Standard error of difference.

M% = Fruit set (%)

T = Number of fruits out of 200

M/t = Mean number of fruits per tree.

Comparing the effect of each treatment on fruit set, there was significant difference between the treatments apart from geitonogamy treatment and the open pollination treatment (table 2-1).

Further analysis using binary logistic modeling on the number of fruits produced showed that the chances that flowers subjected to open pollination setting fruits was 2.9 times higher than those under wind but not insect pollination (Table 2-2). Based on the same procedure flowers under xenogamy treatment were 1.4 times likely to set fruits than those

under the wind but not insect pollination (Table 2-2). These results all consistently suggest that *Ixora scheffleri* subsp *keniensis* is largely outcrossing and natural fruit set is predominantly pollinator-dependent.

Table 2-2 Comparison of the various treatments in the logistic model.
Reference level: Coarse mesh bagging (wind but not insect pollination (B))

Parameter	estimate	s.e.	t pr.	Odds ratio
Constant (B)	0.282	0.152	0.069	
A	-0.523	0.214	0.018	0.5927
C	0.790	0.230	0.001	2.204
D	1.073	0.240	<. 001	2.926
E	-0.836	0.218	<.001	0.4336
F	0.337	0.219	0.12	1.401

2.3.5 Pollen viability

Using both methods, pollen grains lost their viability in about 18 days after anthesis. This was deduced from the fact that pollen stainability was consistent from day one after anthesis to around day 18. The pollen grains also germinated consistently throughout the days that the investigation was carried out.

2.3.6 Stigma receptivity

***In vivo* assessment of stigma receptivity**

The number of fruits set increased from day 4 to day 9 and then started reducing as the days approached 15 onwards. This pattern indicated that the highest percentage of fruits were set between day 4 and day 9. There was no significant difference ($p > 0.05$, one way ANOVA and log linear modeling) in fruiting between these days i.e day 4 to day 9 (Table 2.3 and Figure 2.1).

Table 2-3 Summary of average number of fruits set

DayTotal fruits set out of 100	
1	32
2	42
3	50
4	58
5	56
6	62
7	60
8	62
9	62
10	56
11	50
12	40
13	22
14	22
15	28

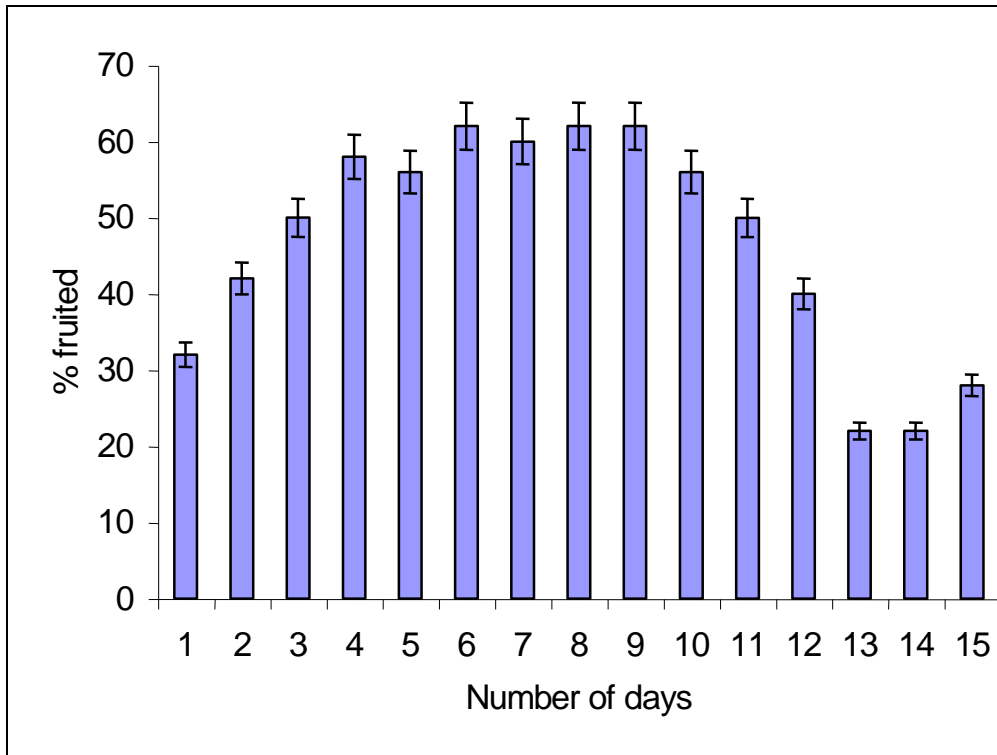


Figure 2-1 Percentage number of fruits set emanating from different days of stigma receptivity. Vertical bars represent standard error of means, n = 100.

Table 2-4 Comparison of fruits set on the various days in the logistic model.
Reference level: Day 1.

Parameter	estimate	s.e.	t pr.	Odds ratio
Constant	-0.754	0.173	<. 001	-
Days 2	0.431	0.238	0.075	1.539
Days 3	0.754	0.236	0.002	2.125
Days 4	1.077	0.238	<. 001	2.935
Days 5	0.995	0.237	<. 001	2.705
Days 6	1.243	0.240	<. 001	3.467
Days 7	1.159	0.238	<. 001	3.187
Days 8	1.243	0.240	<. 001	3.467
Days 9	1.243	0.240	<. 001	3.467
Days 10	0.995	0.237	<. 001	2.705
Days 11	0.754	0.236	0.002	2.125
Days 12	0.348	0.238	0.149	1.417
Days 13	-0.512	0.260	-	-
Days 14	-0.512	0.260	0.054	0.5994
Days 15	-0.191	0.249	0.447	0.8264

Further analysis using binary logistic modeling on the number of fruits set on different days showed that the chances of fruits being set on day 2 were about 1.5 times higher than in day one and 3.5 times higher in day 9 than in day one. On the other hand, the chances of fruits being set on day 15 were less than in day one by about 18% (table 2-4). All these were significantly different ($p < 0.001$) when compared to fruit set in day one.

***In vitro* assessment:**

Pollen tubes were evident in all the pistils examined starting from 10.00 am when the experiment commenced upto 12.00 mid day when artificial pollination was stopped. This shows that stigma in this species is effectively responsive during the period when pollinators are known to be active in most plant species.

2.3.7 Seed germination

No germination of *Ixora scheffleri* subsp *keniensis* seeds was observed either in the field or in the laboratory experiments. In all the treatments, the seeds would rot and dry up in about 2 weeks. In the field all the seeds seen dropped by animals or birds after feeding on the fleshy part of the fruit appeared dried up (plate 2.3). Observations were made in the field of aggregates of intact seeds defecated by monkeys (plate 2-4). The defecated seeds were intact in nthe faecal lump. Its only the fruit coat that had been digested. Seeds from fruits collected at different developmental stages that were used in the germination experiments are shown in plates 2.5 – 2.8. One month old fruitsof this species appear green in colour and some of them have brown patches in their fruit coat. After two months they start ripening and appear reddish purple in colour. At three months the fruits are mainly purple in colour and the fruit coat in many of them apperar cracked (plate 2-

6). Investigations done on seeds at various developmental stages indicated that in all cases, the seeds had well formed embryos (plate 2-9).



Plate 2-3 *Ixora scheffleri* subsp *keniensis* dried drupes and seeds near proximity of mother plants where they have been dropped by frugivorous birds and mammals



Plate 2-4 A faecal lump of *Ixora scheffleri* subsp *keniensis* seeds defecated by a monkey



Plate 2-5 *I. scheffleri* subsp *keniensis* drupes harvested after one month



Plate 2-6 *I. scheffleri* subsp *keniensis* drupes harvested after one and a half months



Plate 2.7 *I. scheffleri* subsp *keniensis* drupes harvested after 2 months



Plate 2-8 *I. scheffleri* subsp *keniensis* drupes harvested after 3 months



Plate 2-9 Embryos extracted from 3-month-old seeds of *I. scheffleri* subsp *keniensis*

2.4 Discussion

In *Ixora* the inflorescences are always terminal. They may however be positioned at the end of axillary short shoots and then give the impression of being axillary. In several Fijian species, the inflorescences are borne on short, defoliate lateral branchlets arising from the trunk (e.g. *I. carewii*, Smith & Darwin, 1988).

Ixora species have the tendency to produce many more flowers than fruits: Abortion levels are high and only a relatively small number of fruits reach maturity (De Block, 1998). However, fruit abortion in *Ixora scheffleri* subsp *keniensis* was minimal. Nillson *et al*, (1990) recorded development into mature fruits of 14.4% of the flowers in an inflorescence. De Block (1998) describes this trend of low reproductivity and noted that it is often found in more primitive groups within the family, notably in many Gardenieae. According to him, contrasting trend of high fruit set occurs in more evolved groups.

The abundance and efficiency of pollinators may affect floral evolution in plant populations by exerting different selective pressures (Galen, 1996). *Ixora scheffleri* in

this study receives a variety of visitors. Bremekamp (1937) listed *Ixora narcissodora* and *Ixora scheffleri* (as *Ixora latituba*) as examples of moth pollinated *Ixoras*. *Ixora coccinea* and *Ixora congesta* are listed as butterfly pollinated.

Ixora scheffleri subsp *keniensis* produced fruits through both self and cross-pollination. The mixed breeding system in the species was revealed by the formation of fruits by both the flowers that were emasculated, selfed and bagged and the ones that were emasculated crossed and bagged. In spite of self-compatibility being a desirable asset in a number of improvement strategies, in several instances, selfing is associated with inbreeding depression. The tree species showed high fruit production under natural, open pollination conditions, a characteristic commonly associated with self-compatible and self-pollinated plant species (Dafni, 1992). However, it is worth noting that bagging treatments increase the temperature, thus, they can negatively affect pollination of flowers. Fruit set was higher from the non-emasculated treatments than from the emasculated ones, indicating a possible negative influence of flower emasculation on fruiting through mechanical injury. Since most insect pollinators visit flowers for pollen grains, they tend to avoid emasculated flowers (Cruden *et al*, 1989, Donnely *et al*, 1998). This can also lead to a low number of fruits among the flowers that were emasculated and left open. In some cases, the stigma and style may function abnormally following emasculation, leading to the formation of lower number of fruits (Guo and Cook, 1990).

Flowering plants possess a wide array of morphological and physiological mechanisms that influence mating patterns, particularly the degree of self-fertilization (Richards, 1986, Eckert and Barrett, 1994). Temporal separation of male and female function within flowers (intrafloral dichogamy) is one of the most widespread morphological

mechanisms and is found in > 75% of cosexual angiosperm species (Bertin and Newman, 1993).

The failure of the seeds of *I. scheffleri* subsp *keniensis* to germinate, probably due to the existence of highly specific germination requirements indicates that seed propagation is not the norm in this species. Monkeys, bush babies and birds were observed feeding on the fruits of this species and consequently it was assumed that they form part of the diet of several frugivorous birds and mammals that abound in the forest. The occurrence of animal mediated seed dispersal in the genus *Ixora* has been noted by Nilsson *et al* (1990). Seed dispersal by animals and especially by birds, which have large home ranges, is generally considered highly efficient (Erickson & Bremer, 1991). Using species number per genus as a measure of success, Eriksson & Bremer (1991) showed that in the Rubiaceae, the possession of drupes that invariably means dispersal by animals, combined with the possession of a shrub habit was an extremely successful strategy. This agrees well with the statement of Snow (1981) who described Rubiaceae fruits as an important food source for frugivorous birds in the tropics. This population of *Ixora scheffleri* subsp *keniensis* must have had the capability of seed germination in the past. It appears that lack of specific germination requirements that existed earlier has contributed to the non-germinability of the seeds. It is also envisaged from this study that effective pollination takes place but germination bottlenecks arises afterwards. More studies are required into this phenomenon and its significance in the reproductive process in *Ixora scheffleri* subsp *keniensis*.

Despite its possession of fleshy fruits and the subsequent endozoochoral dispersal of diaspores, *Ixora* can be considered a valuable indicator for former forest refuge areas

(Robbrecht, 1996). Furthermore, *Ixora* is almost exclusively restricted to the rain forest, which implies narrow ecological conditions. Data on present day distributions of rain forest species can be used to gain insight in the historical position of glacial forest refuge areas (Sosef, 1994.)

CHAPTER 3

3.0 VEGETATIVE PROPAGATION OF *IXORA SCHEFFLERI* SUBSP

KENIENSIS

3.1 Introduction

Vegetative propagation offers the opportunity to rapidly overcome the limitations to domestication imposed by long generation times, irregular fruiting/flowering and out breeding. Vegetative propagation techniques are increasingly being applied to a wide range of tree species, of both moist and dry tropics. Identification of the critical factors determining adventitious root development is crucial to sustained, cost effective propagation, even in species in which these factors are not currently limiting. A wide range of factors influence rooting, including the stockplant growth environment, cutting origin, post-severance treatments applied to cuttings, and the propagation environment. In order to understand the influence of these different factors and their interactions, an appreciation of the physiological, biochemical and cytological processes involved in rooting is required. The primary processes occurring in the leaf are net photosynthesis and transpiration, while those in the stem are starch hydrolysis, translocation of sugars, water and nutrients, respiration, mitosis and cell differentiation. Each of these processes is influenced by a number of environmental, morphological and physiological variables, such as leaf area and thickness, internode length chlorophyll content, stomatal density, stem lignification etc.

Vegetative propagation techniques are increasingly being applied to the domestication of tropical tree species (Leakey *et al* 1990). A range of approaches can be utilized (Hartman

and Kester 1983, Leakey, 1985), including grafting, stem cuttings, marcotting and suckering. Several of these techniques have the disadvantages of a low rate of multiplication, a high requirement for skilled labor, or the need for high capital investment. These problems have been overcome by advances in the development of a low technology propagation system (Leakey *et al* 1990, Newton *et al* 1992), which has enabled the successful propagation of a wide range of species by leafy stem cuttings. This system utilizes non-mist propagators constructed out of cheap and readily available materials, with no requirements for piped water and electricity

Although many species can be propagated easily by leafy stem cuttings, some species are recalcitrant. In such situations, identification of the factors limiting rooting is important even in species which are propagated easily, as small gains in rooting percentage may be of considerable economic value when the species is mass propagated on a commercial scale.

In some circumstances, the rooting ability of cuttings may decline with successive harvests of cuttings from a set of stockplants. This phenomenon can arise when over frequent harvesting of cuttings causes the depletion of the endogenous reserves of the stump, the death of the stockplants fine root system or the reduction in soil fertility. For all these reasons, an understanding of the factors that influence rooting is fundamental to successful and sustained vegetative propagation.

Another key reason for a better understanding of the rooting process is the accumulation of a body of apparently contradictory data in the scientific literature. Most propagation experiments usually consider only one or two of the many factors known to influence rooting, and fail to recognize their interactions. Many investigators also fail to measure

and record sources of variation in rooting ability, which can differ between experiments such as propagation environment and stockplant growth conditions. Consequently, the mechanisms of root formation remain unclear.

In order to understand the interactions between the different factors influencing rooting, an appreciation of the physiological processes involved is required. There has been a number of literature reviews on this topic (e.g. Leakey, 1985; Anderson, 1986; Haisig, 1986; Davis, Haissig and Sakhla, 1988; Thompson, 1992).

The key physiological, biochemical and cytological processes influencing adventitious root development in a leafy cutting are photosynthesis, transpiration, respiration, starch hydrolysis, translocation of sugars, water and nutrients, mitosis, cell differentiation and elongation. While all these processes may operate in different parts of leafy hardwood cuttings, photosynthesis and transpiration primarily occur in the leaf, and mitosis and cell differentiation are generally of greatest importance in the cutting base.

The interactions between these processes and the factors affecting rooting are considered in this chapter.

The aims of the study described in this chapter were to determine the,

- 1) Effect of Indole butyric acid (IBA) on rooting of cuttings
- 2) Effect of Naphthalene acetic acid (NAA) on rooting of cuttings
- 3) Effect of propagation media on rooting
- 4) Seasonal periodicity of rooting
- 5) Effect of leaf area on rooting
- 6) Effect of cutting position on mother plant on rooting
- 7) Effect of cutting length on rooting.

3.2 Materials and Methods

3.2.1 Stock Plant Production

A field survey of *Ixora scheffleri* subsp *keniensis* in its area of endemism in Kenya was carried out from June 2001 to December 2001 to determine the extent of extirpation of the species. To adequately represent the genetic base of the population, all the accessible individuals were considered in the harvesting of cuttings. The cuttings were treated as explained below and used as stockplants in the present trials.

3.2.2 Collection and preparation of cuttings

Single node cuttings were harvested sequentially down the main stem from out planted stockplants and also from Mt Kenya forest. The cuttings were harvested between 12.00 noon and 1.00 pm. Unless otherwise stated, the leaf lamina was trimmed with scissors to an area of 80 cm². All cuttings were treated with Indole-3-butyric acid (IBA) dissolved in industrial methylated spirit. The IBA was applied with a Drummond ‘microcapsule’ disposable micropipette to the base of the cutting as a 10 µl droplet. Except where otherwise stated 50 µg IBA per cutting was applied. Before inserting the cutting in the rooting medium, methylated spirit was evaporated off with a stream of cold air in a lamina flow hood. Cuttings which had a mean length of 5 cm (unless otherwise stated) were set in a specific rooting media in the green house in a completely randomized design.

This study was carried out at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in the central province of Kenya. A propagation unit was

established at the department of Botany green house following the low technology non-mist design described by Leakey *et al* (1990). The propagators were sited in a shade house roofed in transparent plastic sheets providing irradiance inside the propagators of approximately 15 – 30% of that received outside the unit. The cuttings were collected from Mt Kenya forest, near Ragati forest station, the only location where *Ixora scheffleri* subsp *keniensis* singular population is found.

Description of experiments

A number of experiments were conducted as outlined under the following subheadings:

3.2.3 Effect of Propagation Media on Rooting.

The aim of this study was to determine the best propagation media for rooting. Seven rooting media were tested. These were, Forest soil (FRS), Sphagnum moss (SM), Gravel (G), Fine sand (FS), Sawdust (SD). Additional treatments were obtained by producing 50:50 mixtures of gravel: sawdust (G: SD) and forest soil and sand (FRS: S). Forest soil (FRS) and Sphagnum moss were obtained from Mt Kenya forest. Sand was obtained from sand selling locations in Juja town and sieved into 3 grades through meshes of 2, 3 and 5 mm (labeled fine sand (FS), medium sand (S) and gravel (G) respectively. Sawdust was obtained from a local hardware establishment in Juja town, and then sieved through a 5mm mesh. The media was put in polythene bags (10cm diameter x 18cm depth). The media were sprayed with a systemic fungicide prior to insertion of the cuttings. The experiment was established in a Completely Randomized Design (CRD) with each treatment replicated five times. Each experimental unit was assigned 20 cuttings, $n = 100$. The cuttings had their leaves trimmed to 80 cm^2 and the stem base of each cutting was treated with $50 \text{ }\mu\text{g}$ of IBA. Preliminary trials had indicated that 80 cm^2 and $50 \text{ }\mu\text{g}$ were

optimal in promotion of rooting of these cuttings. Evaluation of the rooting success began five weeks after setting the cuttings and involved recording the number of rooted and dead cuttings up to the eighth week. For each rooted cutting, the number of roots more than 2 mm in length was recorded. Cuttings with roots were potted and removed from the experiment.

3.2.4 Effect of leaf area on rooting ability of leafy stem cuttings of *Ixora scheffleri* subsp *keniensis*

Cuttings either from the stock plants growing in the JKUAT botanical garden or directly from Mt Kenya forest were allocated to each of five leaf area treatments: 0, 20, 40, 80, and 100 cm². Using templates cut from graph paper, the leaves were trimmed to size prior to severance. Each cutting was treated with 50 µg IBA as described for experiment 1. The experiment was set as a completely randomized block design just like for experiment 1. The rooting medium was a mixture of forest soil and medium sand in a ratio of 50:50. Percentage of rooting was assessed as in Experiment 1. The rooting media for this and subsequent experiment was FRS: S selected on the premise that it led to optimum root production based on results from section 3.2.3 above.

3.2.5 Effect of different concentrations of IBA and NAA on rooting of leafy stem cuttings of *Ixora scheffleri* subsp *keniensis*.

Single node cuttings were taken and randomly allocated to one of six IBA and NAA treatments namely (0, 10, 45, 55, 65, 75, 150, and 250 µg) IBA or NAA as described in experiment 1. The control treatment was 10ul of industrial methylated spirit only. The leaves of each cutting were trimmed to approximately 80 cm² using paper templates. The

cuttings were inserted in the Forest soil: sand rooting medium and assessed as in experiment 1. The leaf area for this and subsequent experiments was 80 cm² selected on the premise that it led to optimum root production based on results from section 2.3.4 above.

3.2.6 Effect of cutting Position on the mother plant on rooting

Cuttings were collected from two different parts of the crown: (1) the upper third receiving full light during the entire day; this part of the crown consisted of vertical branches and fast growing, bright green shoots and (2) the lower third, which was partly shaded by the surrounding trees; this part consisted of slanting branches, with slower growing dark green shoots. All the cuttings were treated as in 1 above and planted in Forest soil: sand-rooting medium.

3.2.7 Seasonal periodicity of rooting

To determine the best time for rooting, cuttings were taken during the rainy season and the dry season. The rainy season was in mid march. The dry season was in mid November. The mean temperatures for the rainy season were 20 ° C and for the dry season 24° C. The cuttings were treated with 50 µg IBA and all the cuttings were trimmed to have a leaf area of 80 cm². They were planted in Forest soil: sand rooting medium. Percentage of rooting was assessed as in experiment 1.

3.2.8 Effect of cutting length on rooting ability

Shoots were randomly allocated to each of four length treatments: 25, 50, 75 and 100 mm. The cuttings were treated with 50 µg IBA and all the cuttings were trimmed to have

a leaf area of 80 cm². They were planted in Forest soil: sand rooting medium. Percentage of rooting was assessed as in experiment 1.

3.2.9 Description of data and methods of analysis

In all the experiments conducted, the number of cuttings that rooted, root length and survival were assessed as influenced by the factors tested in the experiments outlined above. Assessment on rooting began at week five and ended at week eight.

Descriptive data analysis based on cumulative rooting percentage of *Ixora scheffleri* subsp *keniensis* shoot cuttings was calculated for each propagation media, Indole butyric acid (IBA), Napthalene acetic acid (NAA), seasonal periodicity, leaf area, cutting position on mother plant and cutting length to identify the pattern of rooting as influenced by each factor used in the experiments. Homogeneity of variance for cumulative rooting percentage was evaluated using a Barleit test (Snedercor and Cochran, 1980). Pearson correlation analyses were performed to assess the interrelation of the factors that potentially affect the rooting ability of the cuttings. Comparisons of each propagation media, Indole butyric acid (IBA), Napthalene acetic acid (NAA), seasonal periodicity, leaf area, cutting position on mother plant and cutting length were planned a prior and orthogonal contrasts were used to compare differences in rooting among the potential factors that were associated with rooting ability. Significance levels of ($p < 0.01$ and $p < 0.05$) were used for both main effects and interactions.

Overall, the experiments set generated data that was classified as success or failure to root under different factors. This was classified as binary data hence further inferential statistical analyses on rooting under each factors were carried out using logistic regression model.

All analyses were performed using Genstat release 8.11 statistical software (Genstat, 2005) employing the General Linear model (GLM) procedures. MS Excel for windows 2003 together with Genstat was used in data management procedures and exploratory data analysis. Standard errors and confidence limits of percentages were calculated following the procedure described by Snedecor and Cochran (1980) for binomial data. Analysis of deviance (ANODE) by stepwise regression in GENSTAT 8.11 was applied to assess the effect of both treatment and non-treatment variables on final rooting percentages.

3.2.10 Development of Logistic regression model

To assess and predict the extent at which each propagation media, Indole butyric acid (IBA), Napthalene acetic acid (NAA), seasonal periodicity, leaf area, cutting position on mother plant and cutting length contributed to the rooting ability of *Ixora scheffleri* subsp *keniensis* shoot cuttings, logistic analysis was employed. This model overcomes, most of the problems associated with linear probability models and provides parameter estimates that are symptomatically consistent and computationally easier to use (Pindyck and Rubinfeld, 1981) (See appendix 1)

3.3 Results

3.3.1 Effects of propagation media on rooting of cuttings

The FRS: S rooting medium had the highest number of cuttings that rooted as compared to all other propagation media and this was evidenced in all the weeks. G had shown least germination in week five followed by FS and G: SD (Figure 3.1). The pH of the forest

soil was 6.1, N (%), 0.10, total carbon (TOC, %), 3.5, P(%) 0.17, available Fe (ppm), 24, Zn (ppm), 29, Cu (ppm), 3.1, Mn (ppm), 179.

