SILVICULTURAL AND CONSERVATION TECHNIQUES FOR *Khaya grandifoliola* C. DC. IN SOUTHERN NIGERIA

BY

Anthony Ugochukwu OKERE
B.Sc. (Hons), M.Sc. Forest Biology and Silviculture (Ibadan)
Matric No:-63127

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ABSTRACT

Khaya grandifoliola, an important economic hardwood species, has been severely depleted by overexploitation. This necessitates its e*x-situ* conservation and requires in-depth knowledge of nursery handlings, seed storage, *in-vitro* and *ex-vitro* propagation on which information is sparse. Therefore, silvicultural requirements for conservation and sustainable use of *K. grandifoliola* in some parts of southern Nigeria were investigated in this study.

Seeds of *K. grandifoliola* were purposively sourced from forested areas of Cross River (Boki and Kutia), Ondo (Ayegunle-Akoko, Oba-Akoko and Owo) and Oyo [National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan] states and assessed for length, width and weight. Freshly collected (Control) seeds (n=100) were tested for viability using standard procedure. Seeds (n=100) from each of the sources were planted and 30 competitive seedlings were assessed for growth characters. Seeds (n=2000) were selected and 500 each were stored for 20 weeks at Ambient Room Temperature (ART: 28.0±2.0^{o}C), Short Term genebank (ShT: 24.0^{o}C), Freezer (FrZ: - 6.0^{o}C) and Long Term genebank (LgT: -17.0^{o}C). Monthly, seeds (n=100) were randomly selected from the four storage conditions and sowed in sterilised river sand for viability test. Single node cuttings treated with Indole Butyric Acid (IBA), Napthalene Acetic Acid (NAA) and Indole Acetic Acid (IAA) at (0, 25, 50, 150 and 200 mg/L) using sand, sawdust and 1:1 sand-sawdust were assessed for macropropagation. *In-vitro* culture of embryo on Murashige and Skoog (MS) medium + Benzyl Amino Purine (BAP) + NAA + Adenine Sulphate (ADS) was studied. Deoxyribonucleic Acid (DNA) samples were collected from juvenile leaves of *K. grandifoliola* from the 30 competitive seedlings and tested for molecular genetic diversity using six Random Amplified Polymorphic DNA (RAPD) (OPD-08, OPD-11, OPD-13, OPA-18, OPD-18, and OPD-20) primers. All experiments were laid out in completely randomised design. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

Seed sources significantly affected the seed weight (0.3±0.06g at Owo to 0.5±0.11g at NACGRAB). Seed viability ranged from 88.0% at Oba-Akoko to 98.0% at NACGRAB while seedling height was 21.7±8.27 cm at Owo and 29.9±5.30 cm at NACGRAB; the number of leaves was 30.8±6.51 at Boki and 43.2±12.78 at NACGRAB. After 20 weeks, only ShT seeds had 70.0% viability compared with 98.0% for control. The highest number of roots per cutting

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was observed in cuttings treated with 150mg/L IBA in sawdust. Embryo culture using MS + 0.05mg/L BAP + 0.1mg/L NAA + 10mg/L ADS gave the highest number of nodes (4) and root length (7.5 cm) while pure MS produced 2 nodes and 1.5 cm root length. The RAPD markers revealed genetic similarity among *K. grandifoliola* sources. Ninety-four scorable polymorphic bands were generated from the six primers selected for amplification. An average of 18.2 amplicon per primer was obtained giving amplification products of 4-27 with primer OPD-08 producing the highest.

Viability of *Khaya grandifoliola* seeds was highest at 24.0^{o}C within 20 weeks of storage, while macropropagation was best with stem cuttings treated with 150 mg/L Indole Butyric Acid in cured sawdust. The Random Amplified Polymorphic DNA markers were effective for its molecular characterisation.

Keywords: *Khaya grandifoliola*, Morphological traits, Seed storage, *Ex-situ* conservation, *In-vitro* propagation.

Word count: 500

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DEDICATION

To God the Creator

And

To all who love and conserve nature all over the world

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CERTIFICATION

I certify that this work was carried out by Anthony Ugochukwu OKERE in the Department of Forest Resources Management, University of Ibadan, Nigeria.

Supervisor
Adebola O. Adegeye
B.Sc. Agric. (Ife), M.Phil. (Leeds), Ph.D. (Ib.)

Reader, Department of Forest Resources Management
University of Ibadan, Nigeria.

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ACRONYMS

Å ....ångström
ADS ....Adenine Sulphate
AFLP.. ....Amplified Fragment Length Polymorphism
ART .... Ambient Room Temperature
BAP.. Benzyl Amino Purine
BB ....Binding Buffer
dATP.. deoxy-adenosine triphosphate
dCTP ....deoxy-cytidine triphosphate
dTTP .... deoxy-thymidine triphosphate
dGTP.. deoxy-guanosine triphosphate
DNA ....deoxyribonucleic acid
EDTA.. ethylenediamine tetra acetic acid
FrZ ....Freezer
GPS .... global positioning system
IBA.. Indole Butyric Acid
IAA ....Indole Acetic Acid
KIN .... kinetin
LCD ....liquid crystal display
LgT.. Long Term genebank
MS .... Murashige and Skoog
NAA ....Napthalene Acetic Acid
NACGRAB.. National Centre for Genetic Resources and Biotechnology
NIHORT .... National Horticultural Research Institute
PCR.. polymerise chain reaction
10-Mer ....primer
RAPD.. Random Amplified Polymorphic DNA
RFLP .... Random Fragment Length Polymorphism
RNASE .... Ribonuclease
Rpm.. revolutions per minute
S .... sand

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SD ....sawdust

SDS ....sodium dodecyl sulphate

ShT ....Short Term genebank

TE .... Tris – EDTA

UV .... Ultra violet

WB .... Wash Buffer

% ....percent

^oC ....degree celcius

µ .... micron

U ....units

w/v ....weight per volume

W .... Watts

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

The sub-Saharan Africa is endowed with tropical forests rich in valuable timber tree species, which are of immense benefits to the local people in meeting both their food and medicinal needs. The International Union for Conservation of Nature – IUCN (2009) red list of threatened species listed *Khaya grandifoliola* C.DC. as one of the endangered valuable timber species. Gbile (1998) also listed it as one of the medicinal plant species to be reckoned with. Khaya wood, African mahogany, as it is commercially called, is a high priced wood often used in furniture, cabinetry, paneling, veneer, office and shop fixtures, interior joinery, staircase banisters, handrails and domestic flooring because the wood usually dries well and rapidly with a beautiful sheen when polished (Timber Research and Development Association – TRADA, 2004). The timber is water-resistant and it is therefore used in ship-building.

K. grandifoliola is a medicinal timber species whose seed, leaves and bark are used extensively in West Africa for medicinal purpose. The very bitter bark has a considerable reputation in its natural range as a fever remedy. According to Abbiw (1990), the bark is used for treatment of fevers caused by malaria. A decoction of the bark is also taken for treating stomach problems including gastric ulcers, pain after birth and skin diseases. The bark is also used in traditional veterinary practice, for example for cattle suffering from liver fluke, for ulcers in camels, donkeys and horses, and in horses for internal ailments associated with mucous diarrhoea (Orwa *et al.,* 2009).

The preparations made from the roots have been used against syphilis, leprosy and as an aphrodisiac (World Agroforestry Centre, 2004). However, most of the *K. grandifoliola* exist in the wild state; the regeneration and long term conservation of these species are at the mercy of the vagaries of nature and the profit driven herb collectors and timber merchants. Gbadamosi (2002) reported that the 'wild syndrome' and common property status of forest resources is responsible for the near extinct exploitation of *Enantia chlorantha* Oliv. for malaria treatment in

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Nigeria. The ever-increasing demand for the prepared decoctions and alcoholic products obtained from the harvested stem bark of *K. grandifoliola* has actually endangered the plant species in Ghana (Ameyaw and Ampaw, 2005). In Nigeria, the upsurge in ethno-botanical studies and scientific research into the use of plant species has further enhanced the pressure on populations of medicinal forest species as more people now use plant parts for treating various body ailments. Annan (2000) reported that plant-based medicines provide more than 3 billion people with their primary health. Batta (2012) submitted that more than 70% of the Nigerian population depends on folk medicine for their health.

1.2 Statement of Problem

Seeds are of utmost importance in any reforestation activity and the success or otherwise of plantation establishment or afforestation projects critically depend on the availability of reproductive materials such as seeds, fruits or cuttings adapted to the plantation sites (Finkeldey *et al.,* 2010). Genetically, seeds are 'gene capsules' containing information to be expressed by the resulting seedlings in the next generation. Propagules of tropical tree species are known for their genetic variability due to the out crossing nature of these species (Ward *et al.*, 2005) therefore, the level of genetic erosion taking place in species whose seeds are consumed or used for other purposes is rather great. Traditionally genetic resources have been characterized on the basis of morphological and agronomic traits. Usually, comparisons are made on the growth of materials from different geographical origins in provenance and progeny. The effectiveness of this approach for estimating genetic diversity however has been questioned by several authors (Gottlieb 1977; Brown 1979). However, recently developed molecular techniques – DNA-based procedures for detecting genetic variation enable the extent of genetic differentiation between genera, species and populations to be quantified directly.

The seeds of *K. grandifoliola* are recalcitrant in nature and seed storage technique for this species conservation has not been well developed, coupled with the recent sharp global climatic change that has affected their flowering and fruiting patterns, availability of seeds for meaningful research and development work are ultimately impaired.

Usually timber contractors fell mature and immature stands of the species to feed the highly lucrative market for Khaya timber (Mahogany). On the other hand, herb collectors hinder the growth of the trees in the wild with extensive removal of the bark for sale. Regeneration,

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propagation as well as the improvement strategy is currently a challenge which poses great danger for the continued availability of the species. Even now, plantations of medicinal tree species such as *Azadiracta indica* A. Juss., *Enantia chlorantha* Oliv., *Rauwolfia vomitoria* Afz., *Picralima nitida* (Stapf.), *K. grandifoliola* and others with already established market structure have not been established whereas, exotic tree species used for timber, pulp and paper such as Teak, Gmelina and Pines continue to gain recognition more than the indigenous medicinal/timber tree species in the afforestation and reforestation programmes of both the Federal and State governments in Nigeria.

Vegetative propagation method which offers the tree geneticist the ability to replicate traits/genotypes of particular interest and also to overcome the problem of lack of seeds has not been fully developed for the species. It has been reported that vegetative propagation using leafy stem cuttings has been successful in African mahoganies such as *K. ivorensis* (A. Chev.), *K. anthotheca* (Welw.) C. DC., *Lovoa trichilioides* (Harms) (Opuni-Frimpong *et al.,* 2008), and *K. senegalensis* (A. Juss), (Danthu *et al.,* 2003). The results reported for *K. senegalensis* has been obtained in Thailand on juvenile shoot of hedged seedlings where the effects of hedge height, stump height, and plant growth regulators were evaluated (Ky-Dembele *et al.,* 2011). However, little or nothing has been documented on *K. grandifoliola* in this regards. Meanwhile, it has been hypothesised that, for a species for which little is known about the conditions for rooting, the most important factors to test and optimize are auxin concentration, leaf area, cutting length and node position (Tchoundjeu and Leakey, 1996).

Although plant regeneration from tissues cultured *in vitro* has been accomplished in a range of forest species (Thorpe *et.al.,* 1991), one of the mahoganies species which has been investigated in any detail, however, is *K. ivorensis* (Newton *et al.,* 1994). There is however, no report on successful *in vitro* plant regeneration of *K. grandifoliola.*

1.3.0 Objectives of the Study

1.3.1 Main Objective

The main objective of this research is to generate silvicultural requirement necessary for propagation as well as develop molecular technique for assessing variability with a view to improving the sustainable use and conservation of the species.

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1.3.2 Specific Objectives

i) To assess the genetic diversity in the natural populations of the species in some parts of Southern Nigeria using morphological and molecular techniques.

ii) To develop the seed storage protocol for the species.

iii) To develop the macro (vegetative) propagation protocol for the species and;

iv) To develop the micro (*In vitro)* propagation protocol for the species.

1.3.3 Justification of Study

According to Osemeobo (2005), medicinal timber species contribute to the Nigerian economy with average annual value of harvested wild plant products from the forest (including items consumed, sold, given out to neighbours, and damaged after harvest) per household at one million six hundred and fourteen thousand, one hundred and thirty three naira (₦1, 614,133.00), or US$11,956.54. Hoareau and DaSilva (1999) reported that medicinal plants also contribute to the world economy at large with an estimated market value of plant-based drugs put at US$ 2.5 billion. In fact two out of three people on earth according to Eloff (1998) use plants for primary health care. Therefore, ensuring the availability of these useful plant species through research will be addressing both the timber and health needs of more than half of the world population.

The study of genetic variation within the natural populations of *K. grandifoliola* will facilitate its improvement and conservation. Since variation is the mainstay of biological stability, species tends to adapt to changing environment in order to survive. Therefore, there is need to select elite ecotype that will adapt best to the new environment when establishing arboretum or plantations of *K. grandifoliola*. Owning to dearth of information on the use of deoxyribonucleic acid (DNA) molecular techniques on studying genetic variation and improvement of *K. grandifoliola*, it is therefore, necessary to research into DNA molecular techniques to detect genetic variation in its natural populations.

One of the cheapest and most convenient ways of preserving plant genetic resources is to store the seeds; however, Ng (1991) reported that there is not enough knowledge currently to make general recommendation for storing recalcitrant seeds for long periods. Therefore, there is the need to also document the optimum storage conditions for this species in terms of temperature and storage medium.

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The indiscriminate exploitation of the high priced *K. grandifoliola* for timber and herbal condiments together with the problem of unavailability of planting materials due to lack of seeds resulting from irregular fruiting pattern associated with most rain forest tree species and their difficult terrains, demands that effective and alternative propagation techniques for raising the species is developed to facilitate its multiplication, domestication and subsequent incorporation into our agro ecosystem. Vegetative propagation has proven to be an alternative means hence, juvenile single node stem cuttings were used to produce rooted cuttings as planting stock in this study. In addition, tissue culture has been ascertained to produce clones of true-to-type plantlets for rapid multiplication, therefore, embryo of *K. grandifoliola* seeds were subjected to *in vitro* culture with modified Murashige and Skoog media for rapid multiplication of planting materials for plantation establishment.