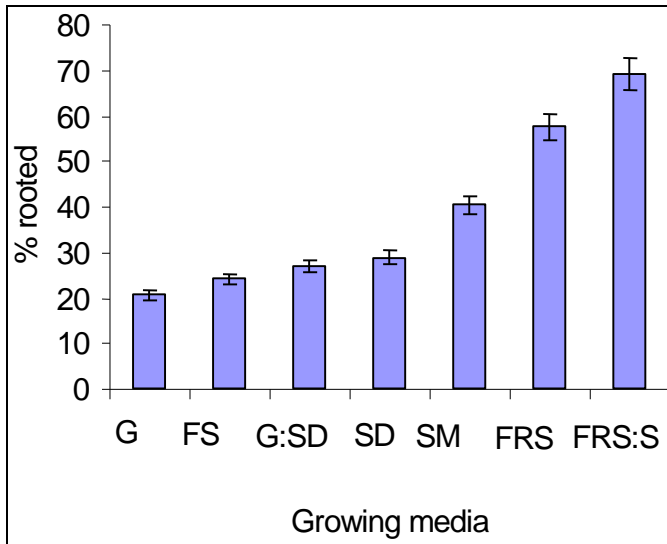


Figure 3-1 Overall percentage rooted at end of 8 weeks. (Vertical bars represent standard error of means, n = 100).

The rooting significantly increased ($p < 0.01$, ANOVA) across the weeks (Appendix 2, Figure 3.2) other than between 7th and 8th week ($p = 0.333$, ANOVA) and varied significantly ($p < 0.01$) among the growing media. Overall the effect of growing media contributed 70.2% of the variance accounted for, time in weeks accounted for 15.5% whereas the interaction between growing media and time accounted for 1.1% which was not significant implying that the rooting under various growing media was independent over time. FRS: S recorded the highest percentage (69%) rooting, followed by FRS and SM with 57.5% and 40.3% respectively (Figure 3.1). Plate 3.1 show uprooted *Ixora scheffleri* subsp *keniensis* seedlings after 3 months



Plate 3-1 Uprooted *Ixora scheffleri* subsp *keniensis* rooted cuttings after 4 Months

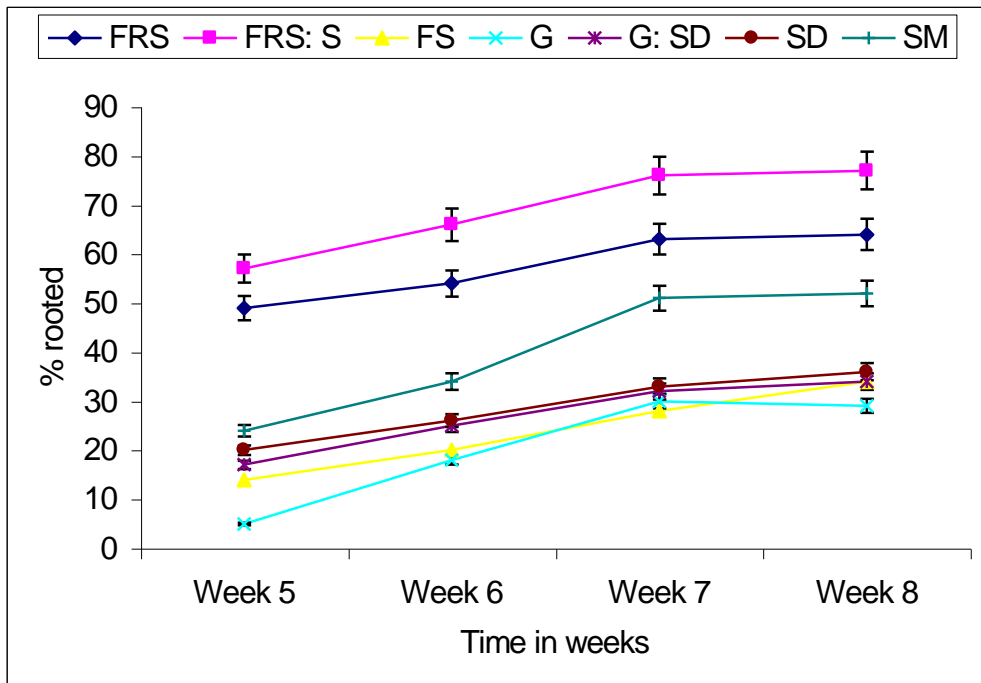


Figure 3-2 Trend of rooting under various growing media (Vertical bars represent standard error of means, n = 100)

In addition, the orthogonal contrasts (Appendix 3) between the weeks showed that rooting was significantly different ($p < 0.01$, ANODE) between 6th and 7th, 6th and 8th weeks respectively. This suggests that maximum rooting is likely to be achieved at 7th week under various propagation media in this investigation.

Further inferential analysis of data by using orthogonal contrasts indicated that there was a significant difference ($p < 0.01$, ANOVA) in rooting between FRS: S and the rest as well as between FRS and the rest. There was also sufficient evidence ($p = 0.011$, ANOVA) to declare a difference in rooting between G and G: SD. On the other hand there was no reason to believe that there was a difference in rooting between FS and G ($p = 0.168$, ANOVA), FS and G: SD ($p = 0.237$, ANOVA) and FS and SD ($p = 0.063$).

Running the logistic regression model for assessing the effect of propagation media and the duration, there was sufficient evidence ($p < 0.01$) that they significantly contributed to rooting of *Ixora scheffleri* subsp *keniensis* cuttings. The fitted terms (constant, growing media and duration in weeks) in the model accounted for 81.6% of the total variance. The interaction term was not fitted in the model because it was not significant meaning that its contribution to rooting is very negligible hence omitted from the model. Table 3.1 shows the comparisons between week five and other weeks. FRS is also compared with all other six growing media.

Table 3-1 Regression analysis results. Reference levels: week 5 and FRS

Terms in the model	s.e	t.prob	Odds ratio (Exp. Estimate.)
Constant	0.112	0.013	0.8
Week 6	0.109	<0.001	1.5
Week 7	0.107	<0.001	2.5
Week 8	0.107	<0.001	2.8
FRS: S	0.132	<0.001	1.7
FS	0.137	<0.001	0.2
G	0.142	<0.001	0.2
G: SD	0.133	<0.001	0.3
SD	0.135	<0.001	0.3
SM	0.128	<0.001	0.5

NB: se = Standard error. T.prob = t probability

Odds ratio refers to how many times the parameter under consideration influences performance in relation to the reference factors. In this case week 5 and FRS.

The results in Table 3-1 shows that the chances that the cuttings rooted at week six was about 1.5 times higher than in week 5. Similarly the chances that the cuttings rooted at week 7 were about 2.5 times higher than in week 5. Equally the chances that cuttings rooted at week 8 were about 2.7 times than in week 5. This confirms the earlier observation and correctly predicts that the appropriate duration *Ixora scheffleri* subsp *keniensis* cuttings would take to have almost maximum rooting under 50ug of IBA for transplanting is about 7 weeks. Figure 3.3 shows the overall cumulative mean percentage of the cuttings that rooted across the 8 weeks beginning from week five.

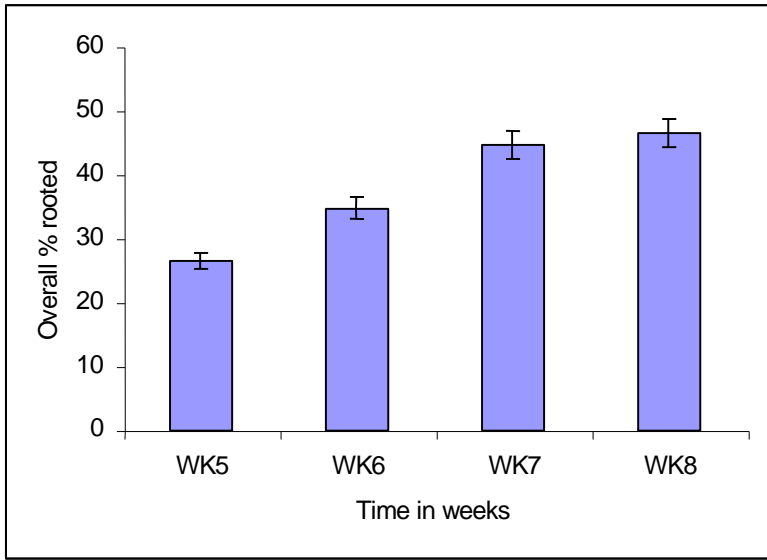


Figure 3-3 Overall percentage rooting across the weeks

Consequently, the chances that the cuttings rooted under FRS: S as compared to FRS was about 1.7 times higher. This implies that the addition of sawdust to FRS significantly ($p < 0.01$) influenced the rooting of *Ixora scheffleri* subsp *keniensis* cuttings. This again confirms the earlier results where the cuttings experimented under FRS: S showed the highest rooting percentage 76% by week seven, 77% by week 8 and overall of 69% rooting in any growing media. In addition the chances that cuttings would root under FRS were about 80% higher than in FS, G: G: SD and SD, but about 52% higher as compared to SM. Subsequently this agrees with the pattern of the data where FRS is the second growing media in which the cuttings rooted better. This implies that FRS can do very well once enriched with sawdust.

3.3.2 Effect of Indole butyric acid (IBA) and Napthalene acetic acid (NAA) on rooting of cuttings

The results of various levels of IBA and NAA are as summarized in Table 3.2 below

Table 3-2 Percentages of the cuttings that rooted under different hormonal concentration

	Week 5	Week 6	Week 7	Week 5	Week 6	Week 7
Conc. (ug)	IBA	IBA	IBA	NAA	NAA	NAA
0	2	3	4	0	0	1
10	19	27	30	12	13	15
45	35	38	40	18	20	23
50	70	73	74	41	45	50
55	67	68	70	36	38	40
65	58	62	64	35	39	40
75	53	55	58	33	33	35
150	42	50	56	30	33	34
200	42	51	54	27	30	32
250	44	47	54	25	29	30

The results in Table 3-2 and figure 3-4 shows that rooting of the cuttings increased from 0 concentration up to 55µg and started decreasing from 65µg to 250µg in both IBA and NAA. However, the general performance showed that the rooting of cuttings under IBA were higher than those under NAA.

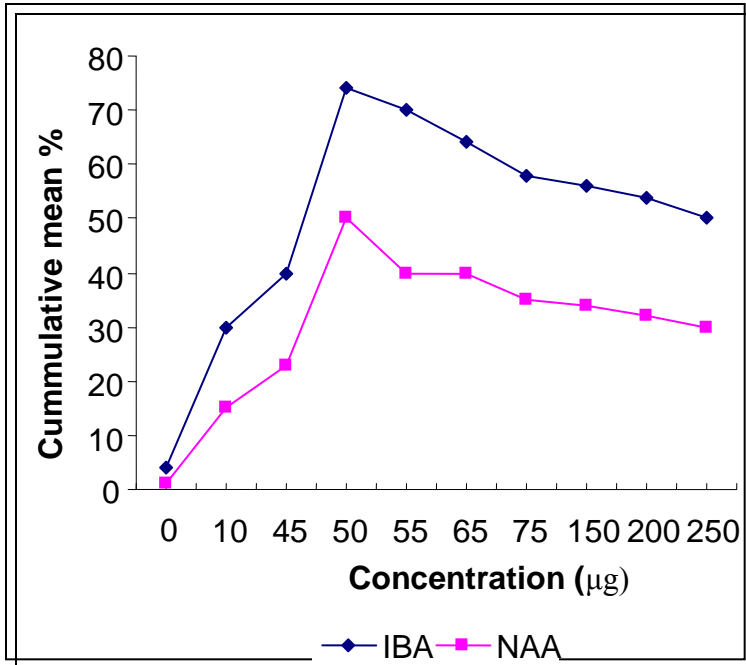


Figure 3-4. Percentage rooting trend for IBA and NAA under different concentrations during the seventh week

It can be deduced further from Figure 3-4 that both IBA and NAA obtained their optimum rooting percentage between the concentrations of 50-55 µg. This range of concentration appears to be optimal for root induction in cuttings of *Ixora scheffleri* subsp *keniensis* cuttings. The overall rooting percentages under various concentrations for the hormones are summarized in figure 3-5 below.

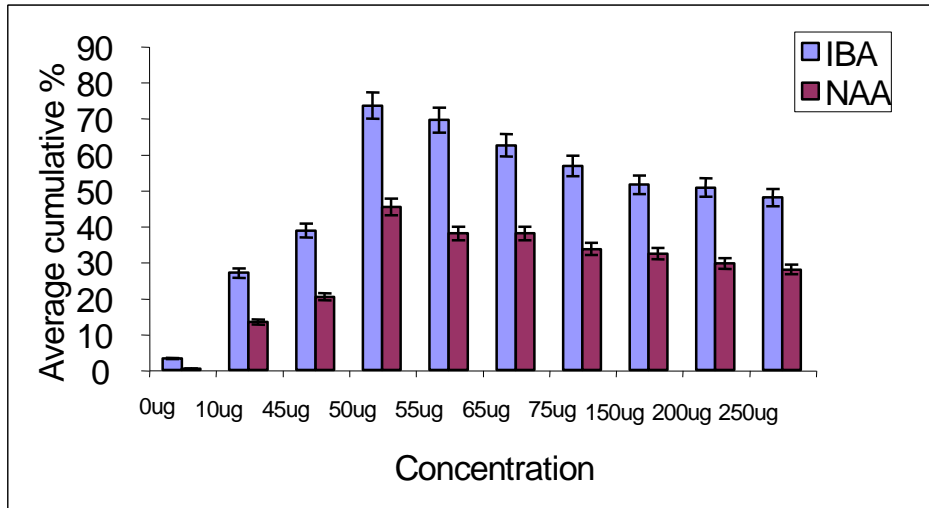


Figure 3-5. Accumulated average rooted cuttings under various concentrations of IBA and NAA. Vertical bars represent the standard error of means, n = 100.

The logistic regression analysis model of the fitted terms (week, replication, hormone, concentration (μg) and the interaction between hormone and concentration) accounted for 95% of the total variance. This implies that the model terms correctly gave the effect of time, hormone and concentration in influencing the rooting of *Ixora scheffleri* subsp *keniensis* cuttings. The concentrations in the analysis of this case were from control up to 75 μg . The other concentration were omitted in this inferential analysis because their effect reduced the percentage rooting of the cuttings significantly ($p < 0.05$). From the accumulated ANODE, Appendix 4, there was significant difference ($p < 0.01$) in rooting among the various hormone concentrations, time, hormones and the interaction between the concentration and hormone. This implied that as from the descriptive analysis, IBA significantly performed better than NAA in influencing the rooting of the cuttings. Equally, the significant interaction showed that the performance of various concentrations highly depended on the type of hormone, where in this case, IBA concentration had high effect on rooting than the ones on NAA. Overall, the hormonal

concentrations accounted for 77% of the total variance whereas hormones accounted for 12.6% and duration 4.2% with interaction accounting for 0.6% of the total variance. This fully supports the findings that the various concentrations significantly influenced the rooting of the cuttings.

Comparisons of the difference from the parameter estimates in the model showed that the chances that the cuttings would root under IBA are about 90% higher than those ones under NAA. This confirms the results that IBA is suitable for enhancing rooting of *Ixora scheffleri* subsp *keniensis* cuttings. Equally, the chances that the cuttings root under 50µg would be about 84.6 times higher and 69.4 times on 55µg than when not completely applying any concentrations (Table 3.3). This affirms the results obtained showing that the appropriate amount of concentration that would significantly influence the rooting lies between 50-55µg even though rooting predicted under 50µg was about 15.2 times higher than in 55µg. This further shows that 50µg can effectively influence the root cuttings than 55µg overall.

Table 3-3 Comparisons of various terms in the logistic model. References; Week5, IBA and 0 μ g

Terms in the model	s.e	Odds ratio (Exp. Estimate)
Constant	0.158	
Week 6	0.0471	1.1
Week 7	0.0470	1.3
Week 8	0.0598	1.4
IBA	0.552	0.1
10 μ g	0.162	11.1
45 μ g	0.160	19.1
50 μ g	0.162	84.6
55 μ g	0.161	69.4
65 μ g	0.160	50.7
75 μ g	0.159	39.8

NB: Odds ratio refers to how many times the parameter under consideration influences performance in relation to the reference level. In this case its week 5 and IBA 0 μ g

3.3.3 Effect of Seasonal periodicity of rooting

Figure 3.6 shows a summary of cumulative average percentage of rooting between dry and rainy season of the year under FRS: S. The cuttings were inoculated with 50ug/l and they had a leaf area of 80 cm².

The rooting as shown did not differ significantly ($p=0.779$) across the seasons of the year but differed significantly ($p<0.01$) across the weeks. This implies that the cuttings rooted equally in both seasons.

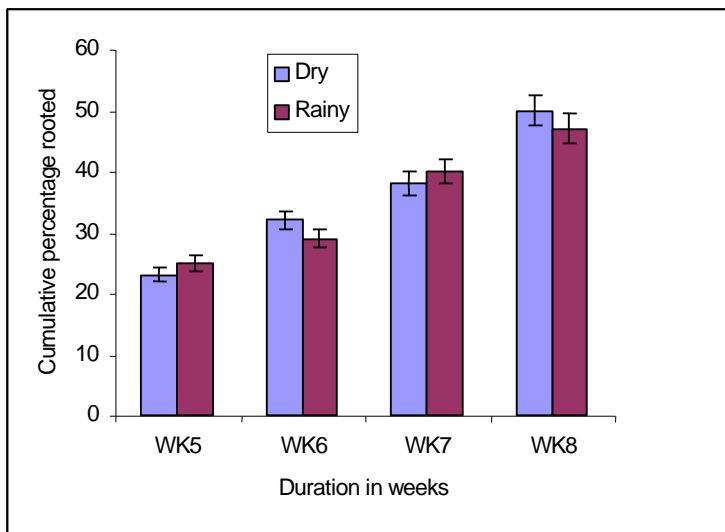


Figure 3-6. Cumulative rooting % under rainy and dry seasons. Vertical bars represent the standard error of means, $n = 100$.

3.3.4 Effect of Leaf Area on rooting

Descriptive analysis (Figure 3-7) showed that the percentage rooting of the cuttings from various leaf areas increased with increase of leaf area from 0-80cm² but reduced as the leaf area increased to 100cm². On the other hand overall rooting increased across the weeks (Figure 3-7).

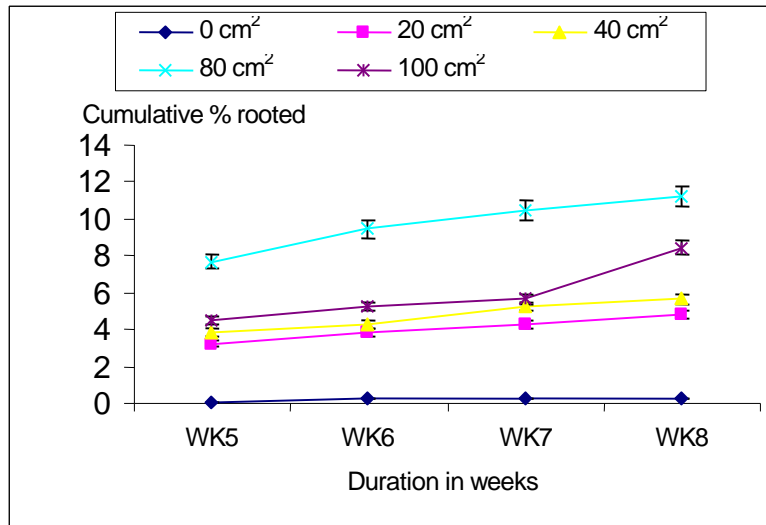


Figure 3-7. Rooting progression across the weeks. Vertical bars represent the standard error of means, n = 100.

The inferential results on this effect using the logistic regression model showed that the rooting significantly increased ($p < 0.01$, Accumulated ANODE) as the leaf area increased from 0 cm^2 to 80 cm^2 but significantly reduced when the area increased to 100 cm^2 . The terms fitted in the model (week, replication and area) accounted 90% of the total variance with leaf area term accounting 81% (Appendix 5). This implied that leaf area has significant effect in influencing the performance of the cutting rooting. In this case the leaf area of 80 cm^2 was found to be the most suitable for propagation. This was further affirmed from the model that the chances of the cuttings rooting under 80 cm^2 would be about 131.5 times higher as compared to 0 cm^2 whereas the chances of the cuttings rooting under 100 cm^2 would be about 57.6 times higher as compared to cuttings of 0 cm^2 (Table 3-4). This shows that indeed the appropriate leaf area for rooting was reached at 80 cm^2 at week 7 or 8. On the other hand there was highly significant difference ($p < 0.01$) in rooting across the weeks with maximum rooting obtained at week 7 or 8.

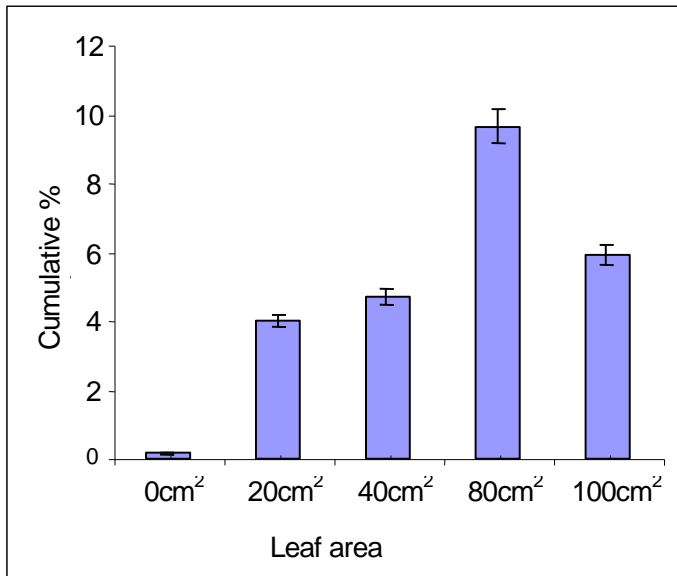


Figure 3-8 Overall percentage of cuttings that rooted under different leaf areas. Vertical bars represent the standard error of means, n = 20.

Table 3-4 Comparisons of various terms in the logistic model. Reference week 5 and 0 cm²

Terms in the model	s.e	t.prob	Odds ratio
Constant	0.389		
Week 6	0.109	0.018	1.3
Week 7	0.440	<0.001	1.6
Week 8	0.677	<0.001	2.0
20 cm ²	0.386	<0.001	34.0
40 cm ²	0.385	<0.001	41.9
80 cm ²	0.383	<0.001	131.5
100 cm ²	0.384	<0.001	57.6

s.e = standard error. T.prob = t. probability.

Odds ratio refers to how many times the factor under consideration influences performance in relation to a specified reference level. In this case its week 5 and 0 cm²

3.3.5 Effect of cutting position on mother plant

The results (Table 3-5, Figure 3-9) showed that cuttings obtained from the lower third (L1/3) of the mother tree significantly ($p < 0.01$, Cumulated ANOVA) rooted better than the ones obtained from upper third (U1/3) of the mother plant under 50ug/l of IBA, 80cm² and FRS: S. This implies that for further propagation of this species cuttings obtained from lower a third can enhance rooting. Overall the chances that the cuttings obtained from lower a third of the mother is about 51% higher than the ones from upper a third.

Table 3-5 Summary of % rooted

Week	L1/3	U1/3
WK5	45	30
WK6	51	35
WK7	56	37
WK8	61	45

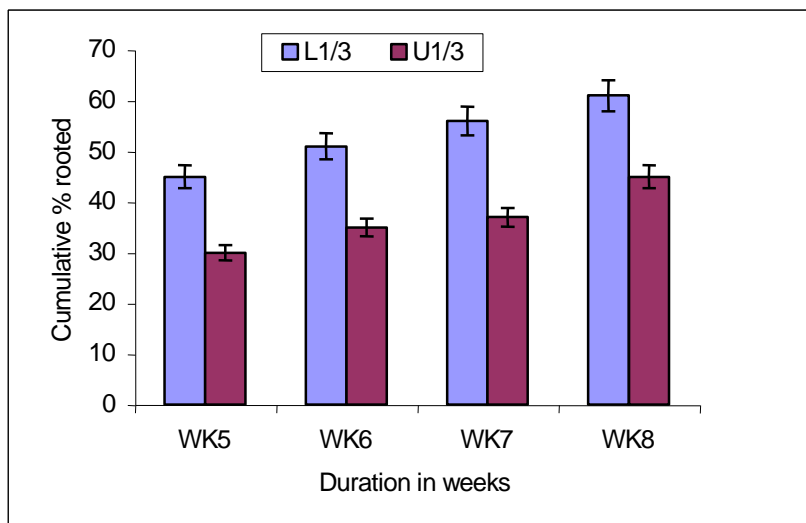


Figure 3-9. Cumulative percentage of cuttings that rooted based on position of cutting on mother plant. Vertical bars represent the standard error of means, n = 100

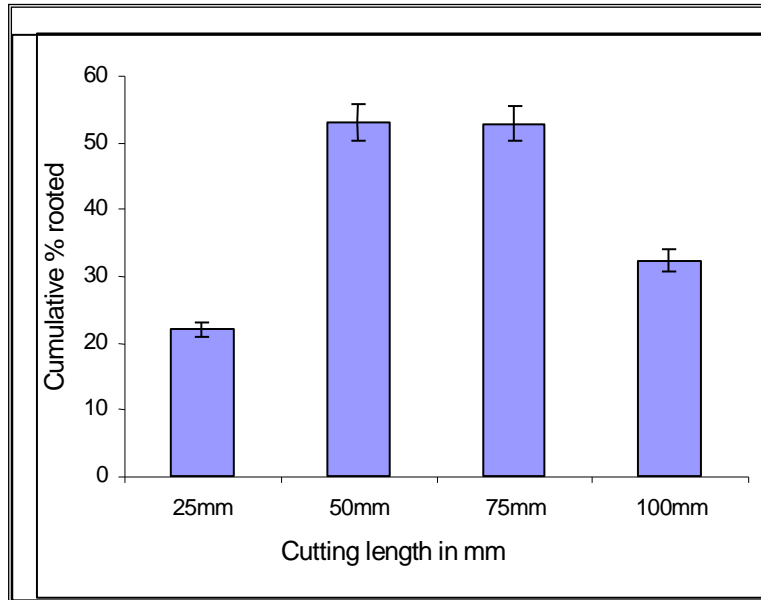


Figure 3-10 Overall cumulative rooting % under different cutting lengths. Vertical bars represent the standard error of means, n = 100

3.3.6 Effect of cutting length on rooting of *I.scheffleri* cuttings

The descriptive results showed that the percentage rooting increased with increase of cut length from 25 –75 mm and decreased when the cutting length was increased to 100 mm.

The rooting percentage also increased across the weeks (Table 3-6, Figures 3-10).

Table 3-6 Summary of % rooted under different cutting length of *I.scheffleri* across the weeks

Percentage rooted under different cut lengths				
Week	25mm	50mm	75mm	100mm
Week 5	17	40	46	26
Week 6	20	50	52	30
Week 7	24	56	54	35
Week 8	27	66	59	38

Further analysis using logistic regression model showed that there was highly significant difference ($p < 0.01$) in rooting as influenced by various terms (weeks, replication, and cutting length) in the model which counted for 85% of the total variance with cutting length accounting for 60% of the total variance. Equally there was significant difference ($p < 0.01$) among the four levels of cutting lengths and duration in weeks. On comparing the differences, the chances that the 50 mm cuttings rooting would be about 2.5 times higher than 100 mm cutting. Also the chances that 75 mm cutting rooting would be about 2.4 times higher than the 100 mm cutting, implying that 50 mm and 75 mm cutting performs almost the same (Table 3-7, Appendix 6).

Table 3-7 Comparisons of various terms in the logistic model. Rerences: 100 mm and week 5

Terms in the model	s.e.	t.prob	Odds ratio
Constant	0.129		
Time (weeks)			
Week 6	0.105	0.010	1.3
Week 7	0.104	<. 001	1.6
Week 8	0.104	<. 001	2.0
Length (mm)			
25 mm	0.111	<. 001	0.6
50 mm	0.101	<. 001	2.5
75 mm	0.101	<. 001	2.4

NB: s.e = Standard error. T.prob = t probability.

Odds ratio refers to how many times the parameter under consideration influences performance under consideration in relation to the reference level. In this case its week 5 and 100mm.



Plate 3-2 Potted *Ixora scheffleri* subsp *keniensis* seedlings. The one on the extreme right is newly potted while the other two are six months old since the commencement of rooting process.



Plate 3-3 Out planted vegetatively propagated *Ixora scheffleri* subsp *keniensis* planted at the *Ixora* conservation plot at JKUAT.

3.4 Discussion

These results clearly indicate that *Ixora scheffleri* subsp *keniensis* may be successfully propagated by leafy stem cuttings, using a non-mist propagation system. This shall go a long way towards re introducing this critically endangered endemic Kenyan hardwood species in its area of endemism (Mt Kenya forest, along the Ragati River). This is also a major breakthrough since propagation of this species through seeds has proved elusive. The fact that maximum rooting percentages of over 70% were achieved in some of the experiments suggests that the species could be multiplied on an operation scale using these techniques. Domestication of species such as *Ixora scheffleri* subsp *keniensis* which produces very high quality timber and whose forage is good fodder for livestock as well as acting as a good ornamental species could contribute greatly to the welfare of rural communities in the tropics by substantially increasing both the quality and quantity of

products available for trade (Leahey and Newton, 1994). However, the pronounced variation in rooting percentage recorded in the different experimental treatments emphasizes the need to adopt appropriate post severance protocols, if rooting percentages are to be optimized.

With respect to propagation medium, *I. scheffleri* subsp *keniensis* displayed contrasting results to a number of other tropical tree species such as *Cordia alliodora*, *Gmelina arborea*, *Vochysia hondurensis* and *Albizia guachapele*, where the highest rooting percentages were respectively recorded in gravel fine sand, gravel and a 50:50 mixture of fine sand and sawdust respectively (Leahey *et al*, 1990; Mesen, 1993). For *I. scheffleri* subsp *keniensis* the highest rooting percentage was recorded in the FRS: S rooting medium by the eighth week. Gravel propagation medium had the least rooting percentage in this experiment. The reasons why different species display contrasting rooting percentages in different rooting media are not well understood (Leahey *et al*, 1990) but may be attributed to variation in the oxygen, water content, pH and porosity of the media. These factors may affect tissue respiration and cell dedifferentiation at the base of the cutting, and subsequent root development (Loach, 1988; Leahey *et al*, 1994). In the experiment described here, the relatively high rooting percentage recorded in FRS: S rooting medium may be attributed to its optimum air: water ratio and water content. Since the forest soil was collected from the area of endemism of this species, other chemical properties e.g. pH optimum for growth of *I. scheffleri* subsp *keniensis* could also have contributed to the high rooting percentage. Contrasting rooting results recorded in different propagation media reflect variation in their physical and chemical characteristics.

Rates and final percentages of rooting in *I. scheffleri* subsp *keniensis* were considerably enhanced by applying auxins, as has frequently been found for some other woody genera. The positive relationship between IBA and NAA concentrations on the number of rooted cuttings parallel results obtained with *Gnetum africanum* (Shiembo *et al*, 1996). The highest rooting percentage in this study was recorded at concentrations of 50-55 µg in 10 µl of industrial methylated spirit for both NAA and IBA. However percentage rooting of a number of other tropical tree species has been shown to be relatively insensitive to applied auxin concentrations, for example *Lovoa trichilioides* (Tchoundjeu, 1989), *Vochysia hondurensis* (Leakey *et al*, 1990) and *Hopea odorata* (Aminah, 1992). Such contrasting results may reflect variation in endogenous auxin contents at the time of severance (Hartman *et al*, 1990). The influence of IBA on adventitious root development in cuttings has been attributed to its effect on the mobilization of carbohydrate reserves by enhancing the activity of hydrolytic enzymes (Middleton *et al*, 1980). Haissig (1986) suggested that the role of auxin in increasing stem respiration rate in cuttings also has a promotive effect on rooting. A decline in percentage of rooting of cuttings was recorded in this experiment at IBA and NAA concentrations higher than 55 µg. Similar deleterious effects of applied auxins at higher concentrations have been noted with other species (Leakey *et al*, 1982). In this study IBA stimulated rooting more than NAA. This kind of a scenario has also been reported by Nanda *et al*, (1968); Hartman and Kester, (1968) and Leakey *et al*, (1982) in other plant species.

The seasonal periodicity of rooting in stem cuttings associated with the growth phase has been established for various tree species (Campuana and Lambardi, 1995), and this periodicity is not entirely eliminated by auxin treatment. In many species cuttings reach a

major peak in rootability during the growing season (Kim and Kim; 1988, Millar, 1987). In this study the rooting percentages did not differ significantly across the seasons. This could be accounted for by the fact that the area where *I. scheffleri* sub sp *keniensis* grows naturally is along the river bank where there is enough soil moisture and optimum relative humidity that stimulates plant growth evenly throughout the year.

With many forest tree species, cuttings from the lower crown root as well as, or better than those from the upper portions (Girouard, 1970; Black, 1973; Wise *et al*, 1985; Tokuoka and Takeoka, 1987). The ontogenetic juvenility of the basal part of the plant is most likely the reason for the high rootability of the material taken from that part of the crown. *I. Scheffleri* subsp *keniensis* cuttings followed this pattern across the weeks. In addition, a natural etiolation of the basal part of the crown may also have played a role in enhancing adventitious root formation.

The fact that there was none or negligible rooting of the leafless cuttings concurs with results from studies with *Terminalia spinosa* (Newton *et al*, 1992) and *Hopea odorata* (Aminah, 1992) where rooting percentages were recorded of 0, 9 and 2%, respectively, in leafless cuttings. The optimum leaf area for propagation of *Ixora scheffleri* subsp *keniensis* under the outlined experimental conditions is 80 cm². The tendency of some species to demonstrate an optimum leaf area for rooting reflects a trade-off between the effects of photosynthesis and transpiration, larger leafed cuttings being more susceptible to water deficits which may hinder carbon fixation and therefore root development (Newton *et al*, 1992).

Investigations with many tree species have shown that rooting percentage is often positively correlated with cutting length (Hoarde and Leakey; 1992., Geary and Harding,

1984). In this study, cuttings having a length of 50 mm had the highest rooting percentage, which was about 2.5 times higher than 100 mm cuttings rooting. Leakey and Storeton (1992) developed the hypothesis that cutting volume determines the capacity of a cutting to store assimilates produced both pre- and post severance. According to the hypothesis, cuttings with a small stem volume may become saturated with starch, which can inhibit photosynthesis and consequently rooting.

The results in this experiment also indicated that cuttings from the apical portions of shoots of *I. scheffleri* subsp *keniensis* displayed higher rooting percentages than those from basal portions. This trend could be ascribed to increasing lignification and secondary thickening from top to base or higher concentrations of endogenous root promoting substances present in the terminal sections of the root (Hartman *et al*, 1990).

CHAPTER 4

4.0 *IN VITRO* PROPAGATION/TISSUE CULTURE OF *IXORA*

SCHEFFLERI SUBSP *KENIENSIS*

4.1 Introduction and Literature Review

4.1.1 A Historical Background

The traditional skills required to propagate plants vegetatively, slowly learned over many centuries, have been improved and extended by modern scientific investigation. But plant propagation by tissue culture is a relatively new and radical departure and utilizes the growth of very small plant organs or pieces of tissue, in aseptic conditions. These are often termed *in vitro* techniques (meaning literally “in glass’ because the cultures are contained within glass or clear plastic vessels. *In vitro* propagation is also called micropropagation because miniature shoots and plantlets are initially derived. It is usually much more rapid than traditional methods.

Rehwald (1927) is thought to have been the first person to have obtained undifferentiated callus tissue in sterile culture, but most would agree that the subject was first put on a scientific footing through the work of Gautheret in France and White in USA. In 1934, White was able to describe a method whereby roots removed from a tomato plant could be made to grow actively in a sterile culture system to form a separate and detached root system. In the same year Gautheret reported that cells from the dividing cell layer (the

cambium) of several tree species would proliferate to a limited extent when pieces of the cambium were placed in a nutrient medium. Organs such as shoots, leaves and flowers can frequently be induced to form adventitiously on cultured plant tissues. The creation of new form and organization, where previously it was lacking, is termed morphogenesis or organogenesis. So far it has been possible to obtain the *de novo* (adventitious) formation of:

- (a). Shoots (caulogenesis) and roots (rhizogenesis) separately.
- (b). Embryos that are structurally similar to the embryos found in true seeds with both a shoot and a root pole, from previously unspecialized tissues. Embryos produced in culture are called somatic embryos (embryoids) and their inception embryogenesis. Shoots, roots and somatic embryos arise from single cells or groups of cells that become induced by the cultural conditions to become centers of active cell division (meristem).

Such morphogenetic meristems can theoretically occur in either of the two ways:

- (a) From the differentiated cells of a newly-transferred piece of whole-plant tissue, without the proliferation of un-differentiated tissue
- (b) From the unspecialized and unorganized cells of callus tissues or suspension cultures.

Hicks (1980) describe these two methods of morphogenesis as direct and indirect organogenesis respectively. In practice it is not always possible to distinguish between them.

There are five main procedural stages recognized in micropropagation: Stage 0, Stage I, Stage II, Stage III and Stage IV; Stage 0 involves the mother plant selection and preparation; Stage I the establishment of aseptic cultures; Stage II shoot multiplication

and Stage III the preparation of shoots or plantlets for growing in the natural environment (in vitro rooting of shoots) and Stage IV when in vitro propagated plantlets are transferred to the natural environment (“weaning” or “acclimatization”). There is no published information on micropropagation of *Ixora scheffleri* and it was therefore essential during this study to first optimize cultural conditions for each of the above stages to facilitate mass propagation of this species.

The generally accepted methods of *in vitro* multiplication of plants are: described in the following section.

4.1.2 Plants from meristems:

In most plants each leaf has an axillary meristem that has the capacity to develop into a shoot identical to the main shoot. Shoot culture establishment and proliferation is promoted in culture by the use of cytokinins. Different cytokinins generally show different activities in affecting axillary shoot formation in vitro (Preece *et al*, 1991). During micropropagation, cytokinins generally support shoot multiplication by releasing apical dominance in individual shoots (De Klerk, 1992). In most cases for woody species, Benzyl adenine (BA), Kinetin and Zeatin have been used to encourage outgrowth of axillary buds. However, in recent years thidiazuron (a substituted phenyl urea) has been shown also to be highly effective for inducing axillary shoot formation in many woody plant species. Therefore, in this study, the effects of this and other cytokinins (both adenine and phenylurea types) were evaluated for their abilities to induce multiple axillary branching in *Ixora scheffleri* *in vitro* shoot cultures. Cytokinins applied to whole plants rarely produce anything more than a small and transient effect on the growth of axillary shoots but its inclusion into nutrient media generally has the marked effect of

promoting the outgrowth of auxillaries in cultured buds (Yeoman, 1986). It was not until the early 1970's that the far-reaching possibilities of axillary shoot enhancement were realized with the reports of Boxus (1974) with strawberry and Murashige et al (1974) with the Transvaal lily (*Gerbera jamsonii*). These plants were rapidly multiplied by serial culture of shoots or small clusters of shoots on medium containing 6-Benzylaminopurine (BAP), and rooting obtained by transfer to medium lacking cytokinins.

4.1.3 Plants from adventitious meristems:

The somatic tissues of higher plants are capable under certain conditions of regenerating adventitious plants via shoot apices or embryos. This phenomenon occurs naturally in many plants and is exploited in conventional propagation by use of cuttings from various organs such as leaf, stem or root (Yeoman, 1986). These observations led to the concept of totipotency, i.e. inspite of their differentiation within the somatic tissues of the plant, all cells retain their capacity to regenerate a complete new plant (Krikorian & Berquin, 1969). Every cell in the plant is ultimately derived from the original zygote by mitotic divisions and should contain the complete genome. The formation of adventitious plants however will depend on the reactivation of the genes concerned with the embryonic phase of development. In practice, this occurs only in certain tissues.

4.1.4 Regeneration directly from explant tissue:

Shoots may be regenerated directly on explants from numerous species (Yeoman, 1986). Plants that are conventionally propagated adventitiously may be proliferated rapidly in vitro by using not only the conventional organ as a source of explants but other tissues not normally associated with vegetative propagation. In conventional propagation, the

main stimulus for adventitious shoot formation arises from the physical separation of the cutting from the parent plant, causing changes in the production and distribution of endogenous hormones. The same applies to explants used for *in vitro* procedures. Adventitious regeneration *in vitro* may give a much higher rate of shoot production than is possible by proliferating axillary shoots. The axillary bud enhancement culture is probably one of the safest methods of micropropagation because it has been shown to produce true to type plants (Murashige 1974; Vasil and Vasil, 1980). Indeed, Hu and Wang (1983) stressed the importance of this method for producing genetically uniform plants, thus avoiding the formation of aberrant ones.

4.1.5 Regeneration of plants from callus:

Levels of growth regulating substances particularly auxin higher than those necessary to stimulate the direct formation of adventitious shoots generally give rise to proliferation of callus from the explant. Callus may be obtained from many different parts of the plant (Yeoman and Macleod, 1977, Evans *et al*, 1981) but younger tissue is more reactive. Adventitious shoots or embryos may be formed from callus if the concentrations of hormones especially auxin are lowered (Becwar *et al.*, 1988). There is mostly no sharp dividing line between the formation of adventitious shoot from intermediate callus. Both processes may occur simultaneously on the same explants or in different explants from the same organ (Yeoman, 1986).

4.1.6 Regeneration from cell suspensions:

When transferred to media with high concentrations of auxin, particularly 2, 4-D, callus may proliferate more rapidly and become friable. Growth of friable callus in agitated

liquid media leads to the formation of cell suspensions. When transferred to media with lower auxin concentrations, various meristem structures may be formed that range from shoot buds to bipolar structures resembling zygotic embryos (Kornar *et al*, 1972, Kohlenbach, 1977, Reineert *et al*, 1977).

4.1.7 Regeneration from protoplasts:

Protoplasts released enzymatically from organ pieces; callus or cell suspensions represent the smallest explant unit for regenerating plants (Gamborg *et al*, 1981, Galun, 1981). Protoplasts plated in agar or held in liquid media may regenerate new cell walls and undergo repeated divisions to form callus.

4.1.8 *In vitro* Propagation of Woody Perennials

The development of suitable procedures for plant regeneration through either organogenesis or somatic embryogenesis is one of the main prerequisites for the potential applications of clonal propagation, genetic transformation and *in vitro* preservation of germplasm of woody plants (Handley, 1995; Park *et al.*, 1998; Minocha and Jain, 2000)

Tissue culture has been used for cloning superior genotypes and in breeding programmes of woody perennials (Karnoski, 1981). Even though some tree species can be micropropagated from tissues collected from mature trees, many others can presently be propagated only from tissues collected from juvenile specimens (Bonga, 1982). Micropropagation of tree species offers a rapid means of producing clonal planting stock, woody biomass production and conservation of elite and rare germplasm (Yeoman, 1986; Bonga, 1987). Micropropagation through tissue culture offers the capacity for clonal propagation of uniform genotypes with multiplication rates higher than macropropagation

and consequent increased production gains (Watt *et al*, 1995). If used in conjunction with conventional methods, *in vitro* culture techniques can also contribute markedly to progress in tree breeding by increasing the number of plantlets produced per parent genotype as well as being potentially important tools for genetic manipulations (Warrag *et al*, 1990). Micropropagation is a viable strategy for mass production of plantlets for use in plantations and/or clonal hedges (Watt *et al*, 1995). The ability to produce through micropropagation large populations of shoots of uniform size from limited source material should be of interest to researchers who have experienced difficulty in obtaining adequate seed-derived material for experimentation (Davey *et al*, 1993). The effectiveness of cytokinin in promoting axillary bud development *in vitro* in forest trees is well documented (Zaer and Mapes, 1982; McCown and Sellmer, 1987). Addition of low level of auxin along with cytokinin has been known to increase percent establishment as well as shoot number in forest trees (Rathore *et al*, 1991). In several tree species the combination of auxin and cytokinin seems to be positive for number and length of shoots whereas in others it was found inhibiting shoot growth and stimulating only shoot multiplication.

4.1.8.1 Examples of woody species successfully cultured *in vitro*

Micropropagation of a range of woody plants has been successfully carried out (Bonga & Von Aderkas, 1992), however numerous forest trees and bushes are still recalcitrant to establishment *in vitro*. Micropropagation of many broad-leaved species including prunus has been accomplished (Chalupa, 1987). Most poplar species of economic importance have been cultured *in vitro* both from juvenile material and from that taken from adult trees (Rutledge & Douglas).

Plantlet regeneration has been reported in neem from leaf explants of a mature tree (Ramesh and Padhya, 1990) and from axillary buds of 2 to 5 year old plants. Similarly, plantlet regeneration via callus was achieved from different explants of 7-day-old seedlings (Salvi *et al*, 2001) and from anther tissue. Neem has also been regenerated from the bud culture method (Afaque *et al*, 2004).

Mbaratha (1985) applied *in vitro* propagation techniques using shoot meristems in three woody species viz: - *Terminalia kilimandscharica*, *T. spinosa* and *T. brownii*. Contamination of the cultures proved a major problem due to unsuccessful attempts to eradicate it. Regeneration was obtained from sterile seed culture.

Njenga *et al* (1999) using shoot tips and uninodal explants regenerated complete plantlets of the gum Arabic tree (*Acacia senegal*) from three provenances in Kenya. *In vitro* performance variation of the *Acacia senegal in vitro* plantlets was also evaluated. Kiriba *et al* (2005) using *Warbugia ugandensis* shoot tips cultured in half-strength MS medium got multiple shoots. However rooting was not achieved. Kuria *et al* (2006) using shoot tips and uninodal explants of *Warbugia ugandensis* regenerated complete plantlets.

Arya and Shekhawat (1986) successfully propagated by tissue culture hardwood trees in the natural environs of Thar Desert. They include *Prosopis cineraria*, *Zizyphus Mauritania*, *Aegle marmelos* and *Tecomella undulata*. Each plant was specific in its requirements of auxins and cytokinins for production of multiple shoots. Other workers have also developed techniques for multiple shoot formation from bud explants and callus culture of *Aegle marmelos* (Surana, 1982), *Tecomella undulata* (Vaishnawa, 1983) and *Zizyphus Mauritania* (Goyal, 1982).

Micropropagation of white pine (*Pinus monticola*) starting with mature embryos has been described by Martin *et al* (1995). Litvay's medium proved to be the best of six media formulations tried. The optimum concentration of N6 Benzyl adenine was 30 μM for 21 days. It was possible to obtain 500 – 600 shoots for every 100 embryos explanted. Twenty to thirty percent of these shoots spontaneously developed roots.

Rajasekaran (1994) developed a micropropagation procedure using explants from mature trees of *Grevilea robusta* A. Cunn. (Silver oak) (Proteaceae). The explants were cultured on woody plant medium (WPM) plus 4.4 μM Benzyladenine and 0.27 μM Naphthalene acetic acid (NAA) for shoot proliferation. Each nodal explant produced three to five shoots within 10 – 12 weeks in three successive transfers. Rooting was obtained by dipping the shoots in 0.54 μM NAA solution.

4.1.8.2 *In vitro* propagation in genus *Ixora*:

Callus cultures were initiated from leaf explants of *Ixora chilensis* taken from expanding young leaves (Noreen *et al*, 2001). Murashige and Skoog (MS) salt mixture containing various concentrations of 2, 4-D was used for callus initiation. Callus initiation was significantly higher in MS medium having 2, 4-D at 3mg/l. Two media (WPM and MS) were tested for embryogenesis. No embryogenesis occurred but callus multiplication was observed. The best combination which showed excellent multiplication was both WPM and MS media supplemented with Gibberelic acid.

None of these studies have described the large-scale multiplication of *Ixoras* nor regeneration from mature trees. Except for limited use, seedling cloning offers very little to the tree breeder who wishes to propagate elite trees not their progeny (Sita, 1986).

Ixora fulgens cultures of proliferating axillary shoots were established on MS medium with different concentrations of either BA alone or in combinations of BA with NAA or IBA with shoot explants from grown mature plants (Amin *et al*, 2002). Nodal segments were found to be the best explants for axillary shoot formation on agar gelled MS medium with 0.5mg/l BA + 0.1mg/l NAA. It was found that 90 % of the plantlets could be established under *ex vitro* conditions when they were transferred on specially made plastic tray containing coco – peat as rooting mix.

The aim of the study in this chapter was to develop a rapid clonal multiplication system of *Ixora scheffleri* subsp *keniensis* using multiple axillary branching and somatic embryogenesis.

4.2 Materials and Methods

4.2.1 Introduction

Most of the studies on *in vitro* techniques were carried out at the Coffee Research Station (CRS) Ruiru, Kenya. CRS lies on latitude 1° 08 minutes south, longitude 36-° 51minutes east and an altitude of 1608 m above sea level. It receives an average rainfall of 1059mm per year. The two wet seasons in a typical year are March to June (The long rains) and October to November (The short rains). The average annual temperature is 19.1 °C with ranges of 11 and 27 °C in the wet and dry seasons, respectively, and the average relative humidity is 80.4% at 0900 h and 1500 h, respectively.