1.4 Scope of Study

The studies were carried out on seeds and seedlings *K. grandifoliola* collected from six different locations in its geographic range in some parts of Southern – Nigeria (Boki and Kutia in Cross River State; Oba-Akoko, Ayegunle-Akoko and Owo in Ondo State and National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan field genebank in Oyo State). Variations in seeds metric characters from six different sources in terms of weight, length and width were assessed. Effect of the six different sources on seed germination were examined while variation in resultant seedlings growth parameters such as height, collar diameter, height of branching, number of branches, internode length and number of leaves were investigated. Molecular marker techniques were used to isolate DNA from young leaves of the seedlings raised from the different collection sources for molecular characterization of the species. Effect of different storage conditions on viability of seeds of *K. grandifoliola* was investigated. The possibility of rapid multiplication of *K. grandifoliola* propagules via macro and micro propagation were equally assessed.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.5 Description and Botanical Features of *Khaya grandifoliola*

Khaya grandifoliola C. DC. occurs from Guinea to Sudan and Uganda and is found mostly in dry semi-deciduous forests and also in gallery forests (Poorter *et al*., 2004). It is a plant Species that belongs to the kingdom Plantae and Phylum Magnoliophyta; It is in the Class Magnoliopsida and Order Sapindales; it belongs to the Family of Meliaceae and Genus Khaya (Hawthorne, 1990; Hall and Swaine, 1981). Five species of this genus occur in Africa and four of them in West Africa. These are *Khaya anthoteca* (Welw.) C. Dc., *K. senegalensis* (Desv.) A. Juss, *K. ivoriensis* A. Chev. and *K. grandifoliola* C. DC. (Hutchinson and Dalziel, 1954). The West African species are commonly referred to as African mahogany. While the Igbos in Nigeria call the species 'ono', the Hausas and Yorubas call them 'madachi' and 'oganwo' respectively. *K. grandifoliola* is easily recognized by its fruit; round or mainly spherical woody capsules which are five-valved and dehiscent (Ibrahim *et al*., 2006).

K. grandifoliola is a deciduous tree attaining between 15 and 20 m tall (Hall and Swaine, 1981), although it may reach up to 40 m on fertile soils. The tree diameter is up to 2.8 m and with 8 -16 m clean bole while buttresses may be prominent or absent in some cases. The bark is dark grey while the slash is dark pink with red latex (Poorter *et al*., 2004). The heartwood is light pink-brown that darkens to reddish brown when cut. Leaves are compound, growing up to 20 cm long paripinnate, with 3-7 pairs of usually opposite leaflets. Each leaflet is 7-12 cm long, 3-5 cm wide, with a grey underside. The flowers are small, about 5 mm, with white petals; unisexual, but with well-developed vestiges of the opposite sex, making it difficult to distinguish between male and female flowers. The growth cycle has a typical rotation of 60 to 80 years while the tree begins to bear seed between 15 and 25 years. The tree fruits from March to July and sometimes later and the fruits apparently remain on the trees throughout the dry season. The fruits are upright, almost spherical, woody capsule and 4-10 cm in diameter with 5 valves that open at maturity. Inside the valves the seeds are arranged in rows with 6-18 seeds per valve. Seeds are brown, broad transversely ellipsoid to flat, about 2 x 2.5 cm with margins narrowly winged. Seeds may be dispersed up to 100 m by prevailing winds.

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2.6 Forest Conservation

Forests are generational heritage bequeathed to man. However, this versatile resource has consistently witnessed abuse. Henne and Thies (2001) reported that of the original forest cover of the world, half is gone and only one-fifth remains as large tracts of ancient forest – that is, forest ecosystems shaped mainly by nature where human impacts have been comparatively small.

Tropical forests are so important because they harbor at least 50 percent, and perhaps more, of the world's biodiversity. Direct observations reinforced by satellite data, documents that these forests are declining. The original extent of tropical rain forests was 15 million square km but it now remains about 7.5-8 million square km. The most recent Food and Agriculture Organization of the United Nations' (FAO) State of the World's Forest report in 2012 estimated world-wide deforestation between 2000 and 2010 at 130 million hectares (about 3.2 % of the total forest area in 2000). Forests in the tropics have suffered most of this devastative exploitation. Despite the pressure of economic development, which elicited the unrestrained exploitation of natural resources for foreign exchange earnings, these resources must be conserved for sustainability and posterity purposes.

Allanby (1993) defined conservation as the planning and management of resources so as to secure their wise use and continuity of supply while maintaining and possibly enhancing their quality, value and diversity. Elliott (1996) described conservation as the prudent use of nature's bounty, in opposition to the unrestrained forest exploitation. Wohlfahrt (1996) attempted to reconcile forest conservation and forest utilization and submitted that the relationship between the two terms is not one of contrasting interests but rather one of reciprocal dependence and support.

Forest resources can either be conserved *in situ* or *ex-situ* (Kio *et al.,* 1985; Ola-Adams, 2000). *In situ* conservation involves maintaining plants and animals in their original habitat and is highly desirable in cases of tree species where there is danger of over-exploitation and where the appropriate silvicultural or propagation techniques for their perpetuation are unknown (Ola-Adams, 2000). *In situ* conservation areas include Strict Nature Reserve, Games Reserve, National Park, Managed Nature Reserve, Botanical Gardens, Arboreta and Anthropogenic Reserves (Sacred Grove or Juju Shrine). *Ex-situ* conservation refers to maintaining organisms outside their original habitat in facilities such as botanical gardens, seed gene banks and field

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gene banks. *In vitro* conservation can either be for short or long duration (Engelmann**,** 1991)**.** For short period of conservation, it involves the storage of plants part in flasks or tubes in artificial media, under controlled environments, normally in sterile conditions. In this case, the aim is to reduce growth, thus increasing intervals between subcultures and it is normally under slow growth storage (SGS) i.e. in conditions of controlled light, temperature and growth retardant chemicals. Cryopreservation is the *in vitro* conservation of plant materials for long-term (LTS), usually for an indefinite period of time in a Liquid Nitrogen (LN) at -196°C.

2.3 Propagation Techniques

Propagation, either sexual or asexual, remains an integral part of a tree improvement and breeding programme either as a means of generating the stock for the study of morphological variations in progenies, or multiplication of planting stock. Quijada (1980) identified the basic difference between sexual and asexual propagation as the process of fertilization that occurs in the first but not in the second method.

Sexual propagation

This involves raising seedlings from seeds. Sexual propagation is a process by which plants reproduce, in which pollen from one plant fertilizes the ovary or ovaries from another, producing one or more seeds. The plants that grow from seed may not be exactly identical to the parent plant. Tompsett (1994) has listed the advantages and disadvantages of raising plants from seeds. Some of the advantages are:-

i. It is a cheap way of growing a large number of plants.
ii. Simple equipment and fewer facilities are required for its use.
iii. Seeds are easy to transport and store.
iv. The variation in the plants produced by seed allows plant breeders to develop new varieties.
v. Plants raised from this method are stronger and more stable and some plants can only be raised by seed, for example, annual plants. These plants grow, seed and die in one year. Examples include some vegetables (lettuce) and flowers (sunflower).

Some of the disadvantages are:-

i. It takes time to establish.
ii. Some seeds do not last well in storage – they may die.

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iii. Some plants do not produce seeds.

iv. The variation in the plants produced by seed might make it difficult to grow a uniform crop to sell.

v. Requires more work and care to handle.

2.3.1 Seed-storage and germination

A seed is an embryonic plant in a resting state, usually, though not always, supplied with food reserves in cotyledons, endosperm or perisperm (Roberts, 1972). Hartmann *et al*., 1990 defined seed as a ripened ovule consisting of an embryo and stored food supply, both of which are encased in a protective covering. However, Oni (1992) defined a seed as a fertilized ovule, essential to the survival of mankind. All these usually contained within protective structures consisting of the testa and possibly other structures formed in a variety of ways. Seeds are crucial to the success or otherwise of both natural and artificial regeneration and often they carry genetic information, which are expressed in growing plant. Seed is expensive, scarce and precious (Evans, 1992).

Two types of seeds have been identified. These are orthodox and recalcitrant seeds (Berjak *et al*., 1989; and Tompsett, 1994). Recalcitrant seeds according to Berjak *et al*. (1989) refer to seeds that undergo no maturation drying as the final phase of their development, tolerate very little post-shedding desiccation and are often chilling sensitive. In other words, recalcitrant seeds are very sensitive to drying and low storage temperatures. Such seeds are un-storable by any of the methods used for air-drying orthodox seeds.

Roberts (1973) based his classification on the physiological behavior of seeds, orthodox seeds according to him, were those seeds that could be dried to low moisture content (5%) and tolerated freezing temperatures while recalcitrant seeds could not be dried below relatively critical moisture content (30%) and could not tolerate freezing temperatures. Williams (1985) observed that recalcitrant seeds present special problems, particularly seeds of many rain-forest species which do not withstand either drying or temperature less than 10°C. Chin and Roberts (1980) noted that recalcitrant seeds could not be satisfactorily dried and then stored for long periods.

However, Tompsett (1987) observed that many recalcitrant species store best at 15-21°C, although he did not indicate for how long the seeds can be stored. Evans (1992) reported that recalcitrant seeds tend to lose viability very rapidly when dried to moisture contents below 35

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percent; hence, they require sustained high moisture content between ripening and germination. Seed moisture is a critical factor determining the viability and longevity of both recalcitrant and orthodox seeds (Chin *et al*., 1989).

Grabe (1989) observed that moisture content is intimately associated with all aspects of physiological seed quality. He noted that proven relationship exist between moisture content and seed maturity; optimum harvest time; longevity in storage; economies in artificial drying; injuries due to heat, frost, fumigation, insects and pathogens; mechanical damage; and seed weight. The survival of a seed in dry storage depends more upon its moisture content than on any other factor (Justice and Bass, 1978). Leopold and Vertucci (1989) reported that deteriorative reaction frequently proceed in the seed more readily if the moisture content is high, and consequently the moisture condition would constitute a threat to longevity of survival.

Furthermore, Hartmann *et al*. (1990) observed that the storage conditions that maintain seed viability are those that slow down respiration and other metabolic processes without injuring the embryo. These conditions are low moisture content of the seed, low storage temperature, and modification of the storage atmosphere. However, of these factors; the moisture-temperature relationships have the most practical significance.

Howland and Bowen (1977) have shown that *Triplochiton scleroxylon* K. Schum. fruits which have a reputation for poor storage can actually be stored for at least 2 years when dried steadily to a moisture content of 8 percent. Oni (1992) reported that seed sources, storage time and temperature had significant effects on the germination of *Terminalia ivorensis* A. Chev. seeds.

However, Chin *et al*. (1989) observed that there are still no rules for drying methods of recalcitrant seeds. UNESCO (1994) gave a two-fold objective of seed storage (i) to improve short-term storage for maintenance of viability during transport and temporary holding for use in species trials and plantation programmes, and (ii) to enable long-term genetic conservation in seed form. Whatever the purpose of seed storage, a good storage method will be that which can maintain seed viability for the longest time. Viability is defined as the capacity to survive or continue developing (Ditlevsen, 1980). This quality of seed (viability) is crucial for germination to occur.

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Germination of Seeds

The activation of metabolic machinery of the embryo leading to the emergence of a new seedling is known as germination (Hartmann *et al*., 1990). Desai *et al.,* (1997) defined germination as an emergence of embryo from the seed through a variety of anabolic and catabolic activities, including respiration, protein synthesis, and mobilization of food reserves after it has absorbed water. Many authors gave different stages for the germination process. Desai *et al.,* (1997) gave two viz: (i) biochemical preparative processes and (ii) emergence of embryo itself. Bewley and Black (1994) gave three overlapping stages of imbibition of water, digestion and translocation and seedling growth.

Ching (1972) listed the three conditions necessary for germination to be initiated; (i) the seed must be viable (ii) appropriate environmental conditions for seeds in terms of available water, proper temperature regime, a supply of oxygen and sometimes light; (iii) removal of any primary dormancy conditions present within the seed.

However, seeds of some species do not germinate even under favourable conditions until they have undergone a physical or physiological change. This state is called dormancy (Ditlevsen, 1980). Turnbull (1975) listed six types of dormancy (a) embryonic dormancy, (b) dormancy of the testa, (c) induced or secondary dormancy, (d) unripe embryo, (e) mechanical resistance of the testa, and (f) double dormancy.

Gbadamosi (1995) noted that dormancy is a potent force of nature in correlating germination with the prevailing environmental condition. He observed that seeds that would have germinated and the resulting seedlings exposed to the harsh and stressful conditions characteristic of dry weather when fruit/seed falls, are protected until the rainy season. However, dormancy often results in delayed and irregular germination in the nursery or forest floors.

Hessou (1984) identified 3 methods of hastening seed germination useful in some tropical tree species viz.:

i. Scarification (mechanical and acid);
ii. Soaking in water (hot, warm or fresh water); and
iii. Low temperature storage.

The type of acid and the time of soaking can affect seed viability and consequently its ability to germinate. Gbadamosi (1995) reported that soaking of seeds of *Xylopia aethiopica* (Dunal) A. Rich in concentrated acid for 2 minutes gave 0 % germination whereas the untreated seeds had 12% germination. The removal of pulp and/or seed coat affects germination in some seeds.

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Okafor (1981); Oni and Gbadamosi (1998) observed that depulping of *Dacryodes edulis* (G. Don) H.J. Lam, seeds before sowing hastened germination.

Asexual Propagation

Two forms of asexual propagation have been identified (Quijada, 1980), these are apomixis and vegetative propagation. Apomixis is propagation through the development of one of the gametes (the ovule) without fertilization, or a cell without reduction, to form seeds or seed-like organs. Vegetative propagation is regeneration from well-differentiated vegetative parts.

2.3.2 Vegetative propagation

Evans (1992) defined vegetative propagation as a way of raising planting stock without involving seed and this includes cuttings, layering, budding, grafting and micro propagation. Vegetative reproduction according to Yanchuk (2001) comprises a broad range of techniques involving the manipulation of plant tissue samples such as sections of stems, leaves, roots, seeds or even cell cultures which ultimately allows for complete vegetative "repropagation" of the whole plant. Vegetative propagation plays an important role in the genetic improvement of timber trees (Gupta, 1988). Leakey and Simons (1998) and Mudge and Brennan (1999) noted that the development of vegetative propagation techniques represents the first step in the process of domestication of a tree species.

Steward and Krikorian (1978) indicated that vegetative propagation is possible because living cells contain genetic information in their nuclei necessary to reproduce the entire plant. This property is called totipotency.

Different methods of vegetative propagation have been identified. Quijada (1980) listed cuttings, layering and grafting; Howland and Bowen (1977) itemized grafting and stem cutting; Garner, 1967 marcotting; Evans (1992) included stump plant, micropropagation using tissue culture; UNESCO (1994) recognized budding, air layering, grafting, leafy stem cuttings and tissue culture. Rooting media, auxin concentration, and leaf area of cuttings influenced rooting ability of juvenile stem cutting (Leakey *et al.,* 1994; Mudge and Brennan, 1999). Rao (1988) reported that as far as vegetative propagation is concerned, the shoots of most of the tropical trees may not root at all, since the tissues are heavily deposited with tannin and other phenolic compounds.

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Bonga (1982) outlined the advantages of vegetative propagation over sexual propagation in large-scale reforestation programmes. These are:

- Cloning of superior trees, resulting in similar superior genetic characteristics.
- Reduction in the unusually long breeding cycles of trees and improvement of planting stock through cloning.
- The genetic uniformity of a clone is generally an asset.
- In some trees, cloned propagules initially grow much faster than seedlings;
- Sometimes, the juvenile phase of development can, if desired, be by-passed; and
- Some valuable tree species e.g. hybrids and polyploids are infertile and can only be propagated vegetatively.

Furthermore, field comparison of both vegetatively and sexually propagated plants with the same genetic content exhibited differential growth rate. For example, Libby and Bolsted (1982); Matheson and Eldridge (1982) observed that cuttings take advantage over time, after a slower initial growth, the cuttings outgrow the seedlings and develop a better form and greater resistance to certain parasites.

UNESCO (1994) observed that vegetative propagation gives the tree breeder the ability to multiply, test, select from and utilize the large genetic diversity present in most tree species. Vegetative propagation is also advantageous for transportation of planting stock. Oporto and Garcia (1999) reported that the transport of properly rooted materials is a better alternative to the shipment of recalcitrant seeds, especially in the absence of refrigerated facilities.

Vegetative propagation techniques have now been successfully applied to many species. Okafor and Lamb (1994) listed 26 species of trees producing edible products, which were raised through budding. Tchoundjeu (1989) raised *Khaya ivorensis* and *Lovoa trichiloides* from stem cuttings of different leaf sizes. Quijada (1980) observed that cuttings put to root in an appropriate medium are the most used method for breeding purposes.

2.3.3 Micropropagation

Advances in biological sciences have opened up an array of options in reproducing and conserving natural resources, one of these is ‘biotechnology’. Yanchuk (2001) noted that biotechnologies used in forestry fall into three main areas: the use of vegetative reproduction methods; the use of molecular genetic markers; and the production of genetically modified organisms (GMOs) or transgenic trees. While forest researchers in Nigeria have used the first

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method extensively, the last two methods have not gained appreciable usage possibly due to the absence of necessary facilities and technical knowhow.

Micropropagation is a sub-set in the wide area of biotechnology. Debergh and Read (1991) defined micropropagation as the true-to-type propagation of a selected genotype using *in vitro* culture techniques. Yanchuk (2001) opined that micropropagation is the development of clonal lines from small tissue samples such as buds, roots and embryos extracted from seeds. More than 60 different media or the combination of media have been used for the *in vitro* propagation of tree species, of which Murashige and Skoog (MS) (1962) medium is the most common (Bonga, 1980; Skirvin *et al.,*1980). Rao (1988) reported however, that there is no single combination of growth substances that works well for all trees and this emphasizes the fact that the biochemical nature of tree tissues and their growth responses to growth substances vary considerably. Rao and Lee (1986) observed that there are many cases where there is hardly any tissue growth irrespective of the kind or quantity of substances used.

Generally, the successful growth of tree tissues or explant *in vitro* is inversely proportional to (a) the age of the mother tree; (b) the position and growth stage of the bud and (c) topophysis which includes juvenility and other abnormal features connected with tissue growth (Rao, 1988).

Philip *et al.,* (1995) indicated that the propagation of selected plant lines through tissue culture is called micropropagation. Jona (1987) reported that the simplest application of tissue culture is rooting a cutting- a structure which was originally devised to bear flowers, fruits and leaves is abruptly induced by cutting to differentiate and bear roots, though originally it was not expected to do so. Generally, micropropagation involves four steps**:** initiation of aseptic cultures; shoot multiplication; rooting of shoots formed in vitro; and transfer of the plantlets into soil (Beelen, 1990). Jona (1987) listed four explant used in tissue culture (i) meristems culture (ii) callus (iii) production of haploids and (iv) protoplasts.

Meristem culture according to (Beelen, 1990) is shoot tip culture. The apical meristem is that part of a shoot distal to the youngest leaf primodium. Meristem culture enables the production of virus-free plants; this is possible because the virus titre increase with increasing distance from meristem tips, which are in general free of virus. Callus consists of a growth of newly developed undifferentiated cells, mostly proliferating from cut tissues at the cut surfaces. Growth regulators can stimulate these. Callus development is affected by the size of the explant, the type of culture, and the polarity of the explant on the medium.

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Haploid plants have a single set of chromosomes and are important in studies on the induction of mutations and also for the production of homozygous plants. Haploid plants are produced by distant hybridization, delayed pollination, application of irradiated pollen, hormone treatments, temperature shocks and culturing of excised anthers.

Protoplasts are plant cells without cell walls (Pierik, 1987). These can be isolated by treating plant tissues, often leaf mesophyll tissue, with a mixture of cell-wall-degrading enzymes in solution, which contain osmotic stabilizers to preserve the structure and viability of the protoplasts (Beelen, 1990).

FAO (1994) reported that the application of micropropagation methods in forest tree improvement permits rapid multiplication of tree species to meet market demand, and in some cases to overcome difficulties in alternative methods such as propagation by cutting. Drew (1993) reported that in pineapple, the release of new varieties typically took 25 years with traditional multiplication procedures, but can be accomplished in one year using micropropagation.

Over 1000 plant species have been micropropagated, including over 100 forest tree species (Bajaj, 1991). Thorpe *et al.,* (1991) listed over 70 angiosperm and 30 gymnosperm tree species for which successful methods for the production of plantlets have been reported while Le Roux and van Staden (1991) listed over 25 species of *Eucalyptus*. Newton *et al.,* (1994) reported a successful micropropagation of some mahogany species (*Cedrela odorata* L.*, Swietania macrophylla* King and *Khaya ivorensis*). Nevertheless, one of the major problems encountered in micropropagation was the initiation of a proliferating culture, owing to variability in the explants (UNESCO, 1994).

2.3.4 Genetic Variation and Tree Improvement

UNESCO (1994) noted that within each species there exists a breadth of variation that has been created and partitioned by evolutionary forces. Variations within the same population of a species have their origin in mutation (Allard, 1960). Basically, three types of variation may be distinguished (Styles, 1976). These are: a component which is fixed, heritable and which is genetic; an environmentally induced, non-heritable component; and a part that is developmental.

Simons (1996) admitted that trees are predominantly out crossing; this results in progeny that segregate with respect to parental traits. This wide genetic variation not only provides buffering against differing environments and management practices but also affords the

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opportunity for selection. Guries (1982) emphasized this viewpoint that variation is a key to long-term species survival and continued evolution. The variation between the individuals of any population is based on three factors (Vavilov, 1951 and Harten, 1988): Environmental modification, Genetic Recombination and Mutation.

Dangasuk *et al*. (1997) reported the existence of a consistent variation in seed length, seed width and seed weight among and within 12 African provenances of *Faidherbia albida* (Del.) A. Chev. He also noted positive correlations in some of the seed characteristics and seedling height among provenances. Likewise, Oni and Gbadamosi (1998) noted a significant variation in all quantitative characters of fruits as well as early growth characters of seedlings in *Dacryodes edulis* from four sources in Nigeria. Gbadamosi (2005) observed marked differences in morphological traits of seeds and seedlings respectively in *Enantia chlorantha* from four provenances in Southwestern Nigeria. Variation is the basic genetic resource information available for domestication which may be improved by selection and breeding. Plant improvement programmes primarily depend on the extent of this genetic variability in the base population of species (Bajaj, 1986; Borojevic, 1990).

Tree improvement harnesses the variation in natural populations of tree species and utilizes this in the enhancement of tree performance in a desired trait. Kieding (1976) defined tree improvement as all activities based on utilizing the genetic potentials of a species. Genetic improvement of metric traits for plant species has relied mainly, if not exclusively, on changes in frequencies for genes with independent and additive effects (Foster and Shaw, 1988). This is so because selection response for traits conditioned by genes with additive effect is predictable, and cumulative gains over generations are easily obtained through a variety of selection and breeding programmes (Foster and Shaw, 1988).

Hanies (1994) observed that the general objectives of a genetic improvement programme is the sustainable management of variation to produce, identify and multiply for the operational planting of well-adapted genotype of the desired quality.

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2.3.5 Molecular Techniques

Traditionally genetic resources have been characterized on the basis of morphological and agronomic traits. In the past, Foresters have greatly relied on morphological characteristics to evaluate genetic variation. The effectiveness of this approach for estimating genetic diversity, however, has been questioned by several authors (Gottlieb, 1977; Brown, 1979). With the advent of molecular marker techniques, DNA based procedures for detecting genetic variation has been proposed to be a better tool in the evaluation of genetic variations and in the elucidation of genetic relationships within and among species (Song *et al.,* 2003). They include restriction fragment length polymorphisms (RFLPs) which have the potential to detect almost unlimited amount of variation. Although chloroplast DNA (Palmer *et.al.,* 1988) and Nuclear RFLPs (Debener *et al.,* 1990) have been used for taxonomic studies, the usefulness of these markers is limited by the fact that they are costly, time consuming and technically demanding.

DNA markers precisely characterize cultivars, provenances or genotypes and measure their genetic relationships. The markers are highly heritable, environmentally stable and exhibit sufficient polymorphism to discriminate very closely related genotypes (Narayanan *et al*., 2007). Both RAPD and ISSR markers are rapid and economical and are extensively used for diversity analysis, mapping and genotype identification of plant species including forest trees (Karp *et al.,* 1998). RAPD (random amplified polymorphic DNA) markers involving PCR (polymerase chain reaction) amplification of genomic DNA with random decamer primers are fast, simple and most effective tools for clone management (Takata and Shiraishi 1996, Watanabe *et al*. 2004). They make precise distinction among clones, varieties and cultivars of many forest trees (Goto *et al.,*1999, Tessier *et al.,* 1999) and have been successfully applied in a range of phylogenetic, taxonomic, and genetic diversity studies (Hammad and Qari, 2010). The RAPD method overcomes many of the limitation of RFLP and has been used for clone identification in cocoa and banana (Wild *et.al.,* 1992), population differentiation in *Gliricidia spp* (Chalmers *et.al.,* 1992), genetic maping in conifers (Carlson *et.al.,* 1991; Roy *et.al.,* 1992). RAPDs have also been applied for the first time to mahoganies in a preliminary investigation to test the applicability of the techniques to these species. A number of different genera were compared, including Khaya, Swietenia and Cedrela *spp.*, and pronounced polymorphisms were detected at the genus level. On the other hand, ISSR (inter simple sequence repeats) markers use short microsatellite motif containing primers anchored at the 3' or 5' end by two to four arbitrary degenerate nucleotides to amplify the DNA sequence lying between two microsatellite regions (Zietkiewicz *et al.,*1994).

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ISSR markers have been successfully used for varietal identification and assessment of genetic relationships in many plant species (Ajibade *et al.,* 2000).

Assessment of genetic variability within a germplasm is of interest for practical applications such as the conservation of genetic resources and for breeding purposes, to predict the ability to combine or to rapidly verify the breeding material. The use of molecular techniques in genetic diversity studies is supported by the finding that evolutionary forces such as natural selection and genetic drift produce divergent phylogenetic branching which can be recognized because the molecular sequences on which they are based share a common ancestor.

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Reconnaissance survey

A reconnaissance survey of the geographical distribution of the species was carried out in the South Western and South Southern part of Nigeria based on the information from Forest Herbarium Ibadan, (FHI) on preliminary survey of the endemic areas of *K. grandifoliola* to ascertain the phenology and the appropriate period for seed collection. Six different sources were selected, Boki and Kutia (Cross River State), Oba-Akoko Ayegunle-Akoko and Owo (Ondo State) and NACGRAB, Ibadan (Oyo State). The selection of the sources used for this study was prompted by the large availability of matured seeds in the same period at the time of seed collection.

Location of Experiments

The experiments on the silvicultural requirements of *K. grandifoliola* were carried out in the nursery, genebank and screen house at the National Centre for Genetic Resources and Biotechnology (NACGRAB), while those on micropropagation and molecular studies were carried out in the Tissue culture and Molecular laboratory, all at NACGRAB, Moor Plantation, Ibadan, Nigeria.

3.2 Description of seed sources

Seeds from mature fruit (Plate 3.1) of *K. grandifoliola* used in this study were collected from six sources along its natural spread in Nigeria. Fig 3.1 shows the location of seed sources in Nigeria.

3.2.1 Boki

Boki local government area is a geographical territory in Cross River State of Nigeria. It is one of the eighteen local government areas of Cross River State and the second largest in terms of landmass (344,952 km^2). It has a population of about 300,000 and it is located between latitude 6°23' N and Longitude 8°45' E. Boki is situated at the southern end of the eastern highlands, a chain of hills along the eastern Nigeria border with Cameroun. The area is bounded to the North by Obudu and Obanliku local government area, to the south by Ikom, to the west by

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Plate 3.1: Mature fruit of *Khaya grandifoliola*

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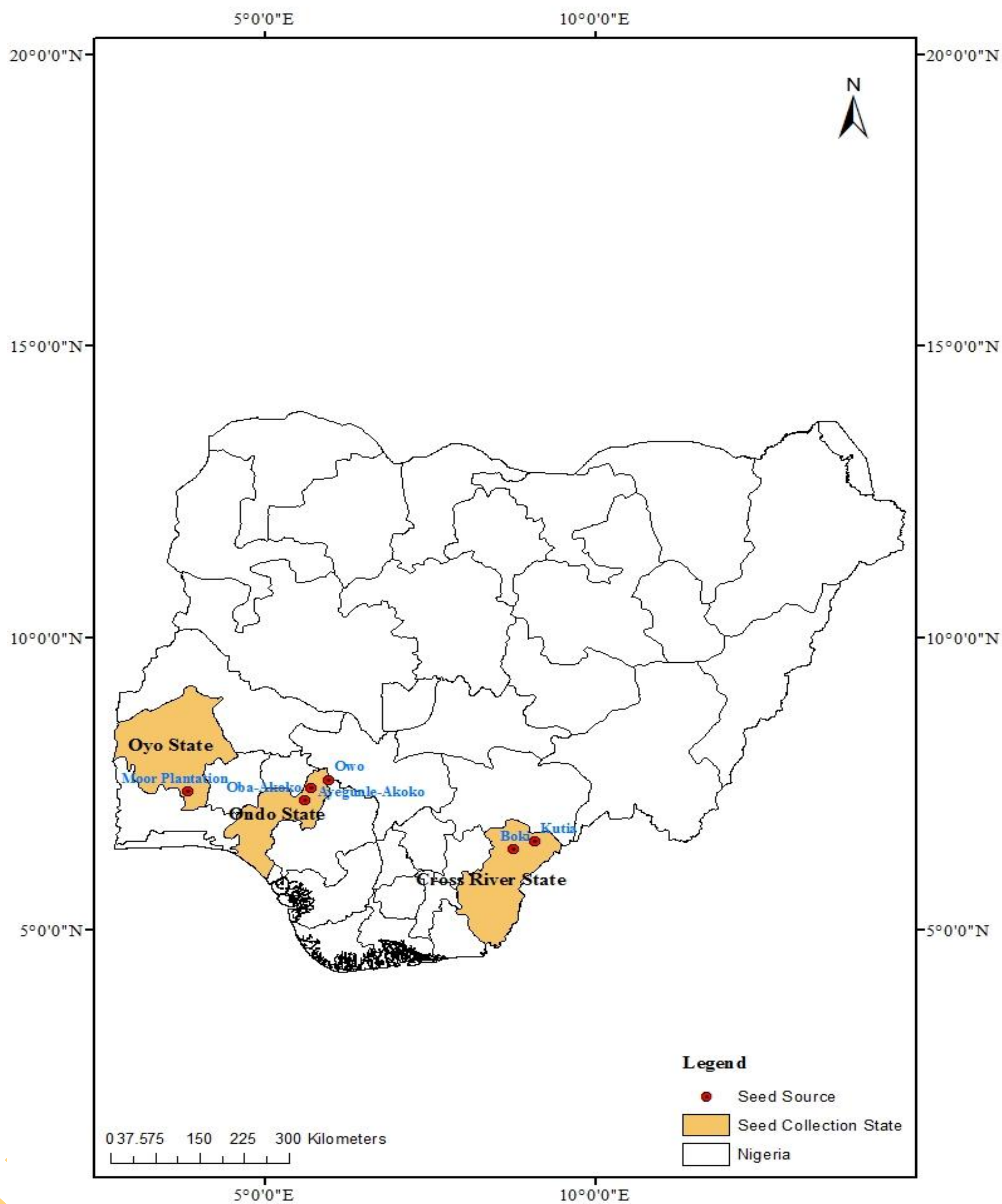

Fig 3.1: Map of Nigeria showing the sources of *Khaya grandifoliola* seeds used for the study.