4.2.2 Plant materials

The explants were obtained from vegetatively propagated *Ixora scheffleri* plantlets grown in the green house. In this study these explants are referred to as first generation

microcuttings. After proliferation of axillary buds from these first generation microcuttings, explants produced *in vitro* from these first generation cuttings were used for the study of rooting. They are referred to as second-generation microcuttings.

4.2.3 Media Preparation

Media were prepared by dissolving the ready-made media salts and other components in double distilled water (DDW). See appendix 1 for the details of composition of media that supported direct organogenesis during the current study. The solutions were stirred until they dissolved and made up to final volume. Media pH was adjusted to 5.8 using either 1 N NaOH or 1 N HCl before Difco Bacto agar at 7g/l (Sigma USA) was added. Media were heated on a hot plate with continuous stirring using magnetic fleas until the agar was dissolved. The media was then dispensed in boiling tubes and autoclaved using an Astell Hearson 2000 series autoclave set to operate at a temperature of 121 °C and a pressure of 1.1 kg/cm² (103.5kpa) for 20 min. All media were autoclaved within 12 h of preparation and when possible freshly autoclaved media were used. However when it was not possible to use the media immediately it was stored in a cold room at 4 °C in darkness for no longer than 2 weeks before use

4.2.4 Plant growth substances

Plant growth substances were weighed and stocks prepared using appropriate solvents (table). The stocks were then clearly labeled and stored in darkness in a cold room maintained at 4 °C.

Table 4-1 Solvents used to solubilise plant growth substances

BAP	0.1 NaOH and heat if required
Kinetin	0.1 N NaOH
2,4-D	Absolute alcohol
NAA	90% alcohol
TDZ	1 N KOH

4.2.5 Sterilisation of thermolabile substances

Thermolabile substances were filter sterilised through 0.22 μm membrane filters (Millipore UK). The filtered solutions were added to the autoclaved media after cooling to ca. 60 °C and mixed in well before the complete media were dispensed to either replidishes or culture tubes.

4.2.6 Sterilization of explant material

Nodal explants shoot tip and leaf explants were transported from the glasshouse in polythene bags. They were then trimmed into single node segments and kept under running tap water for 30 min. The main surface sterilants tested were commercial bleach solutions (Jik) and formaldehyde solutions. The concentrations tested for both sterilants were 10, 15, 20, 25, 30, and 35 (v/v). The time of exposure in the sterilants were 10, 15, 20, 25 and 30 minutes. They were then rinsed twice in sterile DDW followed by a quick immersion (30 sec) in 70% (v/v) ethanol and then rinsed twice in DDW. The explants were then cultured on MS medium supplemented with 100mg/l inositol, 30mg/l cystein, 3% sucrose gelled with 0.3 % Difco Bacto agar.

4.2.7 Aseptic techniques

The process of sterilization and dissection of plant materials to and between culture tubes were carried out under sterile conditions in a laminar flow cabinet. The cabinet was switched on and swabbed down with 70% ethanol using sterile towel paper and was kept running for at least 15 min before any work was started. All dissections were carried out on sterile polypropylene sheets (Cunnings Perry packing, UK) placed on a sterile glass plate. Hands were washed with carex antibacterial moisturizing hand wash (Cussons, UK) before starting any transfer and they were washed with 70% ethanol at suitable intervals while working at protracted periods in front of the cabinets. Following completion of the transfer work, laminar flow cabinets were swabbed down with 70% ethanol. Personal hygiene precautions were observed by wearing clean laboratory coats and gloves while working at cabinets. Control of substances hazardous to health (COSHH) and other regulations were strictly adhered to during handling and disposal of chemicals.

4.2.8 Dissecting tools

All tools were wrapped in an aluminium foil and sterilized in dry heat (Mistral oven) at a temperature of 260 °C for 90 min. During their use in the cabinet, the tools were dipped in 70% ethanol followed by heat sterilization for ca. 20 sec in steribead sterilizer (Simon Keller AG, Switzerland) maintained at 250 °C. In between operations, the tools were frequently sterilized in ethanol and in the steribead sterilizer.

4.2.9 Incubation conditions

For the regeneration of *Ixora* microshoots, the cultures were incubated in growth rooms maintained at 25 °C under 16h photoperiods. Light conditions were provided by fluorescent tubes. These lights produced a broad spectrum light especially in the red wavelength, which promote shoot and leaf development. For the induction and regeneration of *Ixora* somatic embryos, cultures were incubated in the dark at temperature of 25 °C.

4.2.10 Culture methods for direct regeneration of shoots from explants

Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) was used throughout the investigations. Full strength or modified MS (mod MS) was used according to research objectives.

4.2.11 Elongation/multiplication of shoots

For the multiplication of shoots, full or modified MS was used as basal media. The modification consisted of decreasing by half the proportion of macroelements. This has an effect of lowering the total ionic strength of the medium. Bonga and Durzan (1987) underscore the effect of ionic strength (total salt concentration) of a medium on regeneration of explants from woody species. Low ionic strength favours regeneration. Added to the full or half MS basal medium was Benzylaminopurine (BAP), 6-furfurylamino purine (kinetin), 6-(4-hydroxy-3-methyl-but-trans-2-enyl) amino purine (Zeatin) or combinations of these cytokinins with α -naphthalene acetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid at different concentrations. There were no growth regulators in the control medium.

EXPERIMENT 1

The first experiment consisted of 12 treatments in which full MS was augmented with BAP, Kinetin or Zeatin at 0, 0.1, 0.5 or 1mg/l. The treatments had a factorial structure of 3x4 with three cytokinins (BAP, Kinetin and Zeatin) and four cytokinin concentrations.

EXPERIMENT 2

The second experiment consisted of 12 treatments in which modified MS was supplemented with BAP, Kinetin or Zeatin at 0, 0.1, 0.5 or 1 mg/l. The treatments had a factorial structure of 3x4 with three cytokinins (BAP, Kinetin and Zeatin) and four cytokinin concentrations

EXPERIMENT 3

The third experiment consisted of 81 treatments in which modified MS was supplemented with BAP, Kinetin or Zeatin at 0.1, 0.5 or 1mg/l in combination with NAA, IBA or 2, 4-D at 0.01, 0.05 or 0.1mg/l. The treatments had a factorial structure of 3x3x3x3 with three cytokinins (BAP, Kinetin and Zeatin), three cytokinin concentrations, three auxins (NAA, IBA and 2,4-D) and three auxin concentrations.

EXPERIMENT 4

The fourth experiment consisted of 25 treatments in which BAP was combined with TDZ. The BAP concentrations were 0, 10, 15, 20 and 25 μ M. The TDZ concentrations were 0, 0.1, 0.5, 1.0, and 1.5 μ M. The factorial structure was 1 x 5 x 1 x 5, that is BAP with 5 concentrations and TDZ with 5 concentrations.

In each experiment, 20 replicates per treatment were used. Each of the experiments was repeated at least 3 times. Assessments (percent bud sprouting, mean length of shoots (mm) and the mean number of shoots were made after 60 days. Extension growth of

primary buds at stage I was referred to as ‘bud break’ and the resultant shoots at stage I were termed ‘bud shoots’. In addition, the term ‘microshoots’ was used in *sensu strictu* for any subsequent shoot development from the nodal or apical explants or shoots (without roots) produced from buds laid down *in vitro*. The term ‘microplants’ was used to describe microshoots which were subsequently rooted at stage III and weaned at stage IV. All cultures were incubated at 25 °C under 16 h photoperiod in all experiments described in this chapter unless otherwise stated

4.2.12 Rooting of shoots

4.2.12.1 *In vitro* rooting

Rooting was conducted on the second-generation microcutting explants cut in the elongation phase from shoots of first generation microcuttings. The microcuttings used for this study were approximately 2.5cm long. This was done after 60 days of culture of the first generation microcuttings. Two rooting media were tested:

- (i) NAA in modified MS
- (ii) IBA in modified MS

The concentrations of IBA and NAA were 0, 5, 10, 15, 20, 30, 40, 50, 55 and 100 µM. This experiment consisted of 20 treatments. The treatments had a factorial structure of 2x10 with two auxins (NAA and IBA) and 10 auxin concentrations.

4.2.12.2 *Ex vitro* rooting

Vermiculite was used as the rooting medium. Since it had been used previously, it was prepared by washing it several times with tap water to wash away any undesirable chemicals present and enhance its ability to absorb nutrients. The pH was adjusted to 5.8 and steam sterilized by autoclaving at 121 °C and 1.06 kg/cm² for 2 hours. On cooling it

was potted into clean plastic pots that had been sterilized by dipping them overnight in 10 % sodium hypochlorite solution. The pots were covered with polythene bags and placed on the lamina flow hood for inoculation. The microshoots were treated with IBA or NAA (0, 40, 45, 50, 55 and 60 μg) dissolved in industrial methylated spirit. The hormones were applied with disposable micropipettes at the base of the microshoots as a 10- μl droplet. Each concentration was allocated 25 microshoots.

4.2.13 Transplanting and Acclimatization

After *in vitro* rooting, the rooted *in vitro* plants were carefully removed from the growing tubes and transplanted in pots on a substratum consisting of vermiculite. They were watered with half strength MS salt solution and covered with a transparent polythene paper for 3 weeks during which time the plantlets were acclimatized to ambient atmosphere. For about one week, the plantlets were misted with water to maintain a high relative humidity (RH) inside the polythene papers. These polythene papers were opened gradually several hours a day for another two weeks at the end of which the covers were completely removed. The acclimatization process was carried out in growth rooms at 30 ± 2 °C. The plants were then transplanted into polythene bags containing forest soil and transferred to a greenhouse for further growth monitoring.

4.2.14 Induction and regeneration of somatic embryos

Leaf explants from the greenhouse grown plants were surface sterilized using the standard procedure described in section. The explants were induced for 14 d on an induction medium and then transferred to the same medium without growth regulators in the dark at 25 °C. The cytokinins evaluated were Kinetin and TDZ at 0, 0.5, 1.0 and 5 μM

combined with 0, 0.5, 1.0, and 5 μ M IBA. The medium used was MS supplemented with 100 mg/l inositol, 30mg/l cysteine-HCl and 2% sucrose gelled with 0.3 g/l & Difco Bacto agar. Assessment of the embryogenic cultures were carried out after 3 months. The cultures were observed under a dissecting microscope.

4.2.15 Washing up

All glassware and cultures vessels were washed in hot water to which a few drops of detergent had been added. This was followed by three rinses in cold tap water and a final rinse in DDW. Clean glassware were dried in an oven overnight at 60 °C.

4.2.16 Photography

Photographs of the experimental materials were taken using a digital camera

4.2.17 Experimental Design and statistical analysis

All experiments were laid out in a Completely Randomized Design (CRD). The level of replication used per treatment combination varied depending on the availability of experimental materials.

4.3 Results

4.3.1 Shoot elongation

When BAP, KIN or TDZ were used alone at all the concentrations tested, there was absolutely no growth and after about 2 weeks, the explants died. It was only when BAP was combined with TDZ that any growth could be recorded. However even then, very few combinations (BAP: TDZ, 20:1.0, 20:1.5, 25:0.1 and 25:0.5 μM) had an effect on shoot proliferation (Table 4.2). In most of the combinations, the majority of the explants died. The concentration 25 μM BAP: 0.5 μM TDZ had the highest effect on the elongation of the *in vitro* shoots and the highest mean number of microshoots. There was an average of 3.1 new shoots emerging from a single nodal explant. At this rate, 12 plantlets of *Ixora scheffleri* subsp *keniensis* can be regenerated from a single nodal explant in one year (Table 4.2). This level of multiplication can not be achieved through conventional propagation methods.

There was significant difference in shoot length ($p < 0.001$, ANOVA) between BAP: TDZ cytokinin combinations that had an effect on length. However, there was no significant difference between 20:1.5 μM and 25:0.1 μM (Table 4.3). Overall, the BAP: TDZ combination contributed 83.3% of the total variance accounted for implying that they had a strong effect in influencing shoot elongation.

On the other hand, there was a significant difference ($p = 0.039$, log linear modeling procedures for count data) in the number of microshoots that emerged under a concentration of 25: 0.5 and the rest but no significant difference ($p > 0.05$) between 20: 1.0, 20:1.5, and 25:0.1 μM BAP: TDZ combination. In general, the results showed that

25:0.5 μM combination had the optimum effect in microshoot production and microshoot length. Plates 4.1 – 4.5 show *in vitro* shoots at various stages of growth.

Table 4-2 Summary of the effect of combinations of cytokinins on shoot length and number of micro-shoots

Cytokinins combinations (BAP:TDZ μM)	Initial number of explants used	% Survival	Mean shoot length (s.e.d=0.2015)	No. Micro- shoots	Mean no. of microshoots
10,0.1	20	0	0	0	0
10,1.0	20	0	0	0	0
10,1.5	20	0	0	0	0
10,0.5	20	0	0	0	0
15, 0.1	20	0	0	0	0
15, 0.5	20	0	0	0	0
15, 1.0	20	0	0	0	0
20, 0.1	20	0	0	0	0
20, 0.5	20	0	0	0	0
20, 1.0	20	45	1.6	11	1.2
20, 1.5	20	25	1.1	8	1.6
25, 0.1	20	40	0.8	9	1.1
25, 0.5	20	95	2.8	59	3.1
5, 0.1	20	0	0	0	0
5, 1.5	20	0	0	0	0
5,0.5	20	0	0	0	0
5,1.0	20	0	0	0	0
S.e.d			0.2015		
p-value			<0.001		

s.e.d = Standard error of difference

Table 4-3 Mean comparisons of shoot length and no. of microshoots.

Source of variation	d.f	ms	F.prob
Cytokinins combination	3	10.241	<0.001
20, 1.0 vs. 20, 1.5	1	0.992	0.018
20, 1.0 vs. 25, 0.1	1	3.284	<0.001
20 1.0 vs. 25, 0.5	1	8.675	<0.001
20, 1.5 vs. 25, 0.1	1	0.325	0.163
Residual	37	0.161	
Total	40		

NB: df refers to degree of freedom. F.prob refers to F probability.



Plate 4-1 A freshly inoculated shoot tip explant

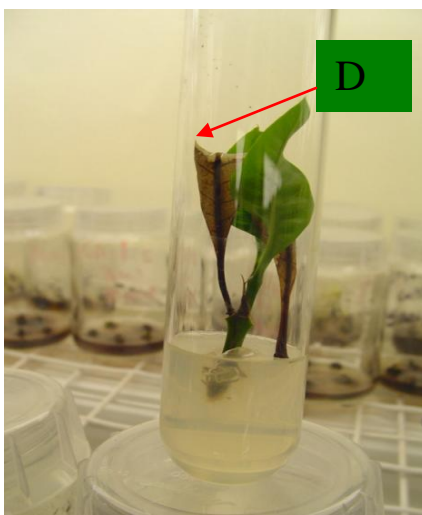


Plate 4.2 An *in vitro* shoot after 2 weeks. Note the drying off (D) of the original leaves and the development of new leaves.



Plate 4-3 An *in vitro* shoot with the original leaves completely fallen off showing the emergence of new leaves.



Plate 4-4a An actively growing *in vitro* shoot after 5 weeks



Plate 4.4b Multiple shoot proliferation after 6 weeks.

4.3.2 Effect of auxins on *in vitro* rooting of *I. scheffleri* subsp *keniensis* second generation microcuttings:

The results showed that both IBA and NAA achieved their optimum concentration at 50 μM for influencing *in vitro* rooting. The trend of rooting under different hormonal concentrations increased slowly from 5 μM in the case of IBA to 50 μM and then started decreasing as the concentration levels approached 100 μM . This confirmed the optimum concentration found. On the other hand there was no clear sequence in rooting for different NAA concentrations other than a decrease in rooting from optimum 50 μM to 100 μM (Table 4-4 and Figures 4-1 & 4-2). Overall results indicated that IBA concentrations influenced more rooting than NAA. Plate 4-5 shows a rooted microplant under 50 μM IBA.



Plate 4-5 A rooted microplant cultured in MS medium supplemented with 50 μ M IBA after 9 weeks



Plate 4-6 A transplanted seedling that flowered after four months.



Plate 4-7 Rooted microplants under green house conditions after one month

Table 4-4 Percentage of *in vitro* shoots that rooted under different hormonal concentrations

Hormone	Concentration μM	No rooted	% Rooted
IBA	0	0	0
	5	4	16
	10	7	28
	15	7	28
	20	5	20
	30	8	32
	40	8	32
	45	11	44
	50	19	76
	55	16	64
	100	4	16
NAA	0	0	0
	5	0	0
	10	3	12
	15	3	12
	20	4	16
	30	6	24
	40	7	28
	45	4	16
	50	10	40
	55	6	24
	100	0	0
s.e.d	1.972		6.540

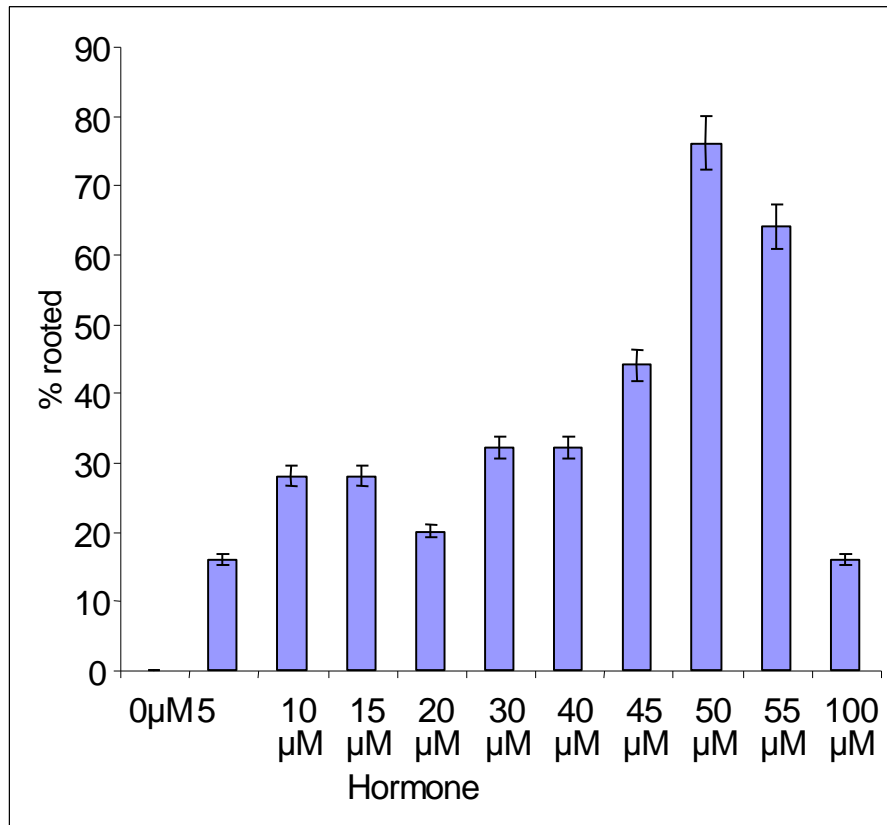


Figure 4-1 Percentage rooting under different IBA concentrations. Vertical bars represent the standard error of means, n = 20

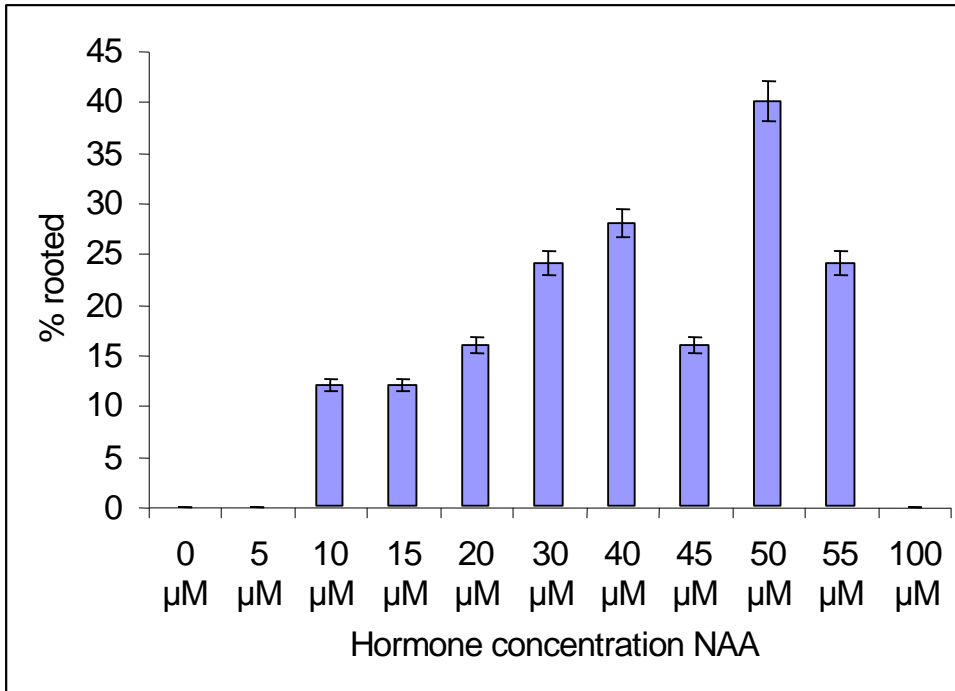


Figure 4-2 Percentage rooting under different NAA concentrations. Vertical bars represent the standard error of means, n = 20

Further analysis showed that there was significant difference ($p < 0.001$, ANOVA based on percentage of second generation microcuttings that rooted) between various hormonal concentrations. Similarly there was significant difference in mean percentage of the microcuttings that rooted ($p < 0.001$) between the two hormones where IBA performed better than NAA. In this case, IBA had an average 32.4 % overall rooting as compared to 15.6% for NAA.

In addition, the effects of hormone per se contributed 15.3% of rooting compared to 64.3% contributed by concentrations. This implies that getting the appropriate concentration has a significant effect on *in vitro* rooting of *Ixora*.

4.3.3 Effect of auxins (NAA and IBA) on *ex vitro* rooting of *Ixora scheffleri* subsp *keniensis* second generation microcuttings

The results showed that there was very negligible rooting influenced by both hormones under different concentrations. Rooting increased under 40 µg to 45 µg for IBA and NAA. However there was higher number of microshoots rooting under IBA (21) than NAA (11) (table 4-5)

Table 4-5 *Ex vitro* rooting results

Hormone	Concentration	%	
		No rooted	Rooted
IBA	0 µg	0	0
	40 µg	2	8
	45 µg	7	28
	50 µg	4	16
	55 µg	4	16
	60 µg	4	16
NAA	0 µg	0	0
	40 µg	1	4
	45 µg	3	12
	50 µg	3	12
	55 µg	2	8
	60 µg	2	8

Further analysis showed that there was significant difference ($p=0.030$, ANOVA based on mean rooting percentage, $s.e.d=2.98$) between the two hormones in inducing rooting where IBA performed well than NAA. Similarly, the effect of different concentrations in each hormone had a significant influence ($p=0.049$, ANOVA based on mean rooting

percentage; s.e.d=7.29) on rooting. Therefore the optimum concentration for rooting under IBA was 45 µg whereas that of NAA was attained at 45 and 50 µg.

The results further showed that there was very minimal number of roots that emerged out of the microshoots that had rooted (table 4-6)

Table 4-6 Summary of number of roots under different concentrations

Hormone	Conc	No.roots	Mean
IBA	0 µg	0	0
	40	3	0.12
	45	13	0.52
	50	10	0.4
	55	8	0.32
	60	6	0.24
NAA	0	0	0
	40	3	0.12
	45	6	0.24
	50	5	0.2
	55	5	0.2
	60	4	0.16

For mean number of roots for hormones s.e.d=0.0708 and for concentration in each hormone, s.e.d=0.1734

4.3.4 Induction and regeneration of somatic embryos

No somatic embryos were induced in this experiment. However callusing of the leaf explants was observed. In all cases callus was evident at the cut edges of the leaf margins. Leaf veins were also evidently enlarged during this period. After about one month, the actively growing callus started turning brown and then dried up in all cases (Plates 4-8, 4-9)

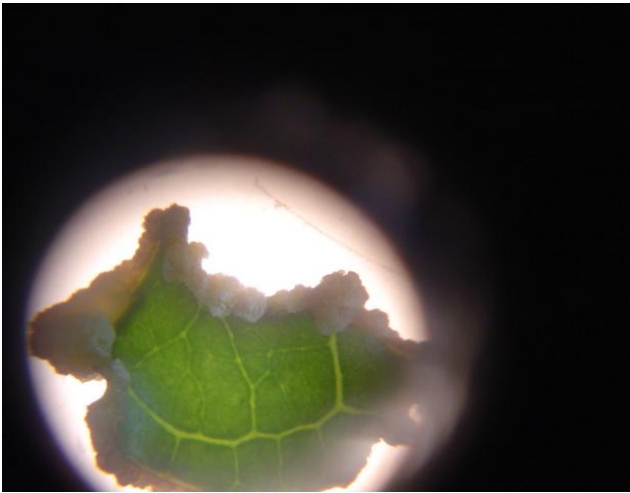


Plate 4-8 Callus induction in *Ixora scheffleri* subsp *keniensis* leaf explant cultured in 1 μ M TDZ: 1 μ M IBA after 4 weeks.