Ogoja and to the East by Cameroun Republic and is known internationally as a commercial centre for agricultural commodities such as cocoa, coffee, timber, and palm products. The

elevation ranges from 150 m to 1000 m above sea level. The most notable are the Boje and Nsadop hills, Erruan Mountains and Mbe Mountains. Boki is well drained with many streams and rivers such as Afi, Okorn and Aren rivers. Minor streams exist across the entire area that constitutes the tributaries of the main rivers. Boki has a tropical climate typified with distinct wet and dry seasons. It has a mean annual temperature of 25°C and annual rainfall of between 2000 m to 3500 mm. The rainfall is of double maxima regime (July and September). The above climatic conditions and rich soils derived from cretaceous and tertiary shale and sand stone produced a Luxuriant vegetation.

Source: http://kekerete.tripod.com/CRSG/boki.html.

3.2.2 Kutia

GPS information indicates that Kutia lies along latitude 6° 31' N and Longitude 9° 5' E and is one of the major communities in Obudu local government area of Cross River state, Nigeria. Kutia climatic conditions are conducive for the cultivation of a large variety of agricultural products. The soil is loamy sand mixed with volcanic ash. Obudu LGA covers an area of 379,164 square kilometres and to the west, it shares a boundary with Bekwarra/Ogoja local Government Areas, to the east with Obanliku Local Government Area, to the South with Boki Local Government Area (all in Cross River State) and to the North with Benue State. Kutia enjoys the influence of the North easterly wind during the dry season and south westerly wind during the wet season with annual mean temperature of 23°C and mean annual rainfall of about 1300 – 2000 mm. The main vegetation characteristic of the area is guinea savannah. The population of Obudu, according to the 2006 census figures was 161,457.

Source: http://dx.doi.org/10.4236/aa.2013.34027

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3.2.3 Oba Akoko

Global Positioning System (GPS) information device indicates that Oba-Akoko is located between Latitude 7° 13' N and Longitude 5° 36' E. Oba Akoko has annual mean temperature of 31.96^{o}C and mean annual rainfall of about 1100 – 1686 mm. It is one of the 40 small Akoko towns, predominantly situated in rocky areas of Ondo state with a tropical rain forest vegetation, highly laterized soil and elevation of 304.7m above the sea level. It is located in Akoko south-west LGA with its headquarters in Oka. Akoko is a large Northeast Yoruba settlement in Yoruba land, the area spans from Ondo state to Edo state in southwest Nigeria. It has an area of 226 km² and a population of 229,486 at the 2006 census.

Source: http://en.wikipedia.org/wiki/Akoko_South-West; Ajayi *et.al.,* 2012.

3.2.4 Ayegunle Akoko

Ayegunle Akoko is located between Latitude 7° 26' N and Longitude 5° 42' E. It is also one of the small Akoko towns in Ondo state south-westhern Nigeria with annual mean temperature of 31.59^{o}C and mean annual rainfall of about 1800 – 2000 mm. The soil type is laterized with a tropical rain forest vegetation.

Source: http://en.wikipedia.org/wiki/Akoko_South-West; Afolayan and Aaderounmu, 2012

3.2.5 Owo

It is a city in the Ondo state south-western Nigeria. Owo is situated at the southern edge of the Yoruba Hills, and at the intersection of roads from Akure, Kabba and Benin City. It is located within the tropical savannah belt of Nigeria and it has a high topography of about 1,200ft above the sea level. It is located between Latitude 7° 34' N and Longitude 5° 57' E. The area has two basic climates: the rainy season which ranges between March and September and dry season between October and February with mean annual rainfall of 1579.283mm, with temperature range of 12.8^{o}C and 42.7^{o}C.

Source: http://en.wikipedia.org/wiki/Owo,_Nigeria; Anifowose A.Y.B. and Adewumi A.J. 2014

3.2.6 NACGRAB (Ibadan)

Ibadan is the capital city of Oyo state and it lies in between latitude 7° 22'N and longitude 3° 50'E. It has a tropical climate typified with distinct wet and dry seasons. The rainfall distribution is bimodal between June, July, August and September, while the dry season is from the end of October to early March. The mean minimum and maximum temperature ranges

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between 23°C to 33°C respectively while the annual rainfall ranges between 2,150 and 2,600 mm. It is situated in the rain forest vegetation with ferric luvisols soil type.

Source: Geographical information system unit of Forestry Research Institute of Nigeria, Ibadan.

3.3 Morphological variation in seeds of *K. grandifoliola*

Due to the outcrossing nature of tropical forest trees, thirty seeds (Plate 3.2) of *K. grandifoliola* were randomly selected from each of the six sources as genetic representatives of each source. The following parameters were measured: length, width and weight. Length and width were measured with a ruler while electronic weighing balance (Model BL-410S, manufactured by Setra, USA) was used to measure the weight of selected seeds in the laboratory (Plate 3.3).

The data collected per seed source were subjected to one-way analysis of variance (ANOVA) to investigate the significance of variability in morphological traits of seeds from the six different sources while the least significant difference was used to separate the significantly different means at $P < 0.05$.

3.4 Germination of seeds of *K. grandifoliola*

From each seed source, one hundred (100) pure seeds (fully formed seeds, that is whole, intact without visible damages from mechanical, insects or diseases – ISTA, 1999) were selected and sown in well-labeled germination trays filled with washed river sand laid out in one of the screen houses at NACGRAB (Plate 3.4). Watering to saturation point of the sown seeds was done daily in the morning using a fine meshed watering can and germination count was also taken daily. Germination was taken to have occurred when the plumule emerged and can be seen above the soil in the germinating tray. Germination was taken to have completed when no additional germination takes place in a week. Data collected were analysed using descriptive statistics.

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Plate 3.2: Seeds of *Khaya grandifoliola*

Plate 3.3: Weighing of *Khaya grandifoliola* seeds

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Plate 3.4: Sowing of *K. grandifoliola* seeds in one of the screen houses at NACGRAB

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3.5 Morphological variation in seedlings of *K. grandifoliola*

At the two-leaf stage (6 weeks from the day of sowing), 30 uniformly growing seedlings from each seed source were picked out of the germination tray and transplanted into medium sized polythene bags (measuring 16cm x 14cm x 12cm) filled with topsoil and arranged under shade to wean for two weeks (Plate 3.5). Thereafter, the seedlings were laid out in an open nursery in three replicates of ten seedlings each in a complete randomized design (Plate 3.6). Additional five seedlings were also transplanted from each seed source as buffer and watering was done to field capacity once daily in the morning.

The following growth traits; seedling height, collar diameter, height of branching, number of branches, internodes length and number of leaves per seedling were measured fortnightly for forty weeks. Total height of the seedling, height of branching and internodes' length were measured using a ruler. The total height was taken from the collar to the top of the apical bud while the height of branching was measured from the collar to the point of first branch. A digital venier caliper was used to measure seedlings' stem collar diameter. The number of branches and leaves were also counted. The data collected was subjected to one way ANOVA to investigate the significance of variability in morphological characteristics of the seedlings from the six different sources.

Morphological characterization was carried out after 40 weeks by subjecting the data collected from the phenotypic traits like height, collar diameter, height of branching, number of branches, internodes length and number of leaves per seedlings from the six sources to Jaccard's similarity coefficient. Estimates of genetic similarity were calculated according to Jaccard's coefficient (Jaccard, 1908). A cluster analysis, using an unweighted pair group method with arithmetic averages (UPGMA) was performed. The analysis was plotted in the form of a dendrogram. All computations were carried out using the NTSYS-Pc, Version 2.02 package (Rohlf, 1993).

Biomass assessment of the seedlings was also carried out at two weeks interval for a period of twelve weeks (6 harvests). The seedlings were allowed a period of 3 months to establish and accumulate biomass before the assessment.

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Plate 3.5: Seedlings of *K. grandifoliola* in the nursery of NACGRAB, Ibadan.

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Plate 3.6: Experimental seedlings of *K. grandifoliola* arranged in a Complete Randomised Design in the nursery of NACGRAB, Ibadan.

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On each occasion, three potted seedlings were randomly selected from each seed source; placed in water each seedling was uprooted by carefully washing off the soil materials. The uprooted seedlings were then divided into root and shoot components. The leaf area meter machine (*Systronics Model* No: *211,* Made in India) of the National Horticultural Research Institute (NIHORT) Ibadan was used in determining the leaf area. The root and shoot portions of each seedling were put into separate envelopes of known weight and labelled adequately for biomass measurement. The fresh weight of the various components was determined by weighing the envelopes containing the root/shoot components using an electronic weighing balance (Model BL-410S, manufactured by Setra, USA) in the laboratory. The envelopes and their contents were then oven dried to a constant weight for 48 hours at 60 ^{0}C (Rutunga *et.al.,* 1999).

The dry weights and the leaf areas were then used to calculate the relative growth Rate (RGR) and the Net Assimilation Rate (NAR) of the seedlings according to the formula used by Oni (1989).

$$\text{Net Assimilation Rate (NAR)}(\text{gcm}^{-2}\text{week}^{-1}) = \frac{W_2 - W_1}{A_2 - A_1} \times \frac{\ln A_2 - \ln A_1}{t_2 - t_1}$$

Where:

A_1 = initial leaf area

A_2 = final leaf area

W_2 = final dry weight

W_1 = initial dry weight

t_2 = final harvest time

t_1 = initial harvest time

ln = natural logarithm

$$\text{Relative Growth Rate (RGR)}(\text{gg}^{-1}\text{week}^{-1}) = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

Where:

lnW_2 = natural logarithm of final dry weight

lnW_1 = natural logarithm of initial dry weight

t_2 = final harvest time

t_1 = initial harvest time

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3.6.0 Genetic variability of *K. grandifoliola* using molecular techniques

3.6.1 Total genomic DNA (deoxyribonucleic acid) extraction

Juvenile leaves were collected from the seedlings raised from the six seed sources and kept in a 1.5ml Eppendorf tube in an ice bucket. Total genomic DNA extraction was later carried out on the collected leaves from each seed source using the modified Dellaporta extraction protocol (Dellaporta *et al.,* 1983). This protocol utilizes *Sodium dodecyl sulfate* (SDS) as detergent and the addition of potassium acetate resulted in the removal of some proteins and polysaccharides as a complex with the potassium-SDS precipitate. The protocol involved the following steps:

1. 0.3 g each of the leaves were weighed out and ground in a mortar and pestle (which were initially preheated at 65°C and placed in the freezer to cool prior to the usage) using a portion of the Dellaporta extraction buffer.
2. 800 µl of the extraction buffer (containing β-Mercapto-ethanol, Sodium chloride, Ethylene-diamine-tetra-acetic acid (EDTA) and Tris-amino-methane – Tris) was quickly added and mixed with pipette tip until the tissue became dispersed in the buffer.
3. Then 100 µl of 20 % SDS was added and mixed thoroughly for another 1 min.
4. Thereafter, this was incubated at 65°C and mixed intermittently 5 – 6 times by vortexing for approximately 10 mins.
5. 300 µl of ice cold 5M potassium acetate was then added and mixed by gently inverting 5 – 6 times and then incubated on ice for 20 mins.
6. This mixture was then centrifuged at 12000 rpm for 10 mins and carefully with a pipette the supernatant was transferred to 2 new 1.5 ml Eppendorf tubes.
7. Later 700 µl of ice cold Iso-propanol was added and mixed by inverting gently 8 – 10 times.
8. This mixture was incubated again on ice for 1hour and centrifuged at 12000 rpm for 10 mins.
9. The supernatant was tipped off and the last drop of isopropanol from the pellet was removed by placing the tubes face down on paper towel.
10. Thereafter, the pellet was re-suspended in 500µl of high TE (Tris – EDTA) salt and 4µl of 10 mg/ml of Ribonuclease (RNASE) and incubated at 37°C in an incubator shaker oven for 30 mins with constant gentle shaking.
11. 500µl of ice cold Isopropanol was added and inverted gently for 5 – 10 mins.

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12. The mixture was incubated on ice for 1hour and later centrifuged at 12000 rpm for 10 mins and the supernatant tipped off and the last drop of isopropanol removed from the pellet by placing the tubes face down on paper towel.
13. 70 % ethanol was then used to wash the pellet twice and spun at 12000 rpm for 20 mins and then the pellets were allowed to dry at room temperature for 1hour.
14. Finally 100 µl of 1 x TE was added to elute (dissolve/suspend) the DNA and then stored at – 40°C.

3.6.2 Purification of extracted DNA

The extracted DNA from the above procedure was purified (Plate 3.7) using Qiagen Spin Column Protocol and it involved the following steps:

1. 500µl of Binding Buffer (BB) was added into a Dneasy Mini spin column, and care was taken not to damage the filter in the process. This was centrifuged for 1min at 8000rpm and the flowthrough discarded.
2. 500µl Wash Buffer (WB) was added into the Dneasy Mini spin column and centrifuged for 3 minutes at 13000 rpm and the flowthrough discarded.
3. Centrifuge was repeated for another 1 minute at 13000 rpm to make sure that the Dneasy membrane was completely dried.
4. The Dneasy Mini spin column was later placed in a clean 1.5 ml Eppendorf tube. With the aid of a pipette, 150µl Elution Buffer was introduced directly onto the Dneasy membrane. This was incubated at room temperature for 15 minutes, and centrifuged for 2 minutes at 8000 rpm to elute the DNA.
5. Finally, the purified DNA samples were stored at – 40°C freezer.

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Plate 3.7: DNA extraction and purification process **(a)** weighing of sample, **(b)** grinding of sample, **(c)** collection of the ground sample into Eppendorf tube, (d) well labelled collected samples **(e)** samples arranged in the centrifuge and **(f)** centrifuged samples.

3.6.3 Verification of DNA quality on a 1.2 % Agarose gel electrophoresis.

This was done to check the integrity and purity of the extracted DNA samples. The gel tray was securely sealed at both sides with a tape to hold and prevent the leakage of the gel while setting. Wells were formed by the combs inserted in them. A 1.2 % Agarose gel was prepared by dissolving 1.2 g Agarose salt with 100ml 1X TBE (Trisma base, Boric acid and EDTA – Ethylene diamine tetra acetate) Buffer. Microwave oven was used to warm and homogenize the medium until all Agarose salt dissolved (about 2-3 minutes). The medium was cooled down to 55°C ± 2 (this is to aid even solidification of the gel and also to avoid damage of the gel tray) by holding under a running cold tap. Once cooled, 3µl of GR-green was added (under a biosafety cabinet) and swirled to mix properly. The gel was poured into the tray and left to stand for at least 30 minutes before loading samples. Combs were carefully and gently removed and the gel placed into an electrophoresis tank. Three microliter (3µl) of Loading Buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 30 % glycerol in water) for each DNA sample was spotted on a strip of parafilm-M. Using a 1- 20 µl range eppendof pipette, 3µl of DNA was mixed with the loading buffer and the mixture was loaded into the well on the gel. This was run for about an hour on 100V voltage and 85 Ma current with electrophoresis power supply device (Plate 3.8 a and b). Thereafter, the gel was viewed with a gel documentation unit made by Beijing Liuyi Instrument factory, China (with the use of eye protection) under the UV viewing carbinet model 9413A and photographs of the gel were taken with the aid of the camera attached and saved.

3.6.4 Spectrophotometry (Spec. check)

A UV – Spectrophotometer model 752 W UV-Grating made by Ocean Med, England was used in this study (Plate 3.8 c and d). This equipment was used to measure the quality and quantity of the DNA; the quantity in terms of the concentration of the DNA in nanogram per microliter (ng/µl) and the quality in terms of the absorbance of ultraviolet light through the cuvette measured in ångström (Å). A prominent accessory is the cuvette which houses the diluents and the DNA. The dilution was in ratio (DNA: diluents; 2:498) and the total solution made 500µl. The results were displayed on the liquid crystal display (LCD) for concentration, absorbance at different wavelengths simultaneously: 260Å, 280 Å and ratio of 260Å /280Å.

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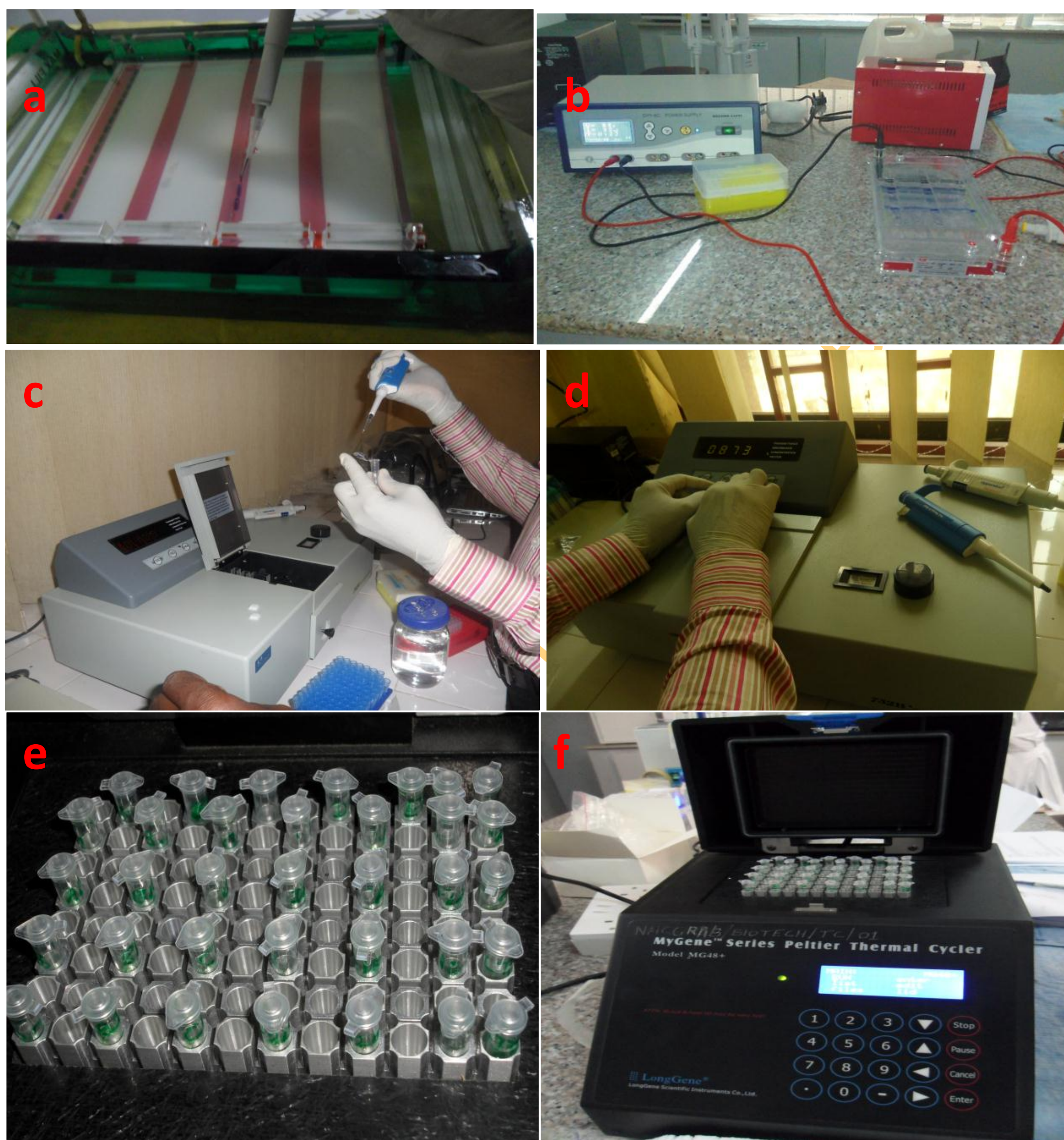

Plate 3.8: DNA quantification and PCR **(a)** DNA loading **(b)** Gel electrophoresis **(c)** loading DNA sample into cuvette **(d)** Spec reading of DNA samples **(e)** loading of DNA samples into PCR machine well **(f)** Running PCR of DNA samples.

3.6.5 Polymerase chain reaction (PCR).

Polymerase Chain Reactions (PCR) is referred to as the enzymatic synthesis of multiple copies of a specific DNA sequence in a cyclical manner. It involves initiation, denaturation, annealing, elongation and final extension of the DNA fragment over varying temperatures of 94°C, 94°C, 42°C, 72°C and 72°C respectively. The reaction mixture is then held at 4°C until electrophoresis was run (Plate 3.8 e and f).

In this study, initial screening was done with thirty RAPD primers (Operon Technologies Inc., USA) using DNA from three collection sources (Kutia, Owo and NACGRAB). Six Operon primers (OPD-08, OPD-11, OPD-13, OPD-18, OPA-18 and OPD- 20) that gave reproducible and scorable amplifications were selected and used in the analysis of all the 6 collections (Table 3.1). Total reaction volume for DNA amplification was 13.5µl containing 10µl of Dream Taq™ Green PCR Master Mix (2x) from Fermentas Life Sciences [consists of Green buffer, $MgCl_2$, mixture of Dntp (deoxyribonucleotide triphosphate); Datp (2-deoxyadenosine 5-triphosphate); Dgtp (2-deoxyguanine 5-triphosphate); Dctp (deoxycytidine 5-triphosphate); Dttp (2-deoxythymidine 5-triphosphate)] and 2µl of x50 dilution ratio of sample DNA. The PCR mixture was run on the Peltier-Based Thermal Cycler (Model MG48+, LongGene Scientific Instruments Co., Ltd, Hong Kong), using the following temperature profile: Initial strand separation step of 3 minutes at 94˚C followed by 35 cycles each consisting of a denaturing step of 1 minute at 94˚C, annealing step of 1minute at 42˚C and an extension step of 1 minute at 72˚C. The last cycle was followed by 10 minutes extension at 72˚C. After amplification, PCR product was stored at 4°C till electrophoresis.

3.6.6 Electrophoresis of PCR product

Reactions products were mixed with 2.5 ml of 10x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose w/v) and spun briefly in a micro centrifuge before loading (Sambrook *et. al.,* 1989). PCR products were then resolved by electrophoresis at 1.2% agarose gel and constant power of 100V for 1hr 30mins followed by staining with ethidium bromide (10mg/ml) for 1 minute and then placed in distilled water to remove excess stain (Plate 3.8 a and b). After the electrophoresis, the PCR products were visualized on the gel and photographed in the Gel Documentation and Analysis Systems (UV Viewing Cabinet Model 9413A).

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3.6.7 Collection and scoring of DNA data

Only distinct, well-resolved and unambiguous bands were scored, discarding faint bands. The assumption was that fragments of equal length had been amplified from corresponding loci. The amplified fragments were scored as 1 at the presence and 0 at the absence of homologous bands. From this binary matrix, similarity matrices were computed using Sequential Hierarchical and Nested (SAHN) clustering option of the NTSYSpc 2.02j software package (Rohlf, 1993). The software generated a dendrogram, which grouped the seed sources using Unweighted Pair Group Method with Arithmetic Average (UPGMA) on the basis of genetic similarity and Jaccard's coefficient.

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Table 3.1: Code, sequence and nucleotide length of selected operon primers used in the study

No	Primer Code	Primer Sequence (5'to 3')	Nucleotide length
1	OPD-08	>GTG.TGC.CCC.A<	10-mers
2	OPD-11	>AGC.GCC.ATT.G<	10-mers
3	OPD-13	>GGG.GTG.ACG.A<	10-mers
4	OPD-18	>GAG.AGC.CAA.C<	10-mers
5	OPA-18	>AGG.TGA.CCG.T<	10-mers
6	OPD-20	>ACC.CGG.TCA.C<	10-mers

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3.7 Effect of Storage Temperature and Duration of Storage on Germination of *K. grandifoliola* Seeds.

This experiment was carried out at the seed genebank unit of NACGRAB where a standard storage facility with regular electricity power supply is available.

From Kutia seed source, two thousand mature pure seeds of *K. grandifoliola* were selected for this study. The seeds were subjected to four different storage conditions viz 28± 2^oC, 24^oC, - 6^oC and -17^oC (representing ambient room temperature (ART), short term genebank condition (ShT), freezer (FrZ) and long term genebank condition (LgT). A total of 500 seeds were randomly allocated to each treatment using complete randomized design. Each seed lot was kept inside air tight plastic containers.

Another sample of 100 seeds each, were drawn from the fresh seeds and sown immediately in washed river sand under a non-mist propagator. This sample of 100 seeds was used to determine the initial germination percentage of the seeds at time T_o. Subsequently, at the end of every month, a sample of 100 seeds each were drawn from each batch under the four storage conditions and germinated as earlier described in 3.2.3. Five sampling periods were observed viz: T_1, T_2, T_3, T_4 and T_5. Percentage germination of the seeds was determined at each sampling occasion and analysis of variance was used to compare longevity of the seeds in storage.

3.8 Macropropagation protocol for *K. grandifoliola*

3.8.1 Effect of growth hormones, rooting media and leaf area on rooting of juvenile stem cuttings of *K. grandifoliola.*

Juvenile leafy stem cuttings were taken from six months old seedlings of *K. grandifoliola* from the six sources with the use of secateurs. The cuttings were collected in the morning hours and were kept moist at all times by placing them under water in a plastic bucket to avoid desiccation. From the scions a total of 432 juvenile single node leafy stem cuttings were obtained from the seedlings. The leaves of two hundred and sixteen stem cuttings were cut horizontally into half (50%) the original leaf size while the remaining two hundred and sixteen cuttings retained their (100%) original leaf size. The cuttings were treated with three types of rooting hormones (Auxins) namely indole-3-butyric acid (IBA), 1- naphthalene acetic acid (NAA) and Indole acetic acid (IAA) at different concentrations of 0, 25, 50, 100, 150 and 200 mg/l using the quick dip method (Oni, 1987). For each of the three different auxins, four cuttings each were

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treated with the auxins. The cuttings were planted in three different rooting media (sterilized washed river sand (S), cured sawdust (SD) and a mixture of sand and sawdust (SSD) at a ratio 1:1 arranged in a 2 x 3 x 3 x 6 factorial design in the non – mist propagator of the screen house at NACGRAB.

Watering was done to field capacity twice daily, morning and evening with the use of a hand sprayer. The cuttings were assessed for the following variables after 60 days.

1. Number of rooted cuttings
2. Number of roots per rooted cuttings
3. Length of longest root per cutting
4. Number of callused cuttings

The data obtained was subjected to two-way analysis of variance to compare the cuttings growth responses to the rooting media, hormones and hormones concentrations. Duncan multiple range test at 5% probability level was used to compare the significantly different means.

3.9 Protocol for *In vitro* Propagation of *K. grandifoliola*

3.9.1 Explant source

Seeds of *K. grandifoliola* used were collected from the Genebank of National Center for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria (07°23.048’N 003°50.431’E).

3.9.2 Disinfection protocol

The ninety (90) seeds used for this experiment were surface cleaned by washing with mild liquid detergent (Tween-20) under running tap water for 10 min. After thorough washing, the seeds were taken into the Laminar Flow Chamber where they were disinfected with 70% (v/v) ethanol for 5 min followed by treatment with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 10 min and then rinsed 3 times in sterile distilled water.

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3.9.3 Culture medium and incubation condition

The embryos were carefully excised together with some endosperm attached and inoculated on to sterilized semi solid basal MS medium (Murashige and Skoog's, 1962) supplemented with 3% w/v sucrose, 0.1 g inositol and gelled with 0.7% w/v agar at various concentration of cytokinins and auxin in a 17 ml test tube. The constituents of MS media are shown in Table 3.2. The cytokinins used were benzylaminopurine (BAP), kinetin (KIN) and Adenine sulphate (ADS) while naphthalene acetic acid (NAA) was the auxin used. All growth regulators were added before autoclaving at 121°C for 15 min and the pH was adjusted to 5.7 ± 0.2. The cultures were incubated in a growth room at 26 ± 2°C under a 16 h photoperiod with cool-white fluorescent light. There were nine treatments (Table 3.3), and ten explants were cultured per treatment and later arranged randomly on the shelves in the growth room. After four weeks, the cultures were evaluated for shoot length, root length, number of nodes and number of roots. The data taken were subjected to statistical analysis using SAS/PC version 9.1 (SAS 1999). The observed means of the characters were subjected to Least Significant Difference (LSD) to show the mean separation.