Plate 4-9 Callus induction in *Ixora scheffleri* subsp *keniensis* leaf explant cultured in 1 μ M TDZ: 1 μ M IBA after 7 weeks. Note the browning of the callus at the edges.



Plate 4-10 Fresh Leaf explants cultured in MS medium supplemented with 2,4-D

Callus cultures were also initiated from leaf explants of *Ixora scheffleri* subsp *keniensis* taken from expanding young leaves using Murashige and Skoog's (MS) salt mixture containing various concentrations of 2, 4-D. Callus initiation was significantly higher in MS medium having 2,4-D at 3 mg/l. No embryogenesis occurred but callus multiplication was observed (Plate 4.11).



Plate 4-11 Callus formed in MS medium supplemented with 2,4-D

4.4 Discussion

Shoot culture establishment and proliferation is promoted in culture by the use of cytokinins. Different cytokinins generally show different activities in affecting axillary shoot formation *in vitro* (Preece *et al.*, 1991). During micropropagation, cytokinins generally support shoot multiplication by releasing apical dominance in individual shoots (De Klerk., 1992). BAP, Zea and Kin are the cytokinins that have been used by many workers in the *in vitro* propagation of woody species. However in recent years thidiazuron (a substituted phenylurea) has been shown also to be highly effective for inducing axillary shoot formation in many woody plant species (Huetteman and Preece, 1993). Therefore in this study, the effects of this and other cytokinins (both adenine and phenylurea types) were evaluated for their abilities to induce multiple axillary branching in *Ixora scheffleri* subsp *keniensis*.

During the current study all the cytokinins tested failed to promote any growth or multiple shoot proliferation except the combination of BAP and TDZ. Even then, a very narrow range of concentrations (20: 1.0, 20:1.5, 25:0.1, and 25:0.5 μM) was able to induce elongation and proliferation of multiple shoots. A concentration of 25:0.5 μM BAP: TDZ induced the highest number of microshoots averaging 3.1 and also the highest microshoot length averaging 2.8 cm. Thidiazuron has been shown to stimulate shoot formation in woody species such as white ash (Bates, 1992) and tea (Mondal *et al.*, 1998). Ellis *et al.* (1991) have suggested that TDZ could be used as a substitute for BA in micropropagation of several woody species. TDZ has been reported to be 2-200 times more effective than BA in shoot formation of *Vitis* spp. (Gray & Klein, 1989). However in the current study, TDZ alone failed to stimulate any growth or multiple shoot

proliferation. The high efficiency of TDZ in stimulating cytokinin-dependent shoot regeneration from a wide variety of plants supports the idea of a modified cytokinin metabolism in TDZ related tissues (Thomas & Katterman, 1986). This is the first report on the successful use of TDZ in combination with BAP for micropropagation of *Ixora scheffleri* subsp *keniensis*.

Rooting may be achieved *in vitro* by placing the excised microshoots from proliferating cultures into auxin supplemented rooting media. Alternatively, microshoots may be placed in vermiculite for *ex vitro* rooting and acclimatization. Auxins such as IBA, NAA, IAA or 2, 4-D are mostly used for the induction of roots (Gaspar and Coumans, 1987).

Results from work on *in vitro* rooting in the current study indicated that supply of an exogenous auxin was essential in rooting of *I. scheffleri* subsp *keniensis* microshoots. IBA was found to be more suitable than NAA. The highest percentage rooting was obtained using 50 µM IBA. The results of the current study are in agreement with those of Boggeti (1997) in rooting cashew nut microshoots. The results also are in agreement with Kahia (1999) *in vitro* rooting experiments of resistant *Coffea arabica* L. Hybrid – Ruiru 11. Of all the auxins, IBA is the most effective for root formation in a wide variety of plants (Hartman *et al.*, 1990). IAA is also used *in vitro*, especially for herbaceous species, while NAA, may cause excessive callusing (Pierik, 1987).

In vitro rooting is comparatively labour intensive and costly. Considerable savings in plant production costs can be obtained if successful *ex vitro* rooting is achieved (Debergh & Maene, 1981). It is estimated that the labour involved in rooting individual shoots *in vitro* can mean that the stage of micropropagation can account for 35-75% of the total costs of plants propagated through tissue culture. *Ex vitro* rooting of microcuttings

eliminates one set of labour costs and overheads as rooting and acclimatization are effectively combined into a single stage of the micropropagation process. This technique is particularly suitable for woody plants in which secondary thickening is important for proper root function (George, 1996).

It was not possible to optimize the cultural conditions required for the production of somatic embryos in this study. Induction and regeneration of direct and indirect somatic embryos was attempted but none was successful. However TDZ: IBA at 1 μ M initiated the highest number of calli within a short time. TDZ has been shown to stimulate somatic embryogenesis in woody species such as white ash (Bates *et al.*, 1992), apples (Daigny *et al.*, 1996) and Cacao (Li *et al.*, 1998). The reason why such comparative low levels of TDZ are effective on some woody plant tissues while adenine-types are not could be due to the fact that TDZ is resistant to cytokinin degrading enzymes (oxidases). TDZ is also stable and biologically active at lower concentrations than the adenine-type cytokinins (Mok *et al.*, 1987). This is the first report on an attempt to induce somatic embryos in *Ixora scheffleri* subsp *keniensis* using TDZ.

The results described in this chapter demonstrated that *Ixora scheffleri* subsp *keniensis* can be propagated using axillary buds proliferation. Although the protocol developed here showed fairly satisfactory regeneration rates, there is still need to optimize other methods of propagation that would give more plants within a short time to provide propagules for both *in situ* and *ex situ* conservation of this species.

CHAPTER 5

5.0 DETECTION AND ANALYSIS OF GENETIC VARIATION IN *IXORA SCHEFFLERI* SUBSP *KENIENSIS* USING RAPD MARKERS

5.1 Introduction

Apart from the fact that the maintenance of existing levels of genetic diversity may be essential for the long-term survival of endangered species, it is also a pertinent factor when one considers reintroduction of plant populations in the wild. In addition, it can be useful to identify interesting genetic traits for future applied uses (Schaal *et al*, 1991). Populations with high levels of genetic variation are valuable since they offer a diverse gene pool from which gene conservation and improvement programs can be made. Furthermore, knowledge of within and among population differentiation will help to develop efficient sampling strategies of genetic resources in rare and/or useful species (Bonnin *et al*, 1996).

Scientific approaches to the conservation and exploitation of plant and animal genetic resources require a detailed knowledge of the amount and distribution of genetic variability within the organism under study. In isolated populations, genetic drift may eventually reduce genetic variation (Lacy 1987, Frankham, 1996) especially because effective population sizes are usually much smaller than the number of reproductive individuals in a population (Frankham, 1995). Increased levels of inbreeding in populations with lower genetic variability may result in reduced fitness because of inbreeding depression (Ellstrand and Ellam, 1993). A positive feedback between genetic

and demographic factors may gradually decrease genetic variability, fitness and population size and thus lead to extinction (Gilp and Soule, 1986). Genetic diversity within populations is considered to be of great importance for possible adaptation to environmental changes and consequently for long-term survival of a species (Vida, 1994). Without an appropriate amount of genetic diversity, species are thought to be unable to cope with changing environments and evolving competitors and parasites (Van Valen, 1973). Thus maintenance of (or at least quantification of) genetic variation is currently regarded as a primary goal in conservation efforts (Falk and Holsinger, 1991, Hoezel, 1992) and accounts for the current utility of genetic information in conservation biology. It is now widely appreciated that understanding patterns of genetic variation is of critical importance to the conservation of threatened taxa (Holsinger and Gotlieb, 1991). Lack of knowledge concerning patterns of diversity in populations of tropical tree species means that our ability to assess the magnitude of the effect of deforestation on diversity loss is severely restricted.

Traditionally, morphological traits have been used to measure genetic diversity. Unfortunately these characters may be influenced by environmental factors and may therefore not reflect true genetic similarities or differences. To overcome these problems biochemical and molecular techniques are being used to monitor genetic variability. Isozymes are used extensively to characterize plant genetic resources (Second, 1982; Transkley and Orton, 1983) but are limited by the relatively low levels of polymorphism detectable.

Different methods of DNA fingerprinting have proved to be a useful tool with a wide range of applications in plant population studies, such as detection of genetic variation

within and between populations, characterization of clones, analysis of breeding systems and analysis of eco-geographical variation (Weising *et al*, 1995). Restriction Fragment Length Polymorphisms (RFLP's) have been used to investigate genetic diversity in cultivated plants and their wild relatives (Song *et al*, 1990, Wang *et al*, 1992). However the RFLP assay requires large quantities of relatively pure DNA and the frequent use of radioactive isotopes in the detection method makes it technically more demanding, laborious and costly to characterize large numbers of samples routinely.

Randomly amplified polymorphic DNA (RAPD) analysis (Williams *et al*, 1990, Welsh and McClelland, 1990) requires only small amounts of starting DNA, does not require prior DNA sequence information, nor involve radioactivity and is simple and quick to perform. The RAPD technique has been previously used for plant germplasm characterization in a large number of studies (e.g. Hu & Quiros, 1991, Wilde *et al*, 1992, Demeke *et al*, 1996, Grando *et al*, 1996). Its utilization in studies of genetic diversity of threatened plant species is not as widespread, although it has been employed in some cases, e.g. *Primula scotica* (Glover & Abbott, 1995), *Iliamna corei* and *I. remota* (Stewart & Porter, 1995), and *Argyroxiphium sandwicense* ssp. *Sandwicense* (Friar *et al*, 1996).

The aim of the study in this chapter was to investigate genetic variability within the singular population of *Ixora scheffleri* subsp *keniensis* at Mt Kenya forest, along the Ragati river basin using RAPD markers. Knowledge of genetic differentiation within this population is critical since pollination ecology and breeding systems studies of this species (Chapter 2) have shown that germination of seeds is a major bottleneck in the recruitment of new individuals in the population. Success in the propagation of this

species through micropropagation and macropropagation (chapter 3 and 4) strongly emphasizes the need to capture the whole range of genetic differentiation available in the population to help develop efficient sampling strategies for propagules to be used in macropropagation and micropropagation ventures. RAPD analysis is hereby used in the initial assessment of levels of genetic variation within the single population of *Ixora scheffleri* subsp *keniensis*.

5.2 Materials and Methods

5.2.1 Plant material:

Twenty plants were studied to assess genetic variability. They were sampled based on the ease of accessibility

5.2.2 DNA isolation

DNA was extracted from young leaves of single individuals. A small amount of tissue was ground to a powder in liquid nitrogen using a mortar, and then 1 ml of extraction buffer was added. The homogenate was incubated with 100ml chloroform/octanol (24:1) at 60 °C for 20-30 minutes, and then centrifuged at 12,000g for 15 minutes. The supernatant was transferred to new Eppendorf tube that was filled in chloroform/octanol (24:1) and then centrifuged at 12,000g for 5 minutes. Once again the supernatant was transferred to a new Eppendorf tube. DNA was precipitated from the aqueous phase by mixing with 1 ml of ice cold 100% ethanol and then pelleted by centrifugation at 12,000 for 15 minutes. After air drying, 1 ml of solution of 0.2M sodium acetate in 76% ethanol was added, allowed to sit for 10 minutes and then spun at 12,000g for 5 minutes. The aqueous phase was removed and 1 ml of 0.01M sodium acetate in 76% ethanol was

added to the pellet. After an incubation period of 10 minutes, it was centrifuged at 12,000g for 5 minutes, the supernatant was removed and the pellet was air-dried. The pellet was redissolved in 150ml TE (10mM Tris-HCl PH 8.0 and 1mM EDTA) to which 15mg RNAase (Sigma) was added.

To obtain highly purified DNA, a phenol extraction was made for all the samples before redissolving the pellet in TE.

5.2.3 RAPD reaction and Gel electrophoresis

Amplification was carried out in a 20 μ l-volume reaction mix, which contained 200 μ M of each of the dNTPs (Life technologies), 1 Taq polymerase buffer (Perkin Elmer), 3 mM MgCl₂ (Perkin Elmer), 0.2 M primer (Life technologies), 2.5 ng l⁻¹ DNA and 0.75 units of Taq polymerase (GoldTM, Perkin Elmer). Amplification conditions were set as, 1 cycle of 15 min at 94 °C, 44 cycles of 1 min at 94 °C (denaturation), 1 min at 36 °C (annealing), 2 min at 72 °C (extension). A final 5 min extension (72 °C) was allowed to ensure full extension of all the amplified products. Amplification products were mixed with 6 x gel loading dye (0.25 % bromophenol blue, 25% xylene cyanol and 30% glycerol) and separated on 2% agarose gel. The ethidium bromide stained agarose gels were visualized under ultra violet light and then photographed using Kodak ID 3.5 gel imaging system (Kodak).

Table 5-1 List of primers screened for the RAPD reaction

CODE	SEQUENCE	CODE	SEQUENCE
KFP-1	GGC TCG TAC C	KFP-19	TCG GAG TGG C
KFP-2	CGT CCG TCA G	KFP-20	CGG GAG ACC C
KFP-3	GTT AGC GGC G	KFP-21	GTA GGC GTC G
KFP-4	CGG AGA GTA C	KFP-22	TAC GCA CAC C
KFP-5	CCT GGC GAG C	KFP-23	GCT CGT CAA C
KFP-6	TCC CGA CCT C	KFP-24	ACT CGT AGC C
KFP-7	CCA GGC GCA A	KFP-25	CTA GGC GTC G
KFP-8	ACG CGC TGG T	KFP-26	CGG AGA GTC C
KFP-9	GAC TGG AGC T	KFP-27	TCC TCG CGG C
KFP-10	ACG GTG CGC C	KFP-28	AAT CGG GCT G
KFP-11	CGC GAC GTG A	KFP-29	TCG CCA TAG C
KFP-12	GAC CCC GGC A	KFP-30	GTG CGG ACA G
KFP-13	CAG TCG GGT C	KFP-31	TCA CCG AAC G
KFP-14	GGG TAA CGC C	KFP-32	GCG ATA CGC T
KFP-15	GTG ATC GCA G	KFP-33	TGA AGG TCC C
KFP-16	GCA TGG AGC T	KFP-34	GTC CGT GCA A
KFP-17	CCG AAG CCC T	KFP-35	CGT AGC CCC G
KFP-18	ACC CAT TGC G	KFP-36	TGC AGG CTT C

5.2.4 Data analysis

Amplification products were scored as presence or absence of a band. Each polymorphic band was considered as a locus, so that every locus has two alleles identified by the presence and absence of the band. The data was then transformed into allele frequencies. The level of genetic diversity was calculated based on Nei's (1973) gene diversity measure and Shannon's information index (Lewontin, 1972). Diversity values were calculated according to Nei's unbiased statistic (Nei, 1987) using POPGENE 1.31 (Yeh *et al*, 1999)

5.3 Results

From a set of 40 primers screened, eight produced clear RAPD patterns consisting of a total of 213 scorable markers 177 of which (80.3%) were polymorphic. Screening of the entire set of samples was repeated with two of the primers to assess repeatability of RAPD profiles and identical RAPD patterns were obtained. Examples molecular profiles obtained using primers KFP01, KF05 and KF07 are shown in plates 5.1 – 5.3. Arrows in the plates indicate some of the polymorphic bands scored in 18 individuals screened..

Table 5-2. Means of Nei's gene diversity and Shannon's information index and standard deviations for the single population of *Ixora scheffleri* subsp *keniensis*.

	na*	ne*	h*	I	
Mean	1.8310	1.3991	0.2438	0.3776	
St. Dev	0.3756	0.3421	0.1735	0.2368	n=20.

* na = Observed number of alleles

* ne = Effective number of alleles [Kimura and Crow (1963)]

* h = Nei's (1973) gene diversity

* I = Shannon's Information index [Lewontin (1972)]

The number of polymorphic loci is: 177

The percentage of polymorphic loci is: 83.10 %

The mean level of genetic diversity within the population based on Nei's (1973) gene diversity measure was 0.2438. The mean based on Shannon's information index (Lewontin, 1972), which is a more sensitive measure, was 0.3776 (table 5.2). The percentage of effective number of alleles in this population was 76.4%.

Analysis of pair-wise RAPD distances by Analysis of Molecular Variance (AMOVA) could not be carried out in this study since we did not have another population of *Ixora scheffleri* sub sp *keniensis* to compare with.

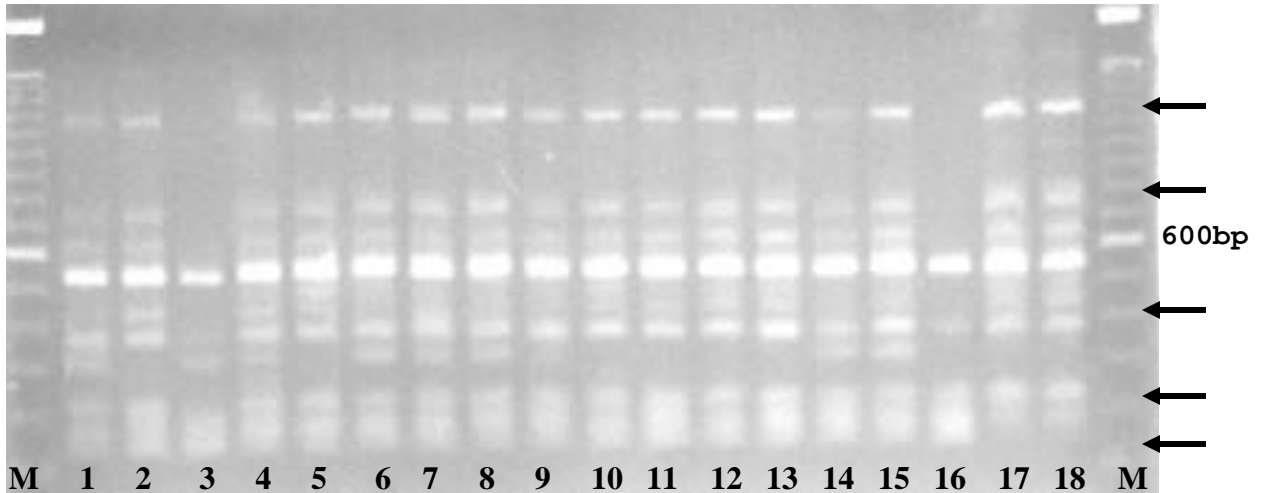


Plate 5.1 An example of an *Ixora scheffleri* subsp *keniensis* population RAPD profile generated using primer KF01. (Profile for 18 individuals sampled). Arrows indicate some of the polymorphic bands scored.

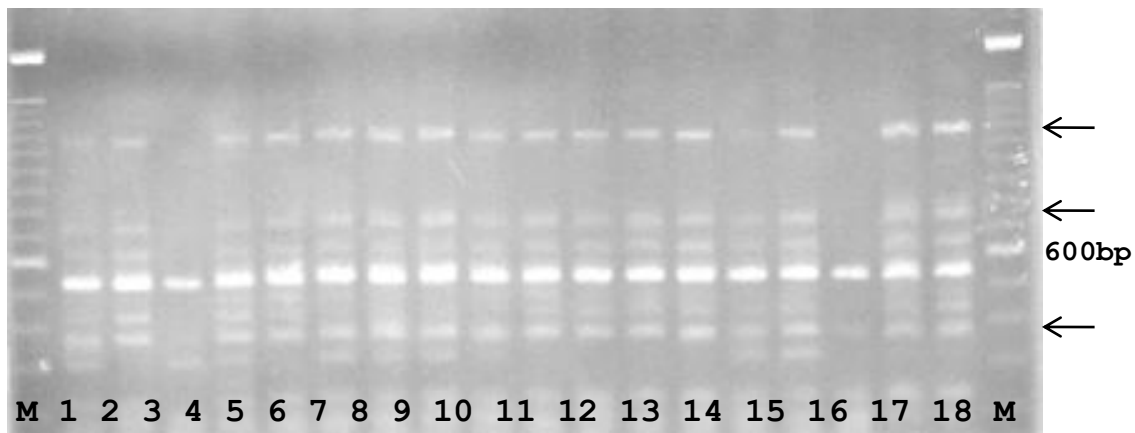


Plate 5.2: An example of an *Ixora scheffleri* subsp *keniensis* population RAPD profile generated using primer KF05. (Profile for 18 individuals sampled). Arrows indicate some of the polymorphic bands scored

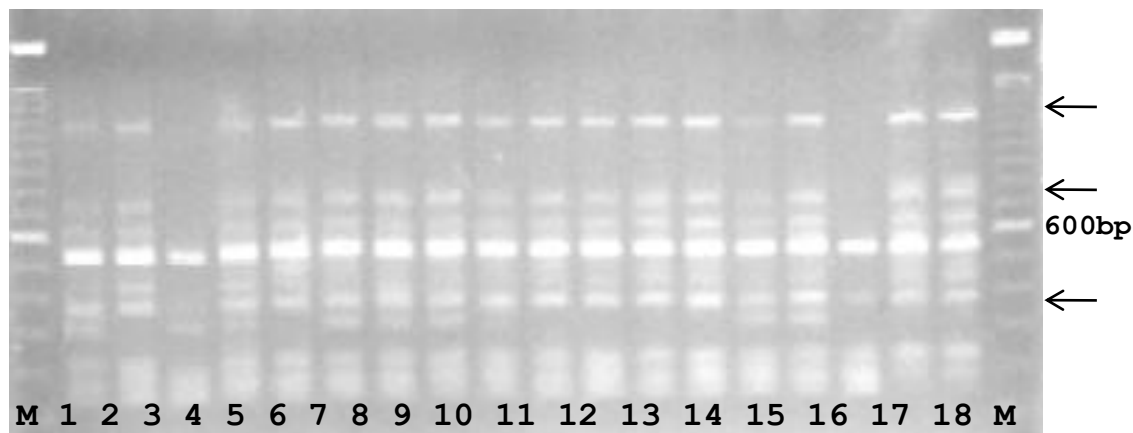


Plate 5.3: An example of an *Ixora scheffleri* subsp. *keniensis* population RAPD profile generated using primer KF07. (Profile for 18 individuals sampled). Arrows indicate some of the polymorphic bands scored

Genetic distance was also not calculated because this is only possible when there are two or more populations that are being evaluated. Genetic distance gives a relative estimate of time that has passed since the populations have existed as single cohesive units (Brent, 1996).

5.4 Discussion

The most striking feature of these results was the relatively high degree of genetic differentiation recorded within this single population of *Ixora scheffleri* sub sp. *keniensis* compared with results obtained for other tree species. For example values of 0.128, 0.144 and 0.070 based on Nei's gene diversity index were recorded for three conifers native to Southern South America, namely *Araucaria araucana* Bekessy *et al* 2002), *Fitzroya cupressoides* (Allnut *et al*, 1999) and *Podocarpus salignus* (Allnut *et al* 2001) respectively. Runo *et al* 2004 obtained mean level of genetic diversity (based on Nei's gene diversity index) within populations of *Melia volkensii* populations in Kenya of 0.0946 which is way below 0.243 recorded in this study. Dawson *et al* (1995) obtained

mean diversity indices ranging from 0.015 to 0.027 in *Hordeum spontaneum* populations. Chalmers and co workers (1992) used RAPDS to study diversity in populations of *Gliricidia sepium*, a multipurpose tree species from Central America and found Shannon's diversity index to vary between 0.160 and 1.754, while another study on *Theobroma cacao* (Russel *et al* 1993) estimated that within population diversity levels ranged between 1.175 and 2.128. The high level of variation found in this single population of *Ixora scheffleri* subsp *keniensis* has profound implications for conservation of this species. Almost all the individuals in existence need to be adequately conserved if the full breadth of genetic variation across the species range is to be maintained. Maintenance of genetic diversity is important as the diversity carries forward both ecological adaptation and microevolution (Machua *et al*, 2007). Dawson and Powel (1996) studied genetic variation in the Afromontane tree *Prunus Africana*, an endangered medicinal species and emphasize the need for determining appropriate approaches for conservation of this species based on RAPD analysis. Cardoso *et al* (1998) using RAPD markers detected high genetic differentiation among remnant populations of the endangered *Caesalpinia echinata* Lam (Leguminosae-Caesalpinioideae). Of the total genetic variability, 28.5% was attributable to differences between two geographical groups, 29.6% to population differences within groups and 42% to individual differences within populations. This study showed that RAPD analysis may be used as an initial approach to assess partitioning of genetic variation in this endangere species.