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Table 3.2 Murashige and Skoog basal media composition used in the study

Ingredients	Amount (mg/l)	Conc. of stock solution (mg/l)
Major salts (Stock 1)		
NH4NO3	1650.00	33,000.00
KNO3	1900.00	38,000.00
CaCl2.2H2O	440.00	8,800.00
MgSO4. 7H2O	370.00	7,400.00
KH2PO4	170.00	3,400.00
Minor salts (Stock II)		
KI	0.83	166.00
H3BO3	6.20	1,240.00
MnSO4.4H2O	22.30	4,460.00
ZnSO4.7H2O	8.60	1,720.00
Na2MoO4.2H2O	0.25	50.00
CuSO4.5H2O	0.025	5.00
CoCl2	0.025	5.00
Iron stock (Stock III)		
FeSO4.7H2O	27.80	5,560.00
NA2.EDTA.2H2O	37.30	7,460.00
B5 Vitamins (Stock – IV)		
Myo-inositol	100.00	20,000.00
Nicotinic acid	1.00	200.00
Pyridoxine HCl	1.00	200.00
Thiamine HCl	10.00	2,000.00
Sucrose (g)	30.00	---
Agar (g)	8.00	---
Growth regulators	As required	As required

Source: Murashige and Skoog, 1962

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Table 3.3: The different media constituents used in the i*n vitro* propagation of *K. grandifoliola*

Treatment	MS Medium Supplements (mg/l)
A	0.125 mg/l (BAP) + 0.01 mg/l (NAA)
B	0.125 mg/l (KIN) + 0.01 mg/l (NAA)
C	0.15 mg/l (BAP) + 0.01 mg/l (NAA)
D	0.15 mg/l (KIN) + 0.01 mg/l (NAA)
E	1mg/l (BAP) + 0.01 mg/l NAA
F	1mg/l (KIN) + 0.01 mg/l NAA
G	0.05 mg/l (BAP) + 0.01 mg/l (NAA) + 10 mg/l (Adenine sulphate)
H	0.05 mg/l (KIN) + 0.01 mg/l (NAA) + 10 mg/l (Adenine sulphate)
CONTROL	MS only

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CHAPTER FOUR

4.0 RESULTS

4.1.0 Morphological Variation in Seeds of *K. grandifoliola*

The weight, length and width of seeds of *K. grandifoliola* varied among the six seed sources in this study. The seed weight ranged between 0.31±0.06g at Owo to 0.52±0.11g at NACGRAB; the seed length 3.10±0.37 cm at Boki to 4.20±0.44 cm at Kutia and seed width 2.44 ± 0.27 cm at Boki to 3.03 ± 0.33 cm at Ayegunle-Akoko (Table 4.1). There were significant differences among the seed weight of the six sources at 0.05 level of probability (Appendix 1).

4.1.1 Seed weight

Seeds from NACGRAB had the highest mean seed weight of 0.52g, followed by seeds from Ayegunle-Akoko with 0.49g while Owo recorded the least seed weight of 0.31g (Table 4.1). There were no significant differences between the mean seed weight of seeds from NACGRAB and Ayegunle-Akoko, similarly, there were no significant difference between the mean seed weight of seeds from Kutia and Oba-Akoko; Owo and Boki. However, there were significant differences between the mean seed weight of seeds from Kutia, Ayegunle-Akoko, Owo, NACGRAB and Boki. In the same vein, there was a significant difference between the mean seed weight of seeds from Ayegunle-Akoko and Oba-Akoko, Owo, Boki.

4.1.2 Seed length

The highest mean seed length of 4.2cm was recorded from Kutia, which was closely followed by those from Ayegunle-Akoko (4.09cm). The lowest mean seed length (3.1cm) was recorded from Boki. There were no significant difference between mean seed length of seeds from Kutia and Ayegunle-Akoko (Table 4.1). Also, the mean seed length of seeds from Ayegunle-Akoko, Oba-Akoko, Owo and NACGRAB were not significantly different from one another. However, there were significant differences among the mean seed lengths of seeds from Kutia, Oba-Akoko, Owo, NACGRAB and Boki. There were also significant differences between Ayegunle-Akoko and Boki.

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4.1.3 Seed width

Ayegunle-Akoko recorded the highest mean seed width of 3.03cm. This was followed by seeds from Oba-Akoko with 2.88cm while the seeds from Boki recorded the lowest mean seed width of 2.44cm. There were no significant difference among the mean seed width of seeds from Kutia, Oba-Akoko and NACGRAB (Table 4.1). Similarly the mean seed width of seeds from Ayegunle-Akoko and Oba-Akoko are not significantly different from each other. However, there were significant differences in the mean seed width of seeds from Kutia, Ayegunle-Akoko, Oba-Akoko, Owo and Boki. There were also significant differences among Ayegunle-Akoko, Owo, NACGRAB and Boki.

4.2 Germination of seeds of *K. grandifoliola*

4.2.1 Seed germination percentage

The germination was hypogeous (or hypogeal). Germination started at the 11th day after sowing and this continued to the 34th day after sowing for each of the seed sources.

The highest germination percentage was recorded for seeds from NACGRAB (98.0%) followed by those from Ayegunle-Akoko (94.0%), Kutia (90.0%), Oba-Akoko (88.0%), Boki (85.0%) and Owo (81.0%), (Fig.4.1).

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Table 4.1: Mean values for seed metric characters of *K. grandifoliola* from six sources in Southern Nigeria

Source	Seed Weight	Seed Length	Seed Width
Kutia	$0.43{\pm}0.08^{b}$	$4.20{\pm}0.44^{a}$	2.53 ± 0.19^{b}
Ayegunle-Akoko	$0.49{\pm}0.09^{a}$	$4.09{\pm}0.44^{ab}$	3.03 ± 0.33^{a}
Oba-Akoko	$0.43{\pm}0.11^{b}$	$3.87{\pm}0.53^{b}$	$2.88{\pm}\ 0.34^{ab}$
Owo	$0.31{\pm}0.06^{c}$	$3.88{\pm}0.40^{b}$	2.49 ± 0.35^{c}
NACGRAB	$0.52{\pm}0.11^{a}$	$3.84{\pm}0.50^{b}$	2.81 ± 0.18^{b}
Boki	$0.32{\pm}0.07^{c}$	$3.10{\pm}0.37^{c}$	2.44 ± 0.27^{c}
LSD	0.06	1.09	0.17

NB: *Means with the same letter under each column are not significantly different from each other at* $P < 0.05$

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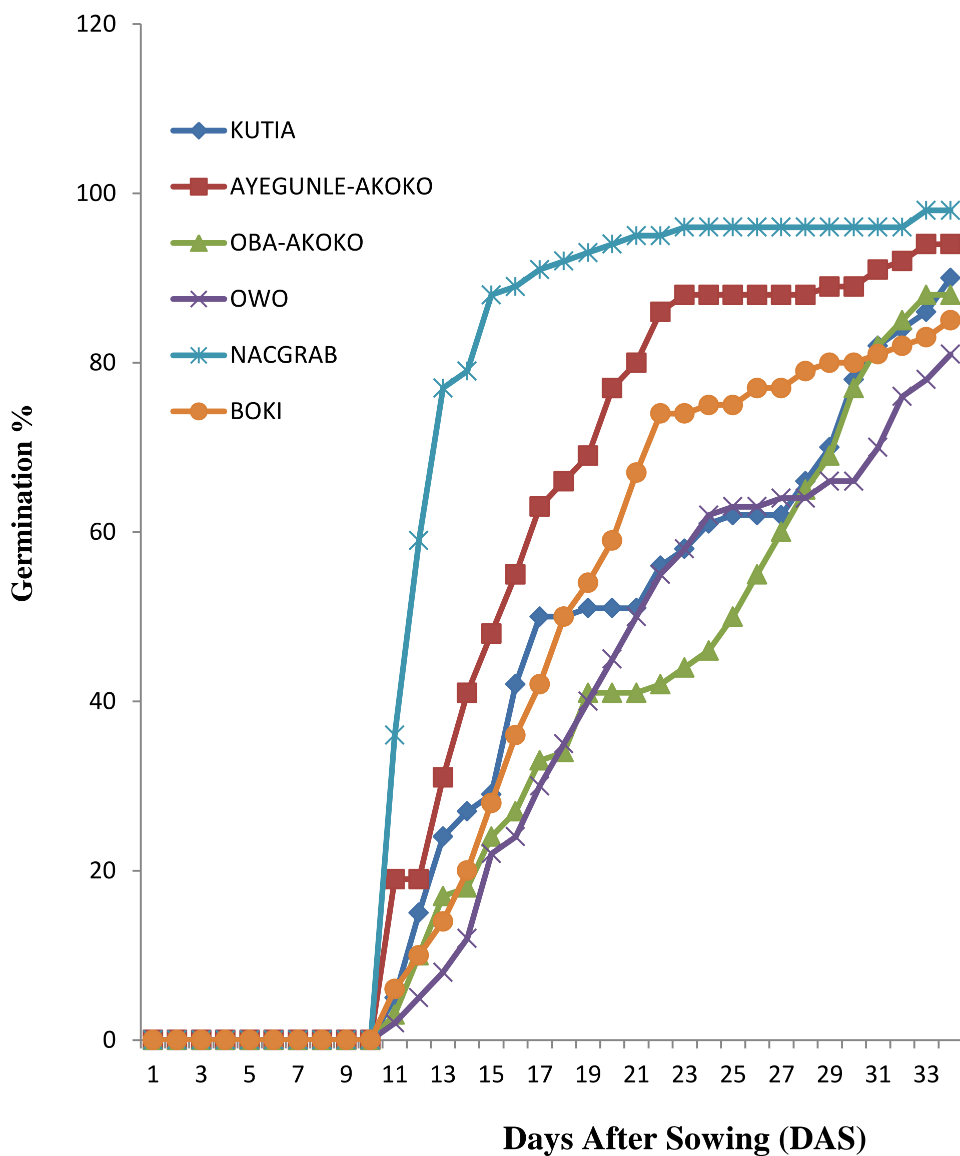

Fig. 4.1: Germination percentage of *K. grandifoliola* seeds from six collection sources in Southern Nigeria.

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4.3.0 Morphological Variation in Seedlings Growth of *K. grandifoliola*

4.3.1 Height of seedling

Seedlings from NACGRAB had the highest value of 29.97cm. This was closely followed by 29.86 cm in seedlings from Ayegunle-Akoko, while the lowest mean seedling height of 21.73cm was recorded for Owo. The mean height of seedlings from Kutia, Ayegunle-Akoko and NACGRAB are not significantly different from each other (Table 4.2). Similarly, the mean height of seedlings from Oba-Akoko and Boki are not significantly different from each other. However, there are significant differences among NACGRAB, Boki and Owo. Generally, there were significant differences in the height of seedlings from all the sources ($P < 0.05$) (Appendix 2).

4.3.2 Collar diameter

Collar diameter of seedlings showed no significant differences among the six sources ($P < 0.05$) (Appendix 2). There were no variation in the collar diameter of seedlings from Kutia, Ayegunle-Akoko, Oba-Akoko, Owo, NACGRAB and Boki (Table 4.2).

4.3.3 Height of branching

The highest value of 2.61 cm was observed in seedlings from NACGRAB. Ayegunle-Akoko had 1.38 cm while seedlings from Kutia recorded 0.00 cm (no branch). The mean height of branching of seedlings from Kutia, Oba-Akoko, Owo and Boki are not significantly different from each other (Table 4.2). Similarly, there are no significant difference between the mean height of branching of seedlings from Ayegunle-Akoko and Oba-Akoko. However, there were significant differences among the mean height of branching of seedlings from Kutia, Ayegunle-Akoko and NACGRAB. In the same vein there were significant differences among the mean height of branching of seedlings from Ayegunle-Akoko, Owo and NACGRAB.

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Table 4.2: Mean values of seedlings metric character of *K. grandifoliola* from six sources in Southern Nigeria

Source	Ht	CD	HB	NB	IL	NL
Kutia	29.61±4.94^{a}	10.14±2.12^{a}	0.00±0.00^{c}	0.00±0.00^{c}	1.17±0.27^{bc}	36.75±5.90^{b}
Ayegunle-Akoko	29.86±3.38^{a}	11.53±1.49^{a}	1.38±1.38^{b}	0.35±0.20^{a}	1.57±0.64^{ab}	43.00±12.74^{ab}
Oba-Akoko	28.07±4.40^{b}	11.52±2.77^{a}	1.02±1.02^{bc}	0.35±0.20^{a}	1.38±0.68^{b}	31.20±10.57^{bc}
Owo	21.73±8.27^{c}	12.10±1.43^{a}	0.68±0.60^{c}	0.20±0.14^{b}	1.35±0.46^{b}	31.38±8.93^{bc}
NACGRAB	29.97±5.30^{a}	12.33±1.46^{a}	2.61±1.44^{a}	0.10±0.10^{bc}	1.64±0.46^{a}	43.20±12.78^{a}
Boki	25.79±9.08^{b}	9.84±2.46^{a}	0.98±0.68^{c}	0.15±0.10^{bc}	1.10±0.57^{c}	30.75±6.51^{c}
LSD	7.93	6.47	2.93	0.57	4.73	10.38

NB: Ht– Height, CD– Collar diameter, HB– Height of branching, NB– Number of Branches, IL – Internode length, NL – Number of leaves (means with the same letter under each column are not significantly different from each other at $P < 0.05$)

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4.3.4 Number of branches

Seedlings from Ayegunle-Akoko and Oba-Akoko recorded the highest value of 0.35 cm followed by 0.2 cm from Owo while seedlings from Kutia had 0.00 cm (no branch). There were no significant difference in the mean number of branches of seedlings from Kutia, NACGRAB and Boki (Table 4.2). Similarly, the mean number of branches of seedlings from Ayegunle-Akoko and Oba-Akoko are not significantly different from each other. However, there were significant differences among the mean number of branches of seedlings from Kutia, Ayegunle-Akoko, Oba-Akoko and Owo.

4.3.5 Internode length

The highest value 1.64cm was obtained in seedlings from NACGRAB. Ayegunle-Akoko had 1.57cm while seedlings from Boki recorded the shortest internode length of 1.10 cm**.** The mean internode length of seedlings from Kutia is significantly different from the mean internode length of seedlings from NACGRAB (Table 4.2). But, there was no significant difference between the mean internode length of seedlings from Kutia, Ayegunle-Akoko, Oba-Akoko and Owo. There were significant differences in the internode length of seedlings from all the sources ($P < 0.05$) (Appendix 2).

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4.3.6 Number of leaves

Seedlings from NACGRAB had the highest mean number of leaves of 43.20 followed by seedlings from Ayegunle-Akoko with a mean of 43.00 (Table 4.2). The least value, 30.75 was recorded from Boki. There were no significant difference among the mean number of leaves of seedlings from Kutia, Ayegunle-Akoko, Oba-Akoko and Owo. However, there is significant difference between the mean numbers of leaves of seedlings from Kutia, NACGRAB and Boki.

4.3.7 Mophorlogical characterization: The morphological similarity among the genotypes of the 6 collections was scaled between 0.0 and 0-1 (Jaccard's similarity coefficient). The unweighted pair group method with arithmetic average (UPGMA) dendrogram produced using neighbour joining method of cluster analysis separated all the 6 collections into 1 cluster and 4 ungrouped at 0.08 similarity coefficient (Fig. 4.2).

Cluster 1 consisted of genotype collections from Ayegunle-Akoko and Oba-Akoko while genotype collection from Kutia, Owo, Boki and NACGRAB were ungrouped. Cluster 1 showed high morphology resemblance at 0.08 similarity coefficient which is evident in the nearness of the collection sources. The dendrogram (Fig. 4.3) further revealed that at 0.11 coefficients of similarity all the six collection sources showed very high morphology distinction, keeping each of the sources as a separate cluster.

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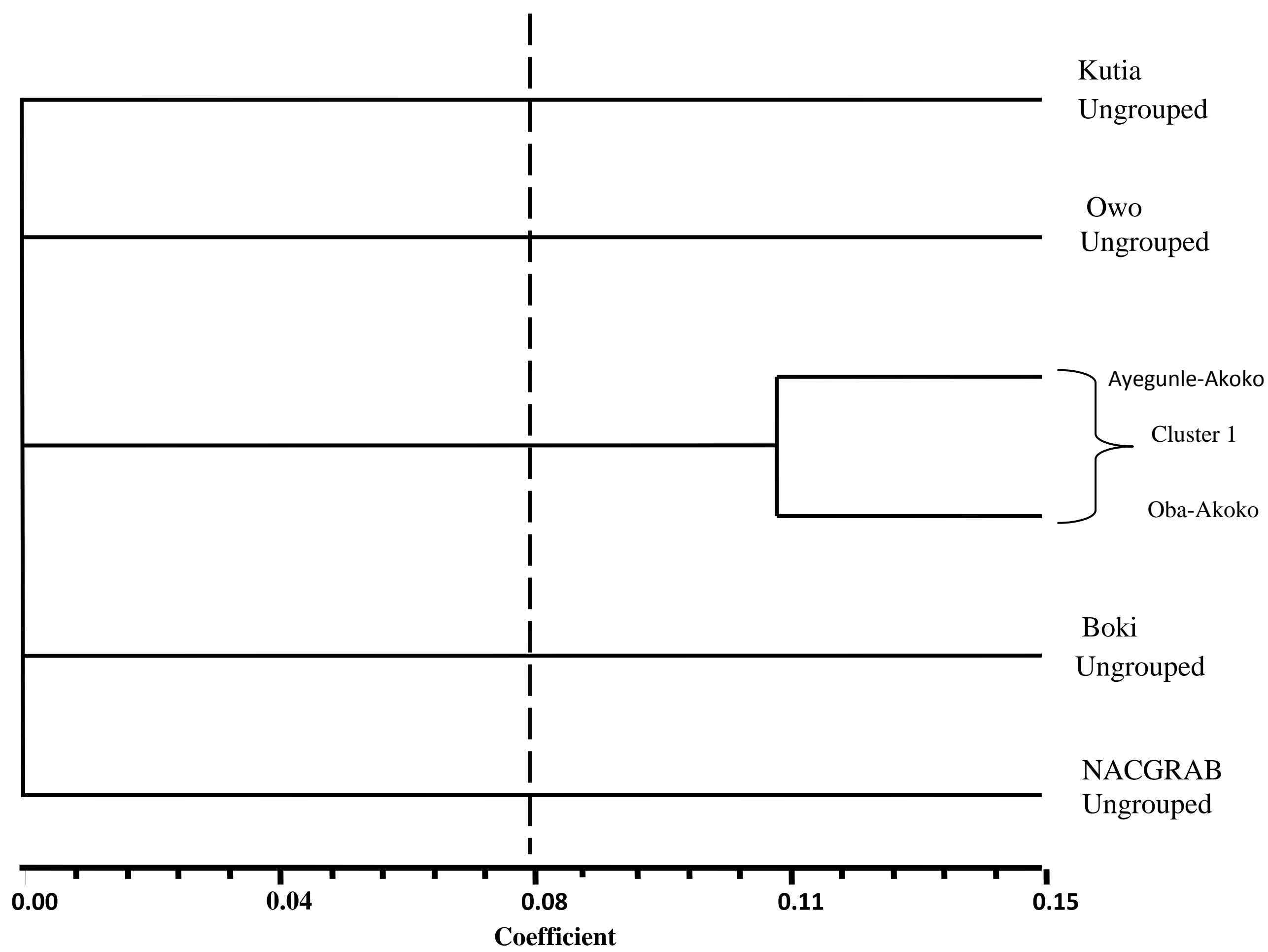

Fig.4.2: Dendogram of the six *Khaya* genotypes collections from Southern Nigeria Showing the morphological variations at 0.08 coefficient of similarity

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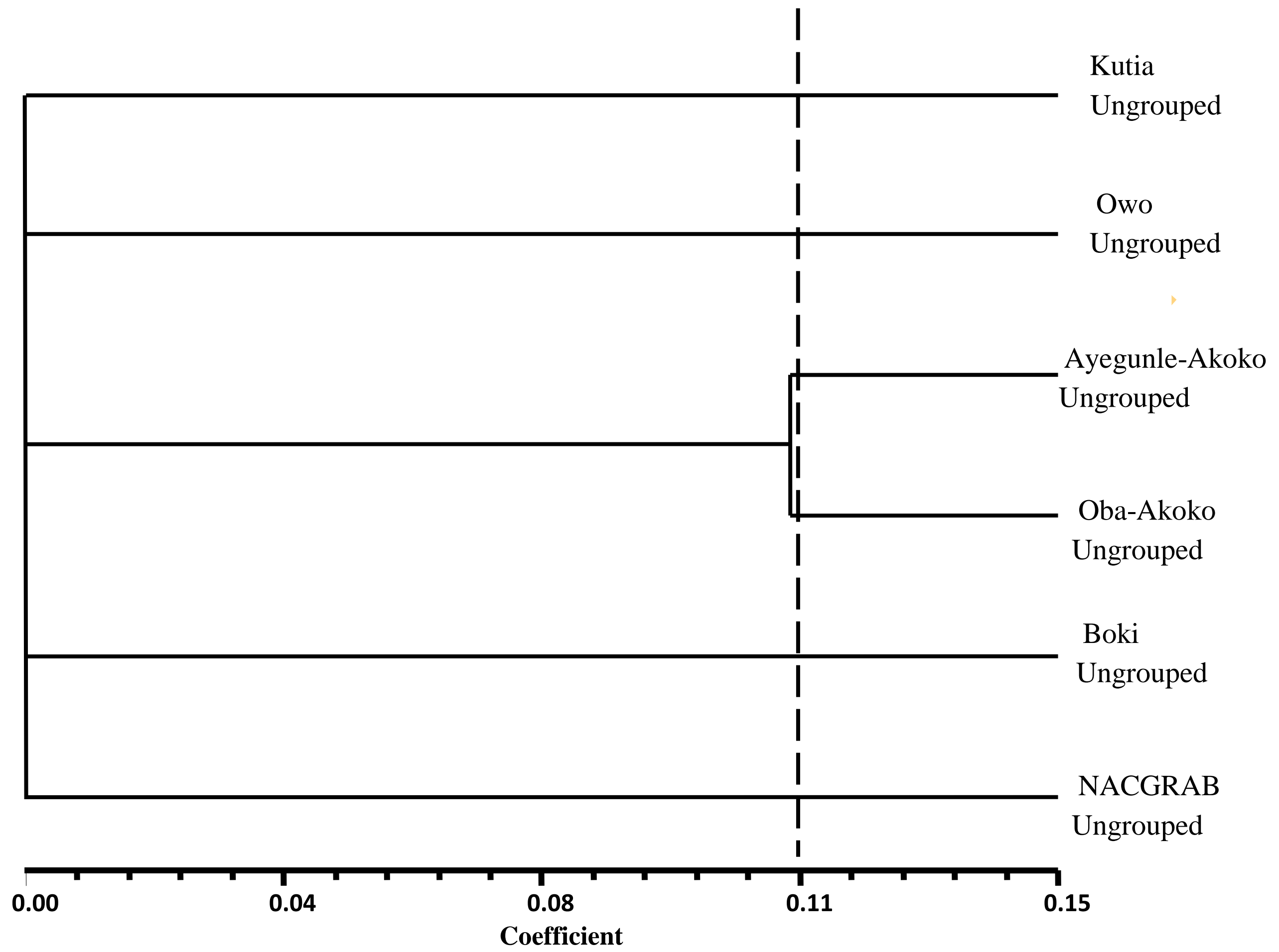

Fig.4.3: Dendogram of the six *Khaya* genotypes collections from Southern Nigeria showing distinction in morphology at 0.11 coefficient of similarity

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4.4.0 Biomass estimation of seedlings from seed sources

4.4.1 Leaf area

Ayegunle-Akoko recorded the highest mean leaf area of 1371.98 m^2 which was closely followed by seedlings from Oba-Akoko (1276.53m^2). The lowest mean leaf area of 949.80m^2 was recorded for seedlings from Kutia (Table 4.3). The mean leaf area of seedlings from Oba-Akoko was higher than the mean leaf area of seedlings from NACGRAB but they were not significantly different from each other. Meanwhile, there were significant differences between the mean leaf area of seedlings from NACGRAB, Boki, Owo, Kutia and Ayegunle-Akoko.

4.4.2 Leaf dry weight

The highest mean leaf biomass of 10.40g was recorded in seedlings from Ayegunle-Akoko. Seedlings from Kutia closely followed with mean value of 10.28g and the least mean value of 5.43g was recorded in seedlings from Oba-Akoko (Table 4.3). There were no significant difference between the mean leaf dry weight of seedlings from Boki and Oba-Akoko; Kutia and Ayegunle-Akoko. However, there were significant differences among the mean leaf dry weights of seedlings from NACGRAB, Boki, Owo, Kutia, Oba-Akoko and Ayegunle-Akoko.

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Table 4.3: Mean values of seedling biomass of *K. grandifoliola* from six sources in Southern Nigeria.

Source	Leaf Area (cm^2)	Leaf Dry Weight (g)	Stem Dry Weight (g)	Root Dry Weight (g)	Total Biomass (g)
NACGRAB	1227.26±173.45^{b}	8.75±0.68^{b}	4.07±0.53^{c}	9.15±1.02^{b}	21.97±2.14^{b}
Boki	978.14±154.22^{d}	6.19±0.76^{d}	3.65±0.45^{c}	4.96±0.66^{c}	14.80±1.02^{c}
Owo	1055.98±159.48^{c}	7.05±0.92^{c}	5.31±0.78^{b}	7.39±1.09^{c}	19.75±2.79^{bc}
Kutia	949.80±140.16^{d}	10.28±0.57^{a}	7.07±0.69^{ab}	10.21±0.91^{a}	27.21±1.19^{a}
Oba-Akoko	1276.53±167.37^{b}	5.43±0.71^{d}	5.27±0.57^{b}	5.97±0.72^{c}	16.67±2.79^{c}
Ayegunle-Akoko	1371.98±216.83^{a}	10.40±0.73^{a}	7.47±0.61^{a}	9.19±1.10^{b}	27.06±2.68^{a}
LSD	327.75	5.27	3.83	4.52	13.62

NB: Means with the same letter under each column are not significantly different from each other at $P < 0.05$

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4.4.3 Stem dry weight

Mean stem biomass of seedlings from Ayegunle-Akoko seeds, 7.47g was higher than the mean stem dry weight of the other five seed sources, but it was not significantly different from those seedlings from Kutia seeds which was 7.07g (Table 4.3). The difference in the mean stem dry weight of seedlings from NACGRAB and Boki was not significant. Likewise there were no significant differences between Owo and Oba-Akoko. However, there were significant differences among the mean stem biomass of seedlings from NACGRAB, Owo, Kutia, Oba-Akoko and Ayegunle-Akoko. Seedlings stem dry weight as affected by seed source was significantly different. Harvest time also had a significant effect on the stem dry weight at $P < 0.05$ (Appendix 3).

4.4.4 Root dry weight

The highest mean value for root dry weight, 10.21g was recorded from Kutia which was followed by a mean value of 9.19g in Ayegunle-Akoko and 9.15g in NACGRAB (Table 4.3). The lowest mean root dry weight of 4.96g was recorded for seedlings from Boki. There were no significant difference between the mean root dry weight of seedlings from NACGRAB and Ayegunle-Akoko. Also there were no significant differences among the mean root dry weight of seedlings from Boki, Owo and Oba-Akoko. However, there were significant difference among the mean root dry weight of seedlings from NACGRAB, Boki, Owo, Kutia and Oba-Akoko.

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4.4.5 Relative growth rate (RGR)

The results of RGR in Table 4.4 followed similar pattern with NAR except for few differences. The seedlings from Kutia recorded negative results in weeks 4-6 while seedlings from Owo recorded negative value in weeks 2-4. Boki recorded negative values in weeks 4-6 and 8-10. The seedlings from Ayegunle-Akoko, Oba-Akoko and NACGRAB did not record any negative values all through the investigation.

4.4.6 Net assimilation rate (NAR)

Net assimilation rate (NAR) varied in all the seedlings from all the seed sources that were studied. The results of the NAR in Table 4.5 revealed that the seedlings from Kutia and Boki had negative value in weeks 4-6, Oba-Akoko and Boki had negative value in weeks 6-8, Owo had negative value in weeks 2-4. The Table further revealed the negative values of seedlings from Boki in weeks 6-8 and 8-10. However, seedlings from Ayegunle-Akoko and NACGRAB recorded no negative NAR values throughout the study

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Table 4.4: Variation in Relative Growth Rate (mg g^{-1} day^{-1}) of seedlings from six sources of *K. grandifoliola* in Southern Nigeria

Source	Harvest Period (Weeks)				
	2-4	4-6	6-8	8-10	10-12
Kutia	9.92×10^{-2}	-3.46×10^{-2}	5.31×10^{-2}	1.53×10^{-1}	3.58×10^{-2}
Ayegunle Akoko	2.20×10^{-2}	1.11×10^{-1}	9.94×10^{-2}	5.17×10^{-2}	1.06×10^{-2}
Oba Akoko	2.09×10^{-2}	1.81×10^{-2}	2.48×10^{-2}	1.14×10^{-1}	1.37×10^{-1}
Owo	-6.29×10^{-1}	9.73×10^{-1}	6.44×10^{-2}	2.49×10^{-2}	2.01×10^{-1}
NACGRAB	1.07×10^{-1}	3.39×10^{-2}	1.42×10^{-1}	1.37×10^{-1}	2.96×10^{-2}
Boki	1.82×10^{-1}	-2.25×10^{-3}	4.89×10^{-1}	-2.89×10^{-2}	1.04×10^{-1}

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Table 4.5: Variation in Net Assimilation Rate ($gm^{-2}day^{-1}$) of seedlings from six sources of *K. grandifoliola* in Southern Nigeria

Source	Harvest Period (Weeks)				
	2-4	4-6	6-8	8-10	10-12
Kutia	5.0×10^{-3}	-9.0×10^{-4}	1.2×10^{-3}	4.2×10^{-3}	7.0×10^{-4}
Ayegunle-Akoko	1.0×10^{-3}	2.3×10^{-3}	2.7×10^{-3}	1.5×10^{-3}	2.0×10^{-4}
Oba-Akoko	4.7×10^{-3}	2.0×10^{-4}	-5.0×10^{-4}	3.1×10^{-3}	2.4×10^{-3}
Owo	-7.8×10^{-3}	1.1×10^{-2}	1.8×10^{-3}	7.0×10^{-4}	3.4×10^{-3}
NACGRAB	1.0×10^{-3}	5.0×10^{-4}	3.3×10^{-3}	4.3×10^{-3}	5.0×10^{-4}
Boki	2.7×10^{-3}	-1.9×10^{-3}	-8.4×10^{-3}	-1.0×10^{-3}	1.6×10^{-3}

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4.5.0 Effect of storage temperature and duration of storage on germination of *K. grandifoliola* Seeds

4.5.1 Storage condition

The germination percentage of freshly collected mature seeds was 98% (Control). After 20 weeks period of storage in different storage conditions, mean germination percentage was highest (81.8%) in seeds stored in short term (ShT) while seeds stored in Ambient Room Temperature (ART) followed closely with 81.0% (Table 4.6) and the lowest mean germination percentage of 78.5% was recorded by the seeds stored in Long Term (LgT) storage condition (Fig. 4.4). There were significant differences in mean germination percentage of seeds stored under ART, Freezer (FrZ) and LgT however; there was no significant difference in the mean germination percentage of seeds stored in ART and ShT (Table 4.6). Seed germination percentage as affected by storage condition was significantly different ($p \leq 0.05$) (Appendix 4).