The high levels of genetic differentiation recorded in the study of *Ixora scheffleri* subsp *keniensis* may have been caused by effective gene flow within populations in the past and high fecundity. Trees that are dispersed by ingestion of seeds by animals have generally

been found more variable within the populations (Hamrick and Godt, 1989). The high rates of gene flow in *Ixora scheffleri* subsp *keniensis* could have been caused by long distance dispersal of pollen and/or seeds. No direct estimates of pollen dispersal are available for this species, but the bagging experiments reported in this study (chapter 2) indicate *Ixora scheffleri* subsp *keniensis* is anemophilous as well as entomophilous. Wind pollination experiment (chapter 2) resulted in successful pollination and 44% fruit set and open pollination (wind and insect pollination) resulted in 79.5% fruit set. Geographical isolation together with some degree of inbreeding may have been important factors in the genetic differentiation observed between the analysed individuals in the single population of *Ixora scheffleri* subsp *keniensis*. Another possible reason for the high degree of genetic differentiation is that over exploitation of this species to provide firewood and charcoal could have led to decimation of individuals with desired characteristics to meet these needs. The end result of such uncontrolled selection is that individuals not deemed to have the desired qualities are left. With time, through outcrossing, a highly differentiated population could have arisen. The presence of 19.7% monomorphic bands in this study suggests that the species may tolerate a degree of inbreeding. The results of the breeding systems study (chapter 2) showed that spontaneous autogamy treatment resulted in 36.5% fruit set. As indicated by Hamrick (1990), inbreeding could also be due to mating between relatives, such as half sibs, rather than to selfing. In addition, there is evidence that outbreeding is not universal among tropical forest plants, as many tree species with bisexual flowers seem to have at least limited self-compatibility (Bawa and Ashton, 1991)

From a conservation point of view the ability to recognize individuals based on RAPD markers will be important in the monitoring of reintroduced populations because it will, for example, enable the assessment of breeding success in the different genotypes (Rosseto *et al*, 1995). The specific characteristics of the RAPD method (random, uncharacterized multiple genomic loci, dominant nature of markers, and possibility of co-migrating non homologous bands) result in limitations for population genetic studies (Lynch and Milligan, 1994). Despite this, RAPD analysis can be used effectively for the initial assessment of levels and partitioning of genetic variation within plant species particularly in tropical tree species for which there is very little other genetic diversity information available.

Genetic analysis can provide valuable insights into the processes influencing extinction (Clarke and Young, 2000), and increasingly, genetic data are being used to define units for conservation management and for inferring changes in population structure and dynamics (Moritz, 1995, Newton *et al*, 1999). Surveys indicate that more than 8000 tree species are currently threatened with extinction worldwide (Oldfield *et al*, 1998). For the vast majority of these species, information on patterns of infraspecific variation is entirely lacking. As a result conservation strategies are generally being developed with little or no reference to genetic information (Allnut *et al*, 2003).

As mentioned earlier (chapter 2), habitat destruction is one of the greatest threats to the conservation of biodiversity. An example is in Australia where the majority of plant extinctions have arisen from habitat destruction associated with grazing and agriculture (Leigh & Briggs, 1992). Population fragmentation may produce an increased susceptibility to both demographical and genetic hazards, which may interact further to

promote high extinction probabilities (Gilpin & Soule, 1986). The evolutionary significance of a reduction in genetic diversity remains uncertain (Charlesworth & Charlesworth, 1987). On theoretical grounds, genetic variation is considered vital for allowing both short term adaptation to environmental fluctuations and long term evolutionary change (Frankel & Soule, 1981., Barrett & Kohn, 1991., Schaal *et al.*, 1991).

CHAPTER 6

6.0 OVERALL CONCLUSIONS AND RECOMMENDATIONS

Ixora scheffleri subsp *keniensis* showed a mixed breeding system that may be an adaptation to maintain fecundity even when mate or pollinator availability is unpredictable (see Table 2-1, chapter 2). The level of selfing and crossing in *Ixora scheffleri* subsp *keniensis* would, therefore, depend on other factors such as population density and the availability of pollinators. Exploitation and reduction in its population size and density are likely to promote selfing and inbreeding. Over time, this could lead to a reduction in genetic variation, inbreeding depression and reduced adaptability of its populations to environmental conditions.

The failure of seeds of this species to germinate either in the field or under laboratory conditions point to the possible existence of germination inhibitors. This observation emanates from the fact that the seeds examined all had well formed embryos but still failed to germinate even after undergoing a wide array of seed germination experiments. Existence of high levels of Absciscic acid (ABA) and other chemical inhibitors in seeds can inhibit their germination.

The results of the present study appear to confirm the hypothesis that high rooting percentages can be achieved in a previously understudied species, by manipulating auxin concentration, leaf lamina area, the cutting length, the cutting position on the mother plant and the season when the cuttings are harvested (See chapter 3). Individually, the optimum treatment for each of the factors studied increased rooting success significantly with indications that interactions can occur between these factors. A better understanding

of these factors appears necessary to maximize the success and cost effectiveness of a vegetative propagation programme. Enhanced rooting is important even in species that are propagated easily as small gains in rooting percentage may be of considerable economic value when the species is mass propagated on a commercial scale.

The results presented here demonstrate that mass propagation of *Ixora scheffleri* subsp. *keniensis* by means of axillary buds is possible. Plantlet production via *in vitro* multiplication is a laborious and relatively expensive process as compared to production of rooted cuttings. The production cost for a plantlet produced via tissue culture is approximately three times that of a rooted cutting (Watt *et al.*, 1995). However, as pointed out by Rockwood and Warrag (1994), a well-devised tissue culture based production strategy can result in the production of one million plants per year from a single explant, yields impossible to realize with vegetative or seed propagation.

In addition, and as reported for other tree species e.g. apple (Webster & Jones, 1989), cuttings from micropropagated plants are easier to root and in some cases produce more shoots than conventional adult sources. In line with this, cost dilution can be achieved through use of micropropagated plants as stock plants from which cuttings can be harvested. If used in conjunction with conventional methods, *in vitro* culture techniques can contribute markedly to progress in tree breeding by increasing the number of plantlets produced per parent genotype as well as being potentially important tools for genetic manipulations (Warrag *et al.*, 1990).

The method adopted and developed in this study for DNA isolation yielded high, pure and adequate DNA for RAPD marker analysis. This method is thus recommended for isolation of high quality DNA for RAPD analysis of *Ixora scheffleri* subsp. *keniensis*.

There is the possibility that generations of loggers felled the straightest, defect free trees because they were of high value and left the crooked, diseased and runty. This could partly explain the high level of genetic differentiation in this population. Genetic resources should be maintained because of their potential utility. No one can predict their actual utility. The genetic resources of non-commercial species may be as important as those of our most valuable timber trees. The new recombinant DNA technology makes it likely that genes can eventually be transferred among individuals and species that do not normally cross. However the genes must be preserved now so that they can be drawn on when needed.

In an attempt to boost the conservation status of this species, we intend to incorporate it in home gardens as an agro forestry species. From the growth habit of the species, it can act as an ornamental plant owing to its well-formed canopy portraying a well laid out leaf mosaic pattern (chapter 1, plate 1.6). As early as 19th century, Don (1834) pointed out that plant in genus *Ixora* are “truly beautiful when in blossom” and recommended their cultivation in every collection made from the wild. David *et al*, 2007 conducted a survey on genus *Ixora* in the Marquesas Island (French Polynesia) and concluded that all the Marquesan taxa are in need of inclusion in the IUCN red list, their status ranging from endangered to critically endangered. This status has arisen as a result of serious threat from human impact, feral animals and weeds. For *Ixora scheffleri* subsp *keniensis*, the near extinction status has arisen mainly from human impact activities.

Domestication in agro forestry systems is seeking to bring a wide range of scientifically overlooked (“Cinderella”) species into cultivation in partnership with farmers in their own farms (Leakey, 2004).

Some mechanical and physical properties of *Ixora scheffleri* subsp *keniensis* evaluated compare favourably with other agroforestry tree species planted in Kenya (Appendix 7). Most of the parameters evaluated, compressive strength, shear strength, hardness, moisture content and density were higher than for *Cupressus lusitanica*. *Croton megalocarpus* recorded higher values than *Ixora scheffleri* subsp *keniensis* for most of the parameters evaluated. It is interesting to note that the modulus of rupture (MOR) for *Ixora scheffleri* subsp *keniensis* is higher than for *Croton megalocarpus* and *Cupressus lusitanica* (See Appendix 7). This clearly indicates that the wood of this species can make hardy tool handles. Currently, the annual consumption of wood carving in Kenya is estimated at about 15,000 cubic meters (Choge, 2002). This could have led to a depletion of their gene diversity. Extensive harvesting of mature trees also affects gene flow, an essential mechanism necessary for maintenance of diversity within and between populations.

6.1 Value for conservation efforts

To most people, biological diversity is undeniably of value but it does not lend itself to the economic valuation upon which policy decisions are usually based. Standard economics has generally failed to assign value to ecological resources, so that the cost of environmental damage or depletion of living resources has usually been disregarded (Begon *et al.*, 1996). A major challenge for the future is the development of a new ecological economics (Costanza, 1991) in which the worth of species, communities and ecosystems can be set against the gains to be made in industrial projects that may damage them. Some feel that this kind of economic valuation is inappropriate because features of the natural world are unique and thus priceless (Ehrenfeld, 1988).

Value can be divided into three main components:

- (i) Direct value of products that are harvested
- (ii) Indirect value where aspects of biodiversity bring economic benefit without the need to consume the resource (McNeeley, 1988)
- (iii) Ethical value.

Of these three main reasons for conserving biodiversity, the first two, direct and indirect economic value has a truly objective basis. The third, ethics, on the other hand, is subjective and is faced with the problem that a subjective reason will inevitably carry less weight with those not committed to the conservation cause. *Ixora scheffleri* subsp *keniensis* being a rare plant with high quality timber and medicinal uses is a priority candidate for conservation based on these features.

Franklin (1980) suggested that an effective population size of about 50 would be unlikely to suffer from inbreeding depression, while 500 might be needed to maintain longer term evolutionary potential. Nunney & Campbell (1993) suggest that the effective population size should be between 2500 – 5000 individuals.

6.2 *Ixora scheffleri* subsp *keniensis* conservations options and management plans

6.2.1 *Ex situ* conservation

Captive propagation is attractive in its directness, its immediacy and the opportunity it provides. However, by itself it clearly offers no long-term solutions. *Ex situ* conservation measures include tree planting (field genebanks), seed stands, storage of seeds and/pollen and *in vitro* culture of reproductive and somatic tissues (Martinez *et al*, 1997).

Specifically, captive propagation can fulfill four functions in conservation of *I. Scheffleri* subsp *keniensis*.

- (i) Providing material for basic research in population biology, reproductive biology, etc.
- (ii) Providing material for basic research in care and management techniques of rare and critically endangered plant species.
- (iii) Providing demographic or genetic reservoirs for enhancing natural populations or establishing new ones
- (iv) Providing a final refuge for the species with no immediate hope of survival in the wild.

Botanical gardens and zoos where captive propagation is normally done have become the centre of a spirited debate. At one extreme, some see botanical and zoological parks as small arks that provide refuge for endangered species from a flood of species extinction; others see them as living museums-once a species enters a botanical garden or a zoo, it is essentially dead (Ginsberg, 1993).

Captive breeding for conservation should involve three stages:

- (i) Planning a conservation strategy
- (ii) Captive breeding
- (iii) Reintroduction. This needs to be integrated with habitat protection and *in situ* conservation. Regrettably, many species are simply inappropriate for captive breeding and eventual reintroduction and available resources are insufficient to build an 'ark' big enough for a significant proportion of the world's biota (Ginsberg, 1993).

For *Ixora scheffleri* subsp *keniensis*, *Ex situ* conservation of the species has been carried out at the following sites with success: Coffee Research Foundation (CRF), Ruiru, Kenya Forestry Research Institute (KEFRI), Muguga and the botanical garden at Jomo Kenyatta University of Agriculture and Technology (JKUAT). The propagules for *ex situ* conservation have been raised through vegetative propagation and *in vitro* propagation protocols developed in this study.

6.3 CONCLUSION

Results from this study indicate that it is a feasible project to use micropropagation in conjunction with macropropagation to start reforestation programmes with *Ixora scheffleri* subsp *keniensis*. Plantations of this species could be established in the vast deforested areas around Mt Kenya, the area of endemism of this species. This would go a long way towards reversing the almost extant status of this critically endangered endemic species. Like all other endangered plant species, *Ixora scheffleri* subsp *keniensis* should be accorded state protection to allow for genetic improvement of the remaining stand. This is the most appropriate form of *in situ* conservation for this rare and critically endangered tree species.

Based on experiences gathered during this study, biological diversity has suffered an attack of such devastating magnitude that our time may well be known as “the dark ages of life on earth”. But I strongly believe that our generation has the opportunity to change course, to initiate an era of enlightenment in the understanding of the relationship between man and nature.

6.4 RECOMMEDATIONS AND WAY FORWARD

- As a preliminary step, a monitoring program should be built for *Ixora scheffleri* subsp *keniensis* in Kenya, including extensive exploration of favourable habitats while looking for additional natural populations. To prevent further habitat loss and population decline, habitat and plants should be protected. Major efforts should be made to develop suitable alternatives for site protection through public or private agencies.
- *In situ* conservation is recommended in order to preserve the existing genetic diversity. In addition *ex situ* cultivation at various sites should be undertaken to enhance recovery of genetic diversity. To be effective, *in situ* conservation of *Ixora* and other vulnerable species needs to be integrated in the overall framework of sustainable forest management. There is need to promote a wider range of uses and values for these resources and to facilitate the access of local people to them. Owing to the high social and economic pressure on forests and lack of information, conservation efforts in the past have rarely targeted forests containing valuable tree species.
- Even though protected areas can play a significant role in conserving critically endangered species like *Ixora*, they need to be complemented by activities outside protected areas, such as sustainably managing forests for multiple uses, including the production of timber and nonwood forest products (FAO, 1993).
- It is recommended that *Ixora scheffleri* subsp *keniensis* be used as a root stock in the propagation of *coffea* hybrids (Ruiru 11) in Kenya especially in those areas where coffee is seriously affected by nematodes which lead to the death of the plants (e.g. Makuyu area of central Kenya). This recommendation emanates from

the fact that *Ixora* is not affected by such nematodes. Trials conducted at JKUAT botanical garden have indicated that grafting is feasible, *Ixora* acting as the rootstock and the *coffea* hybrids being the scion (Plate 6.1). This practice shall go a long way towards helping Kenyan farmers increase coffee production that is greatly hampered by nematode infestations.



Plate 6.1 A *coffea Arabica* (Ruiru 11) scion grafted on *I. scheffleri* subsp *keniensis* rootstock after 2 weeks. Note the buds sprouting from the scion.

- Since the almost extinct status of this species has emanated from overexploitation by the fuelwood gatherer and the slash and burn cultivator in the area of endemism of this species, it is recommended that propagules be reintroduced in these areas and be tended carefully until maturity.
- The species has a well laid out canopy and beautiful flowers. It's recommended that it be introduced in agroforestry, tree cultivation, home gardening and other innovative agrotechnologies that could enable large numbers of cultivators to care for this critically endangered species.

- It's recommended that tree nurseries be established at appropriate locations in the country where propagules of this species raised from *in vitro* propagation and vegetative propagation protocols are tended and sold to the end users. To actualize this proposal, funding shall be sort from agencies such as African Network for Agroforestry Education (ANAFE). Sub-saharan Africa Forest Genetic Resources Network (SAFORGEN) among others. In this proposal, farmers shall be facilitated to establish such nurseries so that they feel part and parcel of the conservation process.
- Kenya's policy statement on natural forests and woodlands of 1994 is that all gazetted natural forests, woodlands and bushlands will remain reserved and will be managed by state approved agencies that will allocate them primarily for: (1). Regulated multipurpose forestry using zoning concepts that do not endanger the conservation functions of forests. (2). Preservation of biodiversity. It's recommended that this policy statement should be adhered to strictly. If this is done, it shall go a long way in conservation of *Ixora scheffleri* subsp *keniensis* and other critically endangered forest species.
- There is urgent need for more and readily accessible information on the status of FGR and activities related to their conservation and use. Such information could support decision-making, enhance regional and international cooperation and facilitate wise management and sustainable use of FGR (Hansen, 1997). Informed decision making for conservation and wise utilization of FGR requires reliable and up to date information systems. These databases can particularly be useful to

scientists and managers for strategically planning conservation, use and identification of priority areas for future work.

- Cryopreservation protocols for seeds, embryos, meristems etc of this species need to be developed. Although many biologists might object to the idea of cryoconservation on ethical grounds, the justification for this option is that we should save as many species of the planet as possible and argue later about whether saving species by artificial means was in their best interest.
- There is need to investigate the effect of global warming on seed germination of this plant species. There is likelihood that this could have affected seed germination adversely.

REFERENCES

- Abbiw D.K (1990)** Useful plants of Ghana: West African uses of wild and cultivated plants: Xii + 337pp. Kew, Royal Botanic Gardens Kew.
- AdAdams W.T (1992).** Gene dispersal within forest tree populations. *New Forests* 6: 217-240.
- Afaque, Q, Koche, V., Sharma, P & Mishra, S.K (2004).** *In vitro* clonal propagation of Neem (*Azadirachta indica*). *Plant cell. Tiss. & Organ Cult*, 73 (3), 281-284.
- Allnut, T.R., Newton, A.C., Lara, A., Premoli, A., Armesto, J.J., Vergara, R., Gardner, M (1999).** Genetic variation in *Fitzroya cupressoides* (alerce), a threatened South American Conifer. *Molecular Ecology* 8: 975-987.
- Allnut, T.R., Newton, A.C., Premoli, A & Lara, A (2003).** Genetic variation in the threatened South American conifer *Pilgerodendron uviferum* (Cupressaceae), detected using RAPD markers. *Biological Conservation*, 114: 245-253.
- Allnut, T.R., Curtis, J.R., Gardner, M & Newton, A.C (2001).** Genetic variation in wild Chilean and cultivated British populations of *podocarpus salignus* D. Don (Podocarpaceae). *Edinburgh Journal of Botany*, 58: 459-473.
- Amin, M.N., Shahrear, A., Sultana, S., Alam, M.R & Azad, M.A.K (2002).** *In vitro* rapid clonal propagation of an ornamental plant *Ixora fulgens* Roxb.. *Online journal of Biological Sciences*, 2 (7): 485-488.

- Aminah, H (1992).** A note on the effect of leaf number on rooting of *Hopea odorata* stem cuttings. *Journal of Tropical Forest Science* **3** (4): 384-385.
- Andersen, A.S (1986).** Environmental influences on adventitious rooting in cuttings of non-woody species. In: *New root formation in plants and cuttings*, edited by M.B Jackson, Dordrecht: Nijhoff. Pp 223-253.
- Anderson, G.J (1995).** Systematics and reproductive Biology. In P.C Hock & A.G Stephenson (eds). *Experimental and molecular approaches to plant Biosystematics*. *Syst Bot*, 25: 263-272
- Arya, H.C & Shekhawat, N.S (1986).** Clonal multiplication of tree species in the Thar desert through tissue culture. *Forest Ecology and Management*, **16**: 201-208.
- Barret, S.C.H & Kohn, J.R (1991).** Genetics and evolutionary consequences of small population size in plants: Implications for conservation. In Falk D.A., Holsinger, K.E (Eds). *Genetics and conservation of rare plants*. Oxford university press, New York, USA, pp 3-30.
- Baskauf, C.J., McCauley, D.E & Eickmeir W.G (1994).** Genetic analysis of a rare and widespread species of Echinacea (Asteraceae). *Evolution* **48**: 180-188.
- Bates, S., Preece, J.E., Navarrete, N.E., Van Sambeek, J.W & Gaffney, G.R (1992).** Thidiazuron stimulates shoot organogenesis and somatic embryogenesis in white ash (*Fraxina Americana* L.). *Plant Cell Tiss. Org. Cult.* **31**: 21-29.

- Bawa K.S & Ashton P.S (1991).** Conservation of rare trees in tropical rain forests: a genetic perspective. In: Genetics and conservation of rare plants (eds Falk D.A, Holsinger K.E) pp 62-71. Oxford university press, Newyork.
- Becwar, M.R., Wann, D.R., Johnson., M.A., Verhagen, S.A., Feirer, R.P & Nagmani, R (1988).** Development and characterization of *in vitro* embryogenic systems in conifers. Pp 1-18. In M.R Ahuja (ed). Somatic cell genetics of woody plants. Kluwer academic publishers, Dordrecht/Boston/London.
- Beentje, H.J (1994).** Kenya Trees, Shrubs and Lianas. National Museums of Kenya, Nairobi, Kenya.
- Begon, M., John, L.H., Colin, R.T (1996).** Ecology, Individuals, Populations and Communities, Blackwell Sciences Ltd, London, pp 945-952.
- Bekessy, S.A., Allnut, T.R., Premoli, A.C., Lara, A., Ennos, R.A., Burgman, M.A., Cortes, M & Newton, A.C (2002).** Genetic variation in the monkey puzzle tree (*Araucaria araucana* (Molina) K. Koch) detected using RAPD. *Heredity*, **88**: 243-249.
- Berry P.E & Calvo R.N (1989).** Wind pollination, self incompatibility and altitudinal shifts in pollination systems in the high Andean genus *Espeletia* (Asteraceae). *American journal of Botany*. **76**: 1602-1614.
- Bertin, R.I & Newman, C.M (1993).** Dichogamy in angiosperms. *Bot. Rev.* **59**: 112-152.
- Bir S.S, Singh G. & Gill B.S (1984).** In: Love A. (ed): Chromosome number reports 82. *Taxon* **33**: 126-134.

- Black D.K (1973).** Influences of shoot origin and certain pre- and post- severance treatments on the rooting and growth characteristics of Douglas-fir stem cuttings. PhD thesis, Oregon State Univ., Corvallis, Oregon, pp 159.
- Boggeti, B. (1997).** Development of micropropagation and potential genetic transformation system for cashew (*Anarcadium occidentale* L.). Ph.D Thesis, University of London.
- Bonga J.M & Von Aderkas P (1992).** *In vitro* culture of trees. Kluwer academy publishers. Dordrecht pp 231.
- Bonga, J.M. (1982).** Vegetative propagation in relation to juvenility, maturity and rejuvenation. In J.M Bonga and D.J. Durzan eds. Tissue culture in forestry. Martinus Nijhoff, The Hague, pp. 384-412.
- Bonga, J.M. (1987).** Clonal propagation of mature trees. Poblems and possible solutions. In Bonga, J.M. & Don, J. Durzan (eds). Cell and tissue culture in forestry, Vol I (1987). Martinus Nijhoff Publishers. Dordrecht. Pp 249-271.
- Bonga, J.M & Durzan, J.D (1987).** Somatic embryogenesis in woody perennials. Cell and tissue culture in forestry vol.2. Martinus Nijhoff publishers. Dordrecht. Pp 61-91.
- Bonnin, I., Huguet, T., Gherardi, M., Prosperi, J.M & Olivieri, I (1996).** High level of polymorphism and spatial structure in a selfing plant species, *Medicago truncatula* (leguminosae), shown using RAPD markers. *American Journal of Botany* **83**: 843-855.

- Boxus, P. (1974).** The production of strawberry plants by *in vitro* micropropagation. *J. Hort. Sci.* **49**, 209-210.
- Bremekamp C.E.B (1937)** The *Ixora* species of Burma and the Andaman Islands. *Jour. Bot. [London]* **75**:108-111.
- Brent, W.M (1996).** The estimation of Genetic Distance and population substructure from microsatellite allele frequency data. McMaster university website on Genetic distance.
- Bridson D. M (1988).** *Ixora*. In: Bridson D.M & Verdcourt B., Flora of East Tropical Africa, Rubiaceae (part 2): 610-617.
- Bridson D.M. & Robbrecht E. (1985).** Further notes on the tribe pavetteae (Rubiaceae). *Bull.Jard. Bot. Nat. Belg.* **55**: 83-115.
- Burkhill H.M (1998)** The useful plants of West tropical Africa. IV. Royal Botanic Gardens Kew.
- Campuana, M & Lambardi, M (1995).** Cutting propagation of common cypress (*Cupressus sempervirens* L.).
- CGIAR (1994).** Keeping faith with the future Forests and their genetic resources. IPGRI, Rome, Italy.
- Chalmers, K.J, Waugh, R, Sprent J. I., Simons, A.J., Powel, W (1992).** Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculate* using RAPD markers. *Heredity*, **69**: 465-472.
- Chalupa V (1987).** European hardwoods. In: Bonga J.M & Durzan D.J (eds), Cell and Tissue Culture in Forestry 3: 224-240.

- Charlesworth, D & Charlesworth B (1987).** Inbreeding depression and its Evolutionary Consequences. *Annual review of Ecological Systematics*. **18**: 237-268
- Choge, S.K (2002).** Study of economic aspects of the woodcarving industry in Kenya: Implications for policy development to make the industry more sustainable. Msc. Thesis, University of Natal, Republic of South Africa.
- Clarke, G.M & Young, A.G (2000).** Introduction: genetics, demography and the conservation of fragmented populations. In: Young, A.G, Clarke, G.M (Eds), *Genetics, Demography and Viability of Fragmented Populations*, Cambridge University Press, Cambridge, pp 1-6.
- Conway, G.W (1991).** The prospects of sustaining species and their evolution. . In: Western, D & Pearl, M (Eds), *Conservation for the Twenty First Century*. Oxford University Press, New York.
- Cooper G.P & Record S.J (1931)** The evergreen forests of Liberia. Yale Univ. School Forestry Bull. 31.
- Costanza, R (1991).** *Ecological Economics: The Science and Management of Sustainability*. Columbia university press, New York.
- Crins, W (1997).** Rare and endangered plants and their habitats in Canada. *The Canadian Field Naturalist* **3**: 506-517.
- Cruden R.W & Layer D.L (1989).** In *The Evolutionary Ecology of Plants* (J.H Bark & Linhart Y.B eds.).pp 71.
- Dafni (1992).** *Pollination Ecology: a Practical approach*. Institute of Evolution, Haifa University, Haifa, Israel.

- Daigny, G., Paul, H., Sangwan-Norrel, B.S (1996).** Factors influencing secondary somatic embryogenesis in *Malus domestica* Borkh. *Plant Cell Rep.* **16**: 153- 157.
- Davey, M.R., Webster, G., Manclers, G., Ringrose, F.L., Power, J.B., & Cocking, E.G (1993).** Effective nodulation of micropropagated shoots of the non-legume *parasponia andersonii* by *Bradyrhizobium*. *Journal of experimental Botany*, Vol **44**, No. 262, pp. 863-867.
- David, H.L., Warren, L.W., Arnaud, M & Jacques, F (2007).** Revision of *Ixora* (Rubiaceae) in the Marquesas (French Polynesia). *Botanical Journal of the Linnean society.* **155 (4)**, 581-597.
- Davis, T.D., Haissig, B.E., & Sankhla, N (1988).** Adventitious root formation in cuttings. Portland, Oregon: Dioscorides press.
- Dawson, I.K., Simons A.J., Waugh R & Powel, W (1995).** Diversity and genetic differentiation among subpopulations of *Gliricidia sepium* revealed by PCR-based assays. *Heredity.* **74**: 10-18.
- De Block P (1998).** The African species of *Ixora* (Rubiaceae – Pavetteae). National Botanic Garden of Belgium pp 1- 218.
- De Klerk, G.J (1992).** Hormonal control of dormancy and apical dominance in tissue cultured plants. *Acta Bot . Neerl.* **41**: 443-451.
- De Wildeman, E (1914).** In. De Block P (1998) Eds. The African species of *Ixora* (Rubiaceae – Pavetteae). National Botanic Garden of Belgium pp 1- 218
- De Wildeman, E (1920).** In. De Block P (1998) Eds. The African species of *Ixora* (Rubiaceae – Pavetteae). National Botanic Garden of Belgium pp 1- 218.

- Deberg, P. & Maene, L.J (1981).** A scheme for commercial propagation of ornamental plants by tissue culture. *J.Sci. Hort.* **14:** 335-345.
- Demeke, T., Lynch, D.R., Kawchuk, L.M., Kozub, G.C., Armstrong, J.D (1996).** Genetic diversity of potato determined by Random Amplified Polymorphic DNA analysis. *Plant Cell Reports.* **15:** 662-667.
- Diamond J (1984)** “Normal” extinctions of isolated populations. In: extinctions, ed. Nitecki M. H, pp 191-246. University of Chicago press, Chicago.
- Diamond J.M & Case T.J (1986)** Introductions, extinctions, exterminations and invasions. In: Community ecology, ed. Diamond J.M & Case T.J, pp 65-79. Harper & Row, New york.
- Don G (1834).** General history of the dichlamydeous pants. London, Rivington. pp 867
- Donnelly S.E, Lorte C.J & Aarssen L.W (1998).** Pollination in *Verbascum thapsus* (Scrophurlariaceae): the advantage of being tall. *American Journal of Botany* **85:** 1618-1625.
- Duke J.A (1965)** Keys for the identification of seedlings of some prominent woody species in eight forest types in Puerto Rico. *Ann. Missouri Bot. Garden* **52:** 314-350.
- Eberhart, S.A., Roos, E.E & Towil, L.E (1991).** Strategies for long-term management of Germplasm collections. In: Western, D & Pearl, M (Eds), Conservation for the Twenty First Century. Oxford University Press, New York.

- Eckert C.G & Barret C.H (1994).** Inbreeding depression in partially self-fertilizing *Decadonverticillatus* (Lythraceae): Population-genetic and experimental analyses. *Evolution* **48**: 952-962.
- Ehrenfeld, D (1988).** Why put a value on biodiversity? In: Biodiversity (E.O Wilson & F.M Peter, eds), pp 212-216. National Academy Press, Washington DC.
- Ehrlich P.R & Ehrlich A.H (1981).** Extinctions: The causes and consequences of the disappearance of species. Random House, New york.
- Ellis, D.D., Barczynska, H.M., McCown, B.H & Nelson, N (1991).** Comparison of BA, zeatin and thidiazuron for adventitious bud formation from *Picea glauca* embryos and epicotyl explants. *Plant. Cell tiss. Org. Cult.* **27**: 281-287.
- Ellstrand, N.C & Ellam, D.R (1993).** Population genetic consequences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics*, **24**: 217-242.
- Erickson O. & Bremer B. (1991)** Fruit characteristics, life forms and species richness in the plant family Rubiaceae. *Am Nat.* **138**: 751-761.
- Evans, D.A., Sharp, W.R. & Flick, C.E. (1981).** Growth and behaviour in cell cultures: Embryogenesis and Organogenesis. In Thorpe, T.A (eds) pp 45-113. Academic Press, New York.
- Faegri K & L Van Pijl (1979).** The principle of pollination Ecology. 3rd ed. Pergamon press, Oxford, UK.

- Fagerlind, F (1937).** In. De Block P (1998) Eds. The African species of *Ixora* (Rubiaceae – Pavetteae). National Botanic Garden of Belgium pp 1- 218.
- Falk D.A & Holsinger K.E (1991).** Genetics and Conservation of Rare Plants. New york. Oxford University Press.
- FAO (1993).** Conservation of genetic genetic resources in tropical forest management. FAO Forestry paper, 107, Rome, Italy.
- Fisher, M., & Mathies, D (1998).** RAPD variation in relation to population size and plant fitness in the rare *Gentianella germanica* (Gentianaceae). *American Journal of Botany* **85**: 811-819.
- Frankel, O.H & Soule, M.E (1981).** Conservation and evolution. Cambridge University Press, Cambridge, UK.
- Frankham, R (1995).** Conservation genetics. *Ann.Rev. Genet.* 29: 305-327.
- Frankham, R (1996).** Relationship of Genetic variation to population size in wildlife. *Conservation Biology*, **10** (6): 1500-1508.
- Franklin, I.A (1980).** Evolutionary changes in small populations. In: Conservation Biology, An Evolutionary-Ecological Perspective (M.E Soule & B.A Wilcox, eds), pp 135-149, Sinauer Associates, Sunderland, M.A.
- Friah, E.A, Robichau, R.H., Mount, D.W (1996).** Molecular genetic variation following a population crash in the endangered Mauna Kea silverwood, *Argyroxiphium sandwicense* spp *sandwicence* (Asteraceae). *Molecular Ecology*, **5**: 687-691.
- Galen, C (1996).** Rates of floral evolution in an alpine wild flower, *polenium viscosum*. *Evolution*, **50**: 120-125.

- Galun, E.W. (1981).** Plant protoplasts as physiological tools. *Ann. Rev. Plant physiol.* **32**: 327-66.
- Gamborg, O.L., Shyluk, J.P., & Shin, E.A (1981).** Isolation, fusion and culture of plant protoplasts. In plant tissue culture: Methods and application in agriculture (ed) Thorpe, T.A pp 115-153. academic press, New york.
- Ganadra, F., Kageyama, P.Y & Ouedraogo, A.S (1997).** Conservation and use of rare tropical forest trees. Pape presented at WCMC regional Meeting, CATIE, Turrialba, Costa Rica.
- Gaspar, T. & Coumans, M (1987).** Root formation. In: *Cell and Tissue culture in Forestry*. Vol. 2. Bonga, J.M and Durzan, D.J. (ed). Martinus Nijhoff, Dordrecht. pp 202-207.
- Geary, T.F & Harding, W.G (1984).** The effect of leaf quantity and trimming on rooting success with *Eucalyptus camaldulensis* Dehn. Cuttings. *Commonwealth Forestry Review*, **63** (3): 225-230.
- Gentry A.H & Dodson C (1987).** Diversity and Phytogeography of neotropical vascular epiphytes. *Ann. Missouri Bot. Gard.* **74**: 325-331.
- George, E.F. & Sherrington, P.D. (1984).** Plant propagation by tissue culture. Handbook and Directory of Commercial Laboratories. Exergetics Limited, UK.
- George, E.F. (1996).** Plant propagation by tissue culture. Part 2. In practice. Exegetics Limited, UK.
- Gilpin, M.E & Soule, M.E (1986).** Minimum viable populations: Processes of species extinction. In Soule, M.E (Ed), Conservation Biology: The Science

of Scarcity and Diversity. Sinauer Associates, Sunderland, MA, USA, pp 19-34.

Ginsberg, J.R (1993). Can we build an Ark? *Trends in Ecology and Evolution*. 8: 4-6.

Giround, R.M (1970). Rooting plain and heel cuttings of spruce. *Plant propagation*. 16: (1): 7-12

Glover, B.J & Abbot, R.J (1995). Low genetic diversity in the scotish endemic *primula scotica* Hook. *New Phytologist*, 129: 147-153.

Goyal, Y. (1982). Clonal multiplication of *Prosopis* and *Zizyphus* through tissue culture. In Arya H.C & Shekhawat, E.S. (eds.). Clonal multiplication of tree species in the desert through tissue culture. *Forest Ecology and Management*. 16: 201-208.

Grando, B.J & Abbot, R.J (1995). Low Genetic Diversity in the Scottish endemic *primula scotica* Hook. *New phytologist*, 129: 147-153.

Gray, D.J & Klein, C.M (1989). *In vitro* micropropagation and plant establishment of “Blanc du Bois” grape. *Proc. State Hort. Soc.* 102: 221- 223.

Guo W.H & Cook C.D.K (1990). The floral biology of *Groelandia densa* (L.) (Potamogetonaceae). *Aquatic Botany* 38: 283-288.

Haissig, B.E (1986). Metabolic processes in adventitious rooting of cuttings. In: *New root formation in plants and cuttings*, edited by M.B Jackson, Dordrecht: Nijhoff. Pp 141-189.

Halle, N (1966). Family Rubiaceae. *The Flora of Gabon*, 12: 1-278.

- Hamrick, J.L & Godt, M.J.W (1989).** Allozyme diversity in plant species. In (eds Brown, A.H.D, Clegg, M.T, Kahler, A.L & Weir, B.S). Plant5 population Genetics, Breeding and Genetic Resources, pp 43-63, Sinauer, Sunderland.
- Hamrick, J.L (1990).** Isozymes and the analysis of genetic structure in plant populations. In: Isozymes in plant Biology. (eds Soltis E.D & Soltis P.S). Chapman and Hall, London. Pp 87-105.
- Hamrick, J.L (1992)** Distribution of Genetic Diversity in Tropical Tree populations: Implications for the Conservation of the Genetic Resources. Paper to IUFRO S2.02-08 conference, breeding tropical trees, Cali and Cartagena, Columbia, 9-18 October, 1992.
- Handley, L.W (1995).** Future uses of somatic embryogenesis in woody plantation species. In: Jain, S., Gupta, P & Newton, R (Eds). Somatic embryogenesis in woody plants, Vol 1. Dordrecht: Kluwer Academic Publishers. pp 415-434
- Hansen, C.P (1997).** Making available information on the conservation and utilization of forest Genetic Resources: The FAO world wide information system on Forest Genetic Resources.
- Hartman, H.T & Kester, D.E (1968).** Plant Propagation: Principles and practices. Prentice-Hall Inc., Englewood Cliffs, NJ, pp 702.
- Hartman, H.T., Kester, D.E & Davies F.T (1990).** Plant propagation: Principles and practice. 5th Edition. Prentice Hall, London.

- Hartmann, H.T & Kester, D.E (1983).** Plant propagation principles and practices. Englewood Cliffs, New Jersey: Prentice Hall.
- Henifin, M.S., Morse, L.E, Griffith, S & Hohn J.E (1981).** Planning field work on rare or endangered plant populations. In Morse L.E and Henifin, M.S eds., Rare plant conservation: geographical data organization, pp 309-312. The New York Botanical Garden, Bronx, New York.
- Hoad, S.P & Leakey, R.R B (1992).** Morphological and physiological factors induced by light quality and affecting rooting in *Eucalyptus glandis*. Pp 51-58 in Mass production technology for genetically improved Fast Growing Forest tree Species. Volume 1. AFOCEL., Nangis, France.
- Hoebel, A.R (1992).** Conservation genetics of whales and dolphins. *Molecular Ecology* 1: 119-125.
- Holsinger, K.E & Gotlieb, L.D (1991).** Conservation of rare and endangered plants. Principles and prospects. In: Falk, A.D & Holsinger, K.E (Eds). Genetics and conservation of rare plants. Oxford university press, Oxford. pp 195-223.
- Hu, C.Y and Wang, P.J. (1983).** Meristem shoot tip culture and bud culture. In: *Handbook of Plant Cell Cultures* Vol 1. Evans, D.A., Sharp, W. R., Ammirato, P.V and Yamada, Y. (eds). MacMillan, New York. Pp 177-227.
- Hu, J & Quiros, C.F (1991).** Identification of broccoli and cauliflower cultivars with RAPD markers. *Plant Cell Reports*, 10: 505-511.

- Hubbel S. P & Foster R.B (1986).** Commonness and rarity in a neotropical forest: Implications for tropical tree conservation. In: Conservation Biology. The Science of Scarcity and Diversity, ed. M.E Soule, pp. 205-231. Sinauer, Sunderland, Mass.
- Huetteman, C.A & Preece, J.E (1993).** Thidiazuron: A potent cytokinin for woody plant tissue culture. *Plant Cell Tiss. Org. Cult.* **33**: 105-119.
- International Board for Plant Genetic Resources (1985).** Hand book of seed Technology for Gene Banks.
- International Union for Conservation of Nature (IUCN) (2004).** 2004 IUCN Red List of Threatened Species. www.redlist.org.
- Jansen, D.H (1988).** Management of habitat fragments in a tropical dry forest. *Annals of Missouri Botanical garden.* **75**: 105-116.
- Janzen D.H (1983).** No park is an Island: Increase in interference from outside as park size decreases. *Oikos*: **41**: 402-410.
- Kahia, J.W (1999).** *In vitro* propagation of the New Disease Resistant Coffea Arabica L. Hybrid –Ruiru 11. Unpublished Ph.D thesis, University of London.
- Karnoski, D.F., (1981).** Potential for forest tree improvement via tissue culture. *Bioscience.* **31**: 114-120.
- Katarina, A & Birgitta, B (2000).** Combined phylogenetic Analysis in the Rubiaceae-Ixoroideae: Morphology, Nuclear and Chloplast DNA data. *American Journal of Botany*, **87** (11): 1731-1748.

- Kato M. (1996)** Plant-pollinator interactions in the understory of a lowland mixed dipterocarp forest in Sarawak. *Am. Journ Bot.* 83:131-155.
- Keay R.W.J (1963).** *Ixora* L. In: Hepper F.N (ed), Flora of West Tropical Africa, ed. 22: 140-144.
- Kim, T.S & Kim, C.S (1988).** Effects of mother tree ages, timing of cutting and growth regulators on cuttings in Korean fir (*Abies koreana* Wlson). Suwon, Korea Republic. Research Report of the Institute of Forest Genetics 24: 47-56.
- Kimura, M & Crow, J.F (1963).** The measurement of effective population number. *Evolution*, 17: 279-288.
- Kiriba, M.G (2005).** *In vitro* and macropropagation of *Warbugia ugandensis* in Kenya. Msc thesis, Kenyatta university, Nairobi.
- Kohlenbach, H.W. (1977).** Basic aspects of differentiation and plant regeneration from cell and tissue cultures. In plant tissue culture and its biotechnological applications. (Eds) Bar, W., Reinhard, E & Zenk, M.H pp. 355-66. Springer-Verlag, Berlin.
- Konar, R.N., Thomas, E., & Street, H.E. (1972).** The diversity of morphogenesis in suspension cultures of *Atropa belladonna*. (L.): *Ann. Bot.* 36: 249-58.
- Krikorian, A.D & Berquin, D.L (1969),** Plant cell and tissue cultures: The role of Haberlandt. *Bot. Rev.* 35: 59-88.
- Kuria, M.W (2006).** Regeneration of the East African Greenheart (*Warbugia ugandensis* (Sprague) subsp. *Ugandensis* through tissue culture.

Unpublished M.Sc. Thesis, Jomo Kenyatta University of Agriculture and Technology, Nairobi

Lacy, R.C (1987). Loss of genetic diversity from managed populations: Interacting effects of drift, mutation, immigration, selection and population subdivision. *Conservation Biology* **1**: 143-158.

Ladd P.G. (1994) Pollen presenters in the flowering plants – form and function. *Bot. Journ. Linn. Soc.* **115**: 165-195.

Laikre, L & Rayman, N (1996). Effects on intraspecific biodiversity from harvesting and enhancing natural populations. *Ambio* Vol 25, No 8 Dec. 1996. pp 504-509.

Leakey, R.R B (1985). The capacity for vegetative propagation in trees. In: *Attributes of trees as crop plants*, edited by M.G.R Cannel & J.E Jackson. Abbots Ripton, Huntingdon: Institute of Terrestrial Ecology. Pp 110-133.

Leakey, R.R B., Mesen, J.F., Tchoundjeu, Z., Longman, K.A., Dick, J.M.C.P., Newton, A., Martin, A., Grace, J., Munro, R.C & Muthoka, P.N (1990). Low-technology techniques for the vegetative propagation of tropical trees. *Commonwealth Forestry Review*, **69** (3): 247-257.

Leakey, R.R.B & Storeton-West, R (1992). The rooting ability of *Triplochiton scleroxylon* cuttings: the interaction between stockplant irradiance, light quality and nutrients. *For. Ecol. Manage.* **49**: 133-150.

Leakey, R.R.B (1991). Low-tech cloning of tropical trees. Botanical Gardens *Conservation News*, **1** (9), 39-40.

- Leahey, R.R.B (2004).** Sustainable agriculture = agroforestry = Organic farming?
 In A. Leu (ed). Organically speaking-soil and society. Proceedings of
 inaugural Queensland organics conference, 31 July-2nd August 2003,
 Mossman, QLD, 4873, Australia, Organic producers Association of
 Queensland.
- Leahey, R.R.B., Chapman, V.R & Longman, K.A (1982).** Physiological studies
 for tropical tree improvement and conservation. Factors affecting root
 initiation in cuttings of *Triplochiton sclereroxylon*. *Forest Ecology and
 Management* **4**: 53-66.
- Leahey, R.R.b., Mesen, J.F., Tchoundjeau,Z., Longman, K.A., Dick, J.McP.,
 Newton, A., Matin, A., Grace, J., Munro, R.C & Muthoka, P.N (1990).** Low-
 technology techniques for the vegetative propagation of tropical trees.
Commonw.For.Rev., **69**(3): 247-257.
- Leahey, R.R.B., Newton, A.C., & Dick, J.McP (1994).** Capture of genetic
 variation by vegetative propagation: processes determining success. In:
 Leahey, R.R.B & Newton A.C (Eds) *Tropical Trees: The potential for
 Domestication and the Rebuilding of Forest Resources*. HMSO, London.
 pp 72-83.
- Leahey, R.R.B & Newton, A.C (1994).** Domestication of ‘Cinderella’ species as
 the start of a ‘woody plant revolution’. Pp 3-6 in Leahey, R.R.B &
 Newton, A.C. (Eds.) *Tropical Trees: The potential for Domestication and
 the Rebuilding of Forest Resources*. HMSO, London.

- Leigh, J.H & Briggs, J.D (1992).** Threatened Australian Plants. Overview and Case Studies. ANPWS, Canberra.
- Lewontin, R.C (1972).** The apportionment of human diversity. *Evolutionary Biology*, **6**: 381-394.
- Li, Z.J., Traore, A., Maximova, S & Gultian, M.J (1998).** Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using Thidiazuron. *In vitro cell Dev. Bio. Plant.* **34**: 293-299.
- Loach, K (1988).** Controlling environmental conditions to improve adventitious rooting. In: Davis, T.D., Haissig, B.E & Sankhla, N (Eds). Adventitious root Formation in Cuttings. Advances in plant science, Volume 2. Dioscorides press, Portland, Oregon. pp 248-272.
- Loveless, M.D & Hamrick J.L (1984).** Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics*, **15**: 65-95.
- Lynch, M & Milligan, B.G (1994).** Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, **3**: 91-99.
- Mabberly D.J. (1987).** The plant book. A portable dictionary of the higher plants: xii + 706 pp. Cambridge, Cambridge university press.
- MacArthur E & Wilson E.O (1967).** The theory of Island Biogeography. Princeton University Press, Princeton
- Machua, J., Muturi, G & Gicheru, J (2007).** Genetic Diversity within *Brachylaena huillensis* (O.Hoffim) populations in Kenya: Implications for

conservation of a wood carving species. *Discov. Innov*; **Vol 19** (RPSUD special edition, No.1). 32-36.

Martin, S.L., Jana, M., & Maxine, C. (1995). Micropropagation of Western White Pine (*Pinus monticola*) starting with mature embryos. *Plant cell. Rep.* **15**: 264-267.

Mbaratha, J.M. (1985). Conventional and non-conventional propagation of tree species: Development of appropriate methods for arid land tree propagation. Unpublished Msc thesis, University of Nairobi.

McCown B.H & Selmer J.C (1987). General media and vessels suitable for woody plant cultures. In: Bonga J.M & Durzan D.J (eds), Cell and Tissue Culture in Forestry. General principles and Biotechnology Vol 2. Martinus-Nijhoff, Dordrecht/Boston/Lancaster. Pp 231-255.

McNeely, J.A (1988). Economics and Biological Diversity: Developing and using Economic Incentives to Conserve Biological Resources. IUCN, Gland, Switzerland.

Mesen, F (1993). Vegetative propagation of Central American Hardwoods. Ph.D Thesis, University of Edinburgh, Institute of Terrestrial Ecology. Edinburgh, Scotland.

Middleton, W., Jarvis, B.C & Booth, A (1980). The role of leaves in auxin and boron dependent rooting of stem cuttings of *Phaseolus agrees*. *The New Phytologist* **84**: 251-259.

Millar, C.I (1987). Experiments in rooting bishop pine (*Pinus muricata* D. Don) cuttings. *New Forests* **1**(3): 231-238.

- Milthorpe, J & Milthorpe, F (1962).** Pre-sowing hardening of plants to drought. *Field crop abstracts*, **15**: 93.
- Minocha, R., Jain, S.M (2000).** Tissue culture of woody plants and its relevance to molecular biology. In: Jain S.M & Minocha, S.C (Eds). Molecular biology of woody plants. Dordrecht: Kluwer Academic Publishers. pp 315-339.
- Mok, C.M., Mok, W.S.D., Turner, J.E & Mujer, C.V (1987).** Biological and biochemical effects of cytokinins-active phenylurea derivatives in tissue culture systems. *J.Hort Sci.* **22**: 1194-1197.
- Mondal, T.K., Bhattacharya, A. Sood, A. & Ahuja, P.S (1998).** Micropropagation of tea (*Camellia sinensis* L) using thidiazuron. *Plant Growth reg.* **26**: 57-61
- Moritz, C (1995).** Use of molecular phylogenies for conservation. Philosophical Transactions of the Royal Society of London Series B: *Biological Sciences*, **349**: 113-118.
- Morse, L.E (1996).** Plant rarity and endangerment in North America. In: D.A Falk., Millar C.I & Ollwell, M. eds. Restoring diversity: Strategies for reintroduction of endangered plants, pp 7-22. Island press, Washington.
- Murashige, T & Skoog, F (1962).** A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 477-497.
- Murashige, T., Serpa, M., & Jones, J.B (1974).** Clonal multiplication of *Gerbera* through tissue culture. *Hortscience.* **9**: 175-80.

- Myers, N (1991).** A major extinction spasm: Predictable and inevitable? In: Western, D & Pearl, M (Eds), Conservation for the Twenty First Century. Oxford University Press, New York.
- Nanda, K.K., Purohit, A.N., Bala, A & Anand V.K (1968).** Seasonal rooting response of stem cuttings of some forest tree species to auxin. *Indian For.* **94**: 154-162.
- National Research Council [NRC] (1993).** Managing Global Genetic Resources: Forest Trees. National Academy Press, Washington, DC.
- Nei, M (1973).** Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences of the USA, 70: 3321-3323.
- Nei, M (1987)** Molecular Evolution Genetics, Columbia university press, New york, USA.
- Newton, A.C., Allnut T., Gillies, A.C M., Lowe, A & Enos, R.A (1999).** Molecular phylogeography, Intraspecific variation and the Conservation of tree species. *Trends in Ecology and Evolution*, **14** (4), 140-145.
- Newton, A.C., Muthoka, P. & Dick,J.Mcp. (1992).** The influence of leaf area on the rooting physiology of leafy stem cuttings of *Terminalia spinosa* Engl.*Trees-Structure and Function*, **6**, 210-215.
- Nilsson L.A., Rabakonandrianina E., Petterson B. & Ranaivo J. (1990).** “Ixoroid” secondary pollen presentation and pollination by small moths in the Malagasy treelet *Ixora platythyrsa* (Rubiaceae). *Pl. Syst. Evol.* **170**: 161-165.

- Njenga, P.K (1999).** *In vitro* propagation of the gum Arabic tree (*Acacia Senegal*) (L.) Willd. In Kenya. Unpublished Msc thesis, Kenyatta University, Nairobi.
- Noreen, R., Aslam, M., Jafar, M & Nazir, H (2001).** Callogenesis and Embryogenesis from leaf disks of *Ixora chilensis*. *International Journal of Agriculture and Biology*, **I**: 65-67.
- NRC (National Research Council) 1991.** Managing global genetic resources: Forest trees. National academy press, Washington, DC.
- Nunney, L & Campbell, K.A (1993).** Assessing minimum viable population sizes: demography meets population genetics. *Trends in Ecology and Evolution*, **8**: 234-239.
- O'Malley, D.M.& Bawa K.S (1987).** Mating system of a tropical rain forest tree species. *Am. j. Bot.* **74**: 1143-1149.
- Oldfield, S (1988).** Rare tropical timbers, IUCN. Gland, Switzerland & Cambridge, UK, pp48.
- Olfeit, J.P., Furnier G.R & Luby J.J (1998).** Reproduction and development of the endangered *sedum integrifolium* subsp *leedyi* (Crassulaceae). *American journal of Botany*. **83**: 346-351.
- Ornduff, R (1969).** Reproductive Biology in relation to systematics. *Taxon*, **18**: 121-133.

- Palmberg-Leche, C (1997).** Conservation and sustainable utilization of forest genetic resources. Presentation notes at COFO meeting 97/5. 10-13 March 1997, FAO, Rome, Italy.
- Park, Y.S., Barrett, J.D & Bonga, J.M (1998).** Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control and stability of cryopreserved clones. *In vitro cell. Dev. Bio. Plant*, **34**: 231-239.
- Perez-Naser, N.L.E Eguiarte & Pineiro D (1993).** Mating systems and Genetic structure of the distylous tropical tree *psychotria faxlucans* (Rubiaceae). *American Journal of Botany*.
- Pierik, R.L.M (1987).** *In vitro* culture of higher plants. Martinus Nijhoff, Dordrecht. pp 20-25.
- Pimm S.L (1979)** Complexity and stability: Another look at MacArthur's original hypothesis. *Oikos* **33**: 351-357.
- Pindyck, R.S & Rubinfeld, P.L (1981).** Econometric Models and Economic Forecasts. Second edition. McGraw-Hill New York
- Preece, J.E, Huettelman, C.A, Ashby, W.C & Roth, P.L (1991).** Micro and cutting propagation of Silver Maple. Results with adult and juvenile propagules. *J. Amer. Soc. Hort. Sci.* **116**: 142-148.
- Preston, E.R (1991).** The intrafloral phenology of *Sterptanthus tortuosus* (Brassicaceae). *Am. J. Bot* **787** (8): 1044-1053.
- Pulliam H.R (1988).** Sources, sinks and population regulation. *Am. Naturalist* **32**: 652-665.

- Ragati Forest Station Annual Report (1998).** Ragati Forest Station Annual Reports on the forest uses and returns. Unpublished report.
- Rajasekaran, P. (1994).** Production of clonal plantlets of *Grevillea robusta* in *in vitro* culture via axillary bud activation. *Plant cell, Tissue and Organ Culture*. **39**: 277-279.
- Ramesh, K & Padhya, M.A (1990).** *In vitro* propagation of neem, *Azadirachta indica* (A.Juss) from leaf discs. *Ind. J.Exp. Biol*, **28** (10) 932-935.
- Rathore T.S., Singh, R.P., Shekhawat N.S. (1991).** Clonal propagation of desert teak (*Tecomella undulata* through tissue culture. *Plant Sci*. **79**: 217-222.
- Raup D.M & Sepkowski J.J (1982).** Mass extinctions in the marine fossil record. *Science* **215**: 1528-1533.
- Reinert, J., Bajaj, Y.P.S & Zbell, B. (1977).** Aspects of organization:- Organogenesis, embryogenesis, cytodifferentiation. Plant tissue and cell culture. Blackwell Scientific publications, Oxford pp 389-427
- Richards A.J (1986).** Plant Breeding systems. Department of Biology, University of Newcastle upon Tyne. Chapman and Hall.
- Robbrecht E (1985).** Further observations on the pollen morphology of the South African genus *Carpacoce* (Rubiaceae – Anthospermae). *Rev. Palaeobot. Palynol.* **45**: 361-371.
- Robbrecht. E (1988).** Tropical woody Rubiaceae: Characteristic features and progressions. Contributions to a new subfamilial classification. *Opera Bot. Belg.* **1**: 1-271.

- Robbrecht, E (1994).** Advances in Rubiaceae macrosystematics. Introduction. *Opera Bot Belg.* 6: 7-8.
- Robbrecht, E (1996).** Geography of African Rubiaceae with reference to glacial rain forest refuges. In Van der Maesen (eds). The biodiversity of African plants. Dordrecht, Kluwer. Pp 564-581.
- Rockwood, D.L & Warrag, E.I (1994).** Field performance of micropropagated, macropropagated and seed derived propagules of three *Eucalyptus grandis* Ortets. *Plant Cell Reports*, 15: 628-631.
- Rosseto, M., Weaver, P.K., Dixon, K.W (1995).** Use of RAPD analysis in devising conservation strategies for the rare and endangered *Grevillea scapigera* (proteaceae). *Molecular Ecology*, 4: 321-329.
- Runo, M.S., Muluvi, G.M & Odee D.W (2004).** Analysis of genetic structure in *Melia volkensii* (Gurke.) populations using random amplified polymorphic DNA. *African Journal of Biotechnology* Vol. 3 (7).
- Russel, J.R., Hosein, F., Johnson, E., Waugh, R & Powel, W (1993).** Genetic differentiation of cocoa (*Theobroma cacao* L.) populations revealed by RAPD analysis. *Molecular Ecology*. 2: 89-87
- Rutledge, C.B & Douglas, G.C (1988).** Culture of meristem tips and micropropagation of 12 commercial clones of poplar *in vitro*. *Physiologia plantarum*, 72 (2): 367-373.
- Salvi, D.N., Singh, H., Tivarekah, S & Eapen, S (2001).** Plant regeneration from different explants of neem. *Plant cell. Tiss. & Organ Cult*, 65 (2): 159-162.

- Schaal, B.A., Leverich W.J & Rogstand S.H (1991).** A comparison of methods of assessing genetic variation in plant conservation biology. In: Genetics and conservation of rare plants (eds Falk D.A, Holsinger K.E) pp 62-71. Oxford university press, New york.
- Second, G (1982).** Origin of the genic diversity of cultivated rice (*Oryza* spp): study of the polymorphism scored at 40 isozyme loci. *Japanese Journal of Genetics*, **57**: 25-57.
- Sharma K.A. & Chatterjee T (1960).** Chromosome studies in *Ixora*. *Genetica*, **31**: 421-447.
- Shiembo, P.N., Newton,A.C & Leakey R.R B (1996).** Vegetative propagation of *Gnetum africanum*, a leafy vegetable from West Africa. *Journal of Horticultural Science*.
- Sita, L.G. (1986).** Choice of the explant and media for culturing sandalwood. In Biotechnology in Agriculture and Forestry. Trees I (ed. By Y.P.S Bajaj). Springer-Verlag, Berlin/Heidelberg pp 363-374.
- Smith A.C & Darwin S.P. (1988).** Family 168. Rubiaceae. *Flora Vitensis Nova*
- Snedecor, G.W & Cochran, W.G (1980).** Statistical Methods. 7th edition. Iowa State University Press, United States of America.
- Snow D.W (1981)** Tropical frugivorous birds and their food plants: a world survey. *Biotropica* **13**: 1-14
- Song, K.M., Osborn, T.C & William P.H (1990).** *Brassica* taxonomy based on nuclear restriction fragment length polymorphism (RFLP's). 3. Genome relationships in *Brassica* and related genera and the origin of *B.*

oleraceae and *B. rapa* (syn *campestris*). *Theoretical and applied Genetics*. **79**: 497-506.

Soule M.E (1986). Conservation Biology: Science of scarcity and diversity. Sinauer, Sunderland, Mass.

Sosef, M.S.M (1994). Refuge begonias. Taxonomy, phylogeny and historical biogeography of *Begonia* sect. *Loasibegonia* and sect. *Scutobegonia* in relation to glacial forest refuges in Africa. *Wagenigen Agric Univ papers*.**94 (1)**: 1-306.

Stebbins, G.L (1980). Rarity of Plant Species: A synthetic viewpoint. *Rhodora* **82**: 77-86.

Stewart, C.N & Porter D.M (1995). RAPD profiling in biological conservation and application to estimating clonal variation in rare and endangered *Iliamna* in Virginia. *Biological Conservation*, **74**: 135-142.

Surana, N.M (1982). Rapid multiplication of an elite of *Aegle marmelos* through tissue growth, differentiation and establishment in culture. In Arya H.C & Shekawat, N.S (ed.). Clonal multiplication of tree species in the Thar Desert through tissue culture. *Forest Ecology and Management*. **16**: 2012-208.

Tanksley, S.D & Orton, T.J (1983). Isozymes in Plant Genetics and Breeding. Elsevier, Amsterdam.

Tanno T. (1981) Plant utilization of the Mbuti pygmies. *African study Monographs* **1**: 1-53.

- Tchoundjeu, Z (1989).** Vegetative propagation of tropical hardwoods, *Khaya ivorensis* A. Chev and *Lovoa trichiloides* Harms. Ph.D thesis, University of Edinburgh, United Kingdom.
- Thomas, J.C & Katterman, F.R (1986).** Cytokinin activity induced by thidiazuron. *Plant physiol.* **81**: 681-683.
- Thompson, D.G (1992).** Current state of the art of rooting cuttings and a view to the future. In: Mass production technology for genetically improved fast growing forest tree species. Nangis.AFOCEL vol 2: 333-349.
- Tokuoka, M & Takeoka, M (1987).** Rooting of *Chamaecyparis obtusa* cuttings collected from upper and lower crowns. Bulletin of the Kyoto Prefectural University Forests **31**: 29-31.
- Vaishnawa, G.R (1983)** Clonal multiplication of *Tecoma undulate* through tissue culture. In Arya, H.C & Shekawat, N.S (ed). Clonal multiplication of tree species in the Thar desert through tissue culture. Forest ecology and management 16: 201-208.
- Van Valen, L (1973).** A new evolutionary law. Evolutionary Theory 1: 1-30.
- Vasil V & Vasil I., K (1980).** Isolation and culture of cereal protoplasts. Part 2: Embryogenesis and plantlets formation from protoplasts of *Pennisetum americanum*. *Theoretical applications Genetics.* **56**: 97-99.
- Vida, G (1994).** Global issues of genetic diversity. In: Loeschke, V., Tomiuk, J., Jain, S.K (Eds). Conservation Genetics. Birkhauser, Basel, Switzerland, pp 9-19.

- Vogel, S (1954).** In. De Block P (1998) Eds. The African species of *Ixora* (Rubiaceae – Pavetteae). National Botanic Garden of Belgium pp 1- 218.
- Von Teichman I. & Van Wyk A.E (1994)** Structural aspects and trends in the evolution of recalcitrant seeds in dicotyledons. *Seed Science Research* **4**: 225-239.
- Wang, Z.Y, Second, G., Transley, S.D (1992).** Polymorphism and phylogenetic relationships among species in the genus *Oryza* as determined by analysis of nuclear RFLPs. *Theoretical and Applied Genetics*, **83**: 565-581.
- Warrag, E.I., Lesney, M.S & Rockwood, D.L. (1990).** Micropropagation of field-tested superior *Eucalyptus grandis* hybrids. *New forests*. **4**: 67-79.
- Watt, M.P., Duncan, E.A., Ing, M., Blakeway, F.C & Herman, B (1995).** Field performance of micropropagated and macropropagated Eucalyptus Hybrids. Suid-Afrikaanse Basboutydskerif-nr. 173.
- Webster, C.A & Jones, O.P (1989).** Effects of sustained subculture on apparent rejuvenation of the apple rootstock M-9 *in vitro* and *in vivo*. *Annales des Foresterieres* **46**: 1875-1895.
- Weising, K., Nybom, H., Wolff, K & Meyer, W (1995).** DNA fingerprinting in plants and fungi. CRC press, Boca Raton.
- Welsh, J & McClelland (1990).** Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*. **18**: 7213-7218.
- Western, D & Pearl, M (1992).** Conservation for the Twenty First Century. Oxford University Press, New York.

- Wilde, J., Waugh, R & Powel, W (1992).** Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA markers. *Theoretical and Applied Genetics*, **83**: 871-877.
- Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V (1990).** DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**: 6531-6535.
- Williamson J. (1955).** Useful plants of Nyasaland: 168 pp. Zomba, Nyasaland, The Government printer.
- Wise, F.C., Blazich, F.A & Hinesley, L.E (1985).** Propagation of *Abie frasseri* by softwood stem cuttings. *Can.J.For.res.* **15**: 1172-1176.
- Yeh, F.C., Yang, R.C & Boyle T (1999).** Popgene version 1.31. University of Alberta, Canada.
- Yeo P.F. (1993)** Secondary pollen presentation. Form, function and evolution. *Pl. Syst. Evol*, Suppl. **6**: 1-268.
- Yeoman, M.M (1986).** Plant cell culture technology. Blackwell Scientific publications, Oxford.
- Yeoman, M.M. & Macleod, A.J (1977).** Tissue (Callus) culture techniques. *In plant tissue and cell culture* (ed. Street, H.E). Blackwell Scientific Publications, Oxford, pp 31-59.
- Zaer, J. B & Mapes, M. O. (1982).** Action of growth regulators. In: Bonga J.M & Durzan D.J (Eds). *Cell and Tissue Culture in Forestry*. Martinus-Nijhoff, Dordrecht/Boston/Lancaster.

APPENDICES

Appendix 1. Development of logistic regression model

The logistic regression model is applicable when we have a binomial observations of the form y_i/n_i , for $i=1,2,\dots,n$ where y_i is number of successes out of n_i Bernoulli trials. The expected value of the random variable associated with the observations y_i is given by $E(Y_i) = n_i p_i$ where p_i is the corresponding response probability and Y_i is the random variable of the y_i success. It then follows that the linear logistic model for the dependence of a p_i on the values x_1, x_2, \dots, x_k of k explanatory variable X_1, X_2, \dots, X_k is such that

$$\text{Logit}(P_i) = \log(p_i/1-p_i) = \beta_0 + \beta_1 x_{1i} + \dots + \beta_k x_{ki} \dots \dots \dots (1)$$

where β_0 is the constant parameter $\beta_1, \beta_2, \dots, \beta_k$ are the parameter coefficient of the corresponding explanatory variables (Collet, 2003, pp 58).

On some re-arrangement

$$p_i = \frac{\text{Exp}(\beta_0 + \beta_1 x_{1i} + \dots + \beta_k x_{ki})}{[1 + \text{exp}(\beta_0 + \beta_1 x_{1i} + \dots + \beta_k x_{ki})]} \dots \dots \dots (2)$$

On fitting the model, the unknown parameter coefficients of the explanatory variable have first to be estimated using the methods of maximum likelihood whose function is given by

$$L(\beta) = \log L(\beta) = \sum_{i=1}^n (n_i C_{y_i} p_i^{y_i} (1-p_i)^{n_i-y_i}) \dots \dots \dots (3)$$

Where \prod =Product and C = combination

This likelihood depends on the unknown success probability p_i , which In turn depends on the parameter coefficient of the explanatory variables. Maximizing the likelihood function and taking the derivatives with respect to β results to a set of $k+1$ unknown

equations equated to zero, which can be solved numerically providing the estimated β coefficients of the explanatory variables. At this stage the estimated coefficient do not directly indicate the effect of change in the corresponding explanatory variable on probability (p_i) of the outcome occurring, rather the coefficients reflects the effect of individual explanatory variable on its log of odds which can be either a positive or negative value. The positive coefficient means that the log of odds increases as the corresponding independent variable increases and vice versa. Consequently if the log of odds is positively (negatively) related to an independent variable both odds and probability p_i of the outcome are also positively (negatively) related to that variable). The only difference is that this relationship is linear for the log of odds and non linear for odds and probability of the outcome.

Following the general overview of the logistic regression model development, data generated from each experiment was modeled independently. For instance the effect of propagation media on rooting was analyzed by letting p_{ij} be the probability of a cutting rooting under i^{th} propagation media at time t_j for $i = 1,2,3\dots7$ and $j = 1, 2,..4$. Then the logistic regression model characterizing the rooting of *Ixora scheffleri* subsp *keniensis* shoot cutting and correctly predicting the category of outcome for individual cases is given by.

$$\text{Logit } (p_{ij}) = \log (p_{ij} / 1-p_{ij}) = \beta_0 + \beta_{1i} \text{Prom.}_{1i} + \beta_{2j} \text{Tim}_{2j} \dots\dots\dots$$

(4)

Where β_0 = constant parameter

β_{1i} = Coefficient parameters associated with various types of propagation media

Prom. = Propagation media

β_{2j} = parameter coefficient associated with time in weeks (week 5, 6, 7 and 8)

Tim= Time.

It then follows that all models fitted for hormonal concentration (IBA and NAA), seasonal periodicity, leaf area, position of cutting and cutting length applied the same procedure as in model 4.

Wald test statistic for testing the statistical significance of each coefficient (β) in the model was used. However, Menard (1995) warns that for large coefficient the standard errors are inflated lowering the Wald statistics (chi- square) value.

Consequently, R- squared (R^2) was used to measure percentage of deviance explained or accounted by the explanatory variables in the model.

Appendix 2. Summary ANOVA table for Mean rooting percentage in the different rooting media across the weeks.

Source of variation	d.f	M.s
Replication	4	192.23
Week	3	3031.90**
Growing media	6	6849.94**
Week. Growing media	18	37.32Ns
Residual	108	63.71
Total	139	

** Significant at $p < 0.01$, Ns Not significant at $p > 0.05$

NB: d.f = degrees of freedom. M.s = Mean sum of squares

Appendix 3. Summary ANOVA table for orthogonal contrasts between the weeks in various rooting media

Source of variation	d.f.	M.s
Replication	4	192.23
Weeks	3	3031.90***
WK6 VS WK7	1	1750.00***
WK6 VS WK8	1	2460.36***
WK7 VS WK8	1	60.36Ns
Growing media	6	6849.94***
FS VS G	1	122.50Ns
FS VS G: SD	1	90.00Ns
FS VS SD	1	225.63**
G VS G: SD	1	422.50*
Residual	108	63.71
Total	139	

*** Significant at ($p < 0.01$), ** significant at $p < 0.05$,

- Significant at $p < 0.1$, Ns not significant at ($p > 0.05$ and 0.1)

Appendix 4. Accumulated ANODE on number of cuttings that rooted across the weeks under IBA and NAA

Source of variation	d.f	Mean deviance
Replication	4	3.052
Week	3	18.616**
Hormone	1	168.828**
Concentration μg	6	171.557**
Hormone. Concentration μg	6	1.268**
Residual	224	0.283
Total	244	

** Significant at (p<0.01)

Appendix 5. Accumulated ANODE on effect of leaf area on rooting

Source of variation	d.f	Mean deviance
Week	3	6.0452**
Replication	4	4.7318
Leaf area	4	79.2415**
Residual	88	0.4308
Total	99	3.9590

** Significant at p<0.01

Appendix 6. Accumulated analysis of deviance (ANODE)

Source of variation	d.f	Mean deviance
Week	3	7.002**
Replication	4	8.566**
Cutting length	3	42.423**
Residual	69	0.455
Total	79	2.708

** Significant at ($p < 0.01$)

Appendix 7. Mechanical and physical properties of *Ixora scheffleri* subsp *keniensis* compared to *Cupressus lusitanica* and *Croton megalocarpus* (Measurements were done at KEFRI wood center, Karura forest, Kenya).

Species		<i>Ixora scheffleri</i>	<i>Cupressus lusitanica</i>	<i>Croton megalocarpus</i>
Static bending	MOE* (Nmm ²)	10994.67	6724.00	11243.00
	MOR* (Nmm ²)	86.31	54.24	83.77
Compressive strength (Nmm ²)		32.44	29.91	41.01
Shear strength (Nmm ²)	Tangential	11.11	8.22	11.43
	Radial	10.18	7.23	10.73
Hardiness (KN)	Tangential	4.79	2.47	6.05
	Radial	4.74	2.75	6.68
Moisture content percent		73.70	78.07	61.86
Density (Kgm ⁻³)		647.00	455.75	674.25

- MOR = Modulus of rupture.
- MOE = Modulus of elasticity

Definitions:

1. Modulus of rupture: Maximum surface stress in a bent beam of wood at the instant of failure.
2. Modulus of elasticity: Also called young's modulus. It indicates by how much the material will yield when subjected to a given force per unit area. The amount the material yields (elongation per unit length) is called strain and the force per unit area is called stress.
3. Shear strength: Maximum load required to shear a specimen in such a manner that the resulting pieces are completely clear of each other. The maximum compressive load (sustained by a specimen during a compression test) divided by the original cross sectional area.
4. Hardness: pressing diamond or a hardened steel ball into its surface measures the hardness of a material. The hardness is defined as the indenter force divided by the projected area of indent

Appendix 8. The GPS coordinates for the 29 individuals located in study area.

Individual	Elevation (asl)	Latitude	Longitude
1	2113m	S 00°21'28.6''	E 037 09'47.6''
2	2111m	S 00°21' 28.5''	E 037 09'48.4''
3	2120m	S 00° 21' 08.3''	E 037 09' 42.8''
4	2118m	S 00° 21' 07.7''	E 037 09' 42.2''
5	2121m	S 00° 21' 05.9''	E 037 09' 43.1''
6	2126m	S 00° 21' 01.7''	E 037 09' 48.8''
7	2131m	S 00° 21' 01.2''	E 037 09' 50.6''
8	2118m	S 00 °21' 08.3''	E 037 09' 41.8''
9	2111m	S 00° 21' 08.4''	E 037 09' 41.5''
10	2111m	S 00°21' 08.6''	E 037 09' 41.4''
11	2112m	S 00° 21' 08.9''	E 037 09' 41.6''
12	2121m	S 00° 21'12.3''	E 037 09' 42.3''
13	2120m	S 00° 21' 12.8''	E 037 09' 42.4''
14	2118m	S 00° 21' 16.0''	E 037 09' 39.4''
15	2115m	S 00° 21' 16.9''	E 037° 09' 39.8''
16	2114m	S 00° 21' 16.4''	E 037° 09' 40.4''
17	2113m	S 00°21' 16.4''	E 037° 09' 40.4''
18	2113m	S 00° 21' 16.1''	E 037° 09' 40.7''
19	2110m	S 00° 21' 16.9''	E 037° 09' 40.9''
20	2110m	S 00° 21' 17.3''	E 037 °09'41.0''
21	2109m	S 00° 21' 17.3''	E 037° 09' 41.4''
22	2105m	S 00° 21' 17.4''	E 037° 09' 41.9''
23	2102m	S 00 °21' 17.5''	E 037° 09' 42.3''.
24	2100m	S 00° 21' 17.3''	E 037° 09' 42.9''
25	2101m	S 00° 21' 17.1''	E 037° 09' 43.0''
26	2102m	S 00° 21' 16.7''	E 037° 09' 43.4''
27	2105m	S 00° 21' 16.2''	E 037° 09' 43.7''
28	2106m	S 00° 21' 15.6''	E 037°09' 44.3''
29	2106m	S 00° 21' 15.7''.	E 037° 09' 44.9''