4.5.2 Storage duration

After 4 weeks of storage, 94% of germination was recorded from seeds stored in the LgT. This was followed by seeds stored in FrZ (92%) and the lowest was seeds stored in ShT (90%). At 8 weeks after storage the highest germination percentage (87%) was recorded from seeds stored in ShT, closely followed by LgT (86%) while seeds stored both in ShT and FrZ recorded the least germination percentage (85%). However, at the end of 5 months study period of the seeds, those stored in ShT recorded the highest (70%) followed by the seeds stored in ART (67%) while those stored in LgT recorded the least (55%) (Table 4.7).

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Table 4.6: LSD tests for seed germination percentage of *K. grandifoliola* seeds in storage

Storage Condition	Mean
ART	81.3[a]
ShT	81.8[a]
LgT	78.5[c]
FrZ	80.0[b]
LSD	42.4

NB: Means with the same letter under each column are not significantly different at P < 0.05, ART – Ambient Room Temperature, ShT – Short Term, LgT – Long Term, FrZ - Freezer

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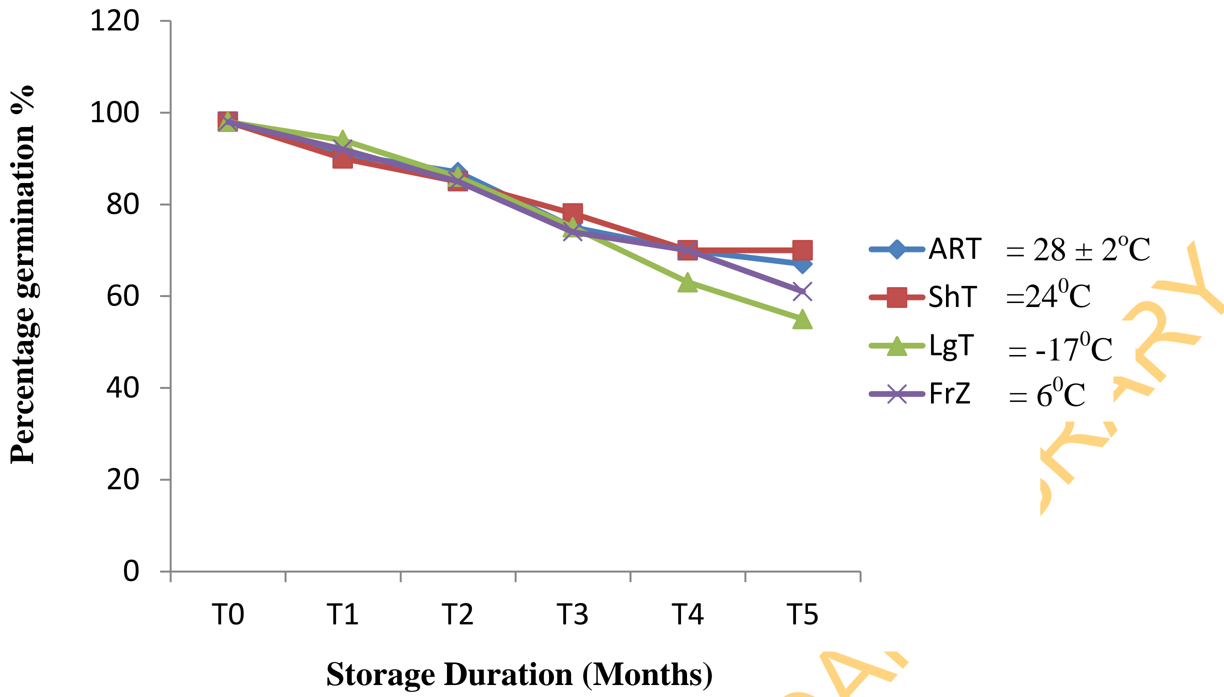

Fig. 4.4: Germination percentage of *K. grandifoliola* seeds stored in different temperature conditions.

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Table 4.7: Percentage germination of *K. grandifoliola* seeds under different storage conditions

Measurement time	Storage duration (Wks)	Storage conditions			
		ART (%)	ShT (%)	LgT (%)	FrZ (%)
T_0	0	98	98	98	98
T_1	4	91	90	94	92
T_2	8	87	85	86	85
T_3	12	75	78	75	74
T_4	16	70	70	63	70
T_5	20	67	70	55	61
Mean		81.33	81.83	78.5	80.0
LSD		9.33	0.11	20.67	11.33

NB: ART – Ambient Room Temperature, ShT – Short Term, LgT – Long Term, FrZ – Freezer; T_0 – Initial germination percentage, T_1, T_2, T_3, T_4 and T_5 – Sampling periods.

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4.6.0 Vegetative propagation of *K. grandifoliola*.

4.6.1 Effect of growth hormones, rooting media and leaf area on the rooting of juvenile stem cuttings of *K. grandifoliola*

4.6.2 Number of rooted cuttings

The rooting medium did not have any significant effect on the number of rooted cuttings. The interaction between rooting media and concentration of hormones; and rooting media, leaf area, growth hormones and concentration of hormones had significant effect on the number of rooted cuttings. Half leaf area had the higher mean value of 1.75 for number of rooted cuttings while full leaf area had 0.5 (Table 4.8). IBA recorded higher mean value (3.53) for number of rooted cuttings while NAA had 2.86 as the mean value for number of rooted cuttings (Table 4.11). Cuttings treated with 150 mg/l concentration of hormone recorded the highest mean value of 2.04 for number of rooted cuttings followed by 100 mg/l with 1.73, while the control (no hormone) recorded the least of 0.5 (Table 4.8). The interaction between rooting media and hormone concentration revealed that Sawdust x 150 mg/l had the highest mean number of rooted cuttings of 1.25 (Table 4.9). Interaction between Sand/Sawdust x 150mg/l had 0.25 while interaction between Sand/Sawdust x Control had no rooted cuttings. Leaf area, growth hormones and concentration of hormones had significant effect on the number of rooted cuttings ($P < 0.05$) (Appendix 5).

4.6.3 Number of root per rooted cuttings

Interactions between rooting media, leaf area, growth hormone and concentration of hormones also had significant effect on the number of root per rooted cuttings. Sawdust had the highest mean value of 2.0 for number of root per rooted cuttings (Plate 4.1) while sand and mixture of sand and sawdust had 1.75 and 1.05 respectively (Table 4.10). Half leaf area had a higher mean value of 1.92 for number of root per rooted cuttings while full leaf area had 0.75 (Table 4.8). The highest mean value (3.07) for number of root per rooted cuttings was recorded for IBA while NAA followed with a mean value of 1.75 (Table 4.11). The 150 mg/l concentration of hormone had a value of 2.45, 100 mg/l had 1.51 while the control (no hormone) recorded the least of 0.5 (Table 4.8). Rooting media, leaf area, growth hormones and concentration of hormones had significant effect on the number of root per rooted cutting ($P < 0.05$) (Appendix 6).

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Table 4.8: Effect of interaction between leaf area and hormone concentration on rooting of juvenile stem cuttings of *K. grandifoliola*

Treatment	Number of rooted cutting	Number roots per rooted cutting	Length of longest root per cutting	Number of callused cutting
Half leaf	1.75^{b}	1.92^{b}	1.05^{b}	2.25^{b}
Full leaf	0.50^{c}	0.75^{d}	0.41^{c}	1.37^{c}
Auxin Concentration				
Control	0.50^{c}	0.67^{d}	0.21^{c}	3.50^{a}
25mg/l	1.25^{b}	1.00^{c}	0.73^{c}	1.55^{c}
50mg/l	1.34^{b}	1.25^{c}	1.01^{b}	2.45^{b}
100mg/l	1.73^{b}	1.51^{b}	1.05^{b}	1.75^{c}
150mg/l	2.04^{a}	2.45^{a}	3.05^{a}	2.95^{b}
200mg/l	1.51^{b}	1.24^{c}	1.45^{b}	1.02^{c}
LSD	0.97	1.01	0.76	1.31

NB: Means with the same letter under each column are not significantly different at $P < 0.05$

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Table 4.9: Effect of interaction between rooting media and auxin concentration on rooting of juvenile stem cuttings of *K. grandifoliola*

Rooting medium/hormone concentration	Number of rooted cutting	Number roots per rooted cutting	Length of longest root per cutting	Number of callused cutting
Sand × control	0.00^{d}	0.00^{d}	0.00^{g}	2.35^{b}
Sand × 25mg/l	0.25^{c}	0.50^{c}	1.45^{f}	1.87^{c}
Sand × 50mg/l	0.25^{c}	0.75^{c}	3.25^{d}	2.50^{b}
Sand × 100mg/l	0.50^{b}	1.25^{b}	2.55^{e}	2.72^{b}
Sand × 150mg/l	0.75^{b}	0.50^{c}	1.25^{f}	2.75^{b}
Sand × 200mg/l	0.25^{c}	0.75^{c}	1.05^{f}	2.10^{b}
Sawdust × control	0.00^{d}	0.00^{d}	0.00^{g}	2.49^{b}
Sawdust × 25mg/l	0.75^{b}	0.50^{c}	3.65^{d}	2.00^{c}
Sawdust × 50mg/l	0.75^{b}	1.00^{b}	4.13^{c}	3.63^{a}
Sawdust × 100mg/l	1.00^{a}	1.50^{b}	7.15^{b}	2.54^{b}
Sawdust × 150mg/l	1.25^{a}	4.45^{a}	9.65^{a}	3.95^{a}
Sawdust × 200mg/l	0.25^{c}	0.75^{c}	4.65^{c}	2.05^{c}
Sand/sawdust × control	0.00^{d}	0.00^{d}	0.00^{g}	1.55^{d}
Sand/sawdust × 25mg/l	0.00^{d}	0.00^{d}	0.00^{g}	2.65^{b}
Sand/sawdust × 50mg/l	0.25^{c}	0.75^{c}	1.50^{f}	1.75^{d}
Sand/sawdust × 100mg/l	0.50^{b}	0.50^{c}	2.05^{e}	1.95^{d}
Sand/sawdust × 150mg/l	0.25^{c}	0.75^{c}	3.65^{d}	2.05^{c}
Sand/sawdust × 200mg/l	0.25^{c}	0.50^{c}	1.50^{f}	1.25^{d}
LSD	0.13	0.47	1.25	1.92

NB: Means with the same letter under each column are not significantly different at $P < 0.05$

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Table 4.10: Effect of rooting medium on rooting of juvenile stem cuttings of *K. grandifoliola.*

Rooting medium	Number of rooted cutting	Number roots per rooted cutting	Length of longest root per cutting	Number of callused cutting
Sand	1.75^{b}	1.75^{b}	4.87^{b}	3.86^{a}
Sawdust	2.00^{a}	2.45^{a}	6.48^{a}	2.73^{b}
Sand/sawdust	1.05^{b}	1.25^{b}	3.55^{c}	2.51^{c}
LSD	1.23	1.47	3.06	2.17

NB: Means with the same letter under each column are not significantly different at P < 0.05

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Table 4.11: Effect of growth hormone on rooting of juvenile stem cuttings of *K. grandifoliola.*

Growth hormone	Number of rooted cutting	Number roots per rooted cutting	Length of longest root per cutting	Number of callused cutting
Control	0.00^{d}	0.00^{d}	0.00^{d}	3.58^{a}
IBA	3.53^{a}	3.07^{a}	3.17^{a}	2.58^{b}
IAA	2.17^{c}	1.04^{c}	1.76^{c}	2.15^{c}
NAA	2.86^{b}	1.75^{b}	2.10^{b}	2.01^{d}
LSD	2.13	1.02	0.86	1.02

NB: Means with the same letter under each column are not significantly different at $P < 0.05$

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4.6.4 Length of the longest root per cuttings

There was no significant difference in the interactions between rooting media and growth hormone; rooting media, leaf area and hormones; rooting media, leaf area and concentration of hormone; and leaf area, growth hormones and concentration. Sawdust had the highest mean value of 6.48 cm (Plate 4.2) while sand and mixture of sand and sawdust had 4.87 cm and 3.55 cm respectively (Table 4.10). Half leaf area recorded a higher mean value of 1.05 cm (Table 4.8). Cuttings inoculated with IBA had the highest mean value of 3.17 cm (Table 4.11) while cuttings inoculated with 150mg/l had the highest mean value of 3.05 cm (Table 4.8). Rooting media, leaf area, growth hormones and concentration of hormones significantly affected the length of the longest root per cutting ($P < 0.05$) (Appendix 7).

4.6.5 Number of callused cuttings

There were significant differences in the various interactions of all the factors on the number of callused cuttings. Sand had the highest mean value of 3.86 (Plate 4.3) while mixture of sand and sawdust had the least mean value of 2.51 (Table 4.10). Cuttings with half leaf area recorded the highest mean value of 2.25 while full leaf area had 1.37 (Table 4.8). The control experiment (zero concentration of hormone) had the highest mean value of 3.50. Cuttings treated with 150 mg/l concentration of hormone and 200 mg/l concentration of hormones had values of 2.95 and 1.02 respectively (Table 4.8). Rooting media, leaf area, growth hormones and concentration of hormones significantly affected the number of callused cuttings ($P < 0.05$) (Appendix 8).

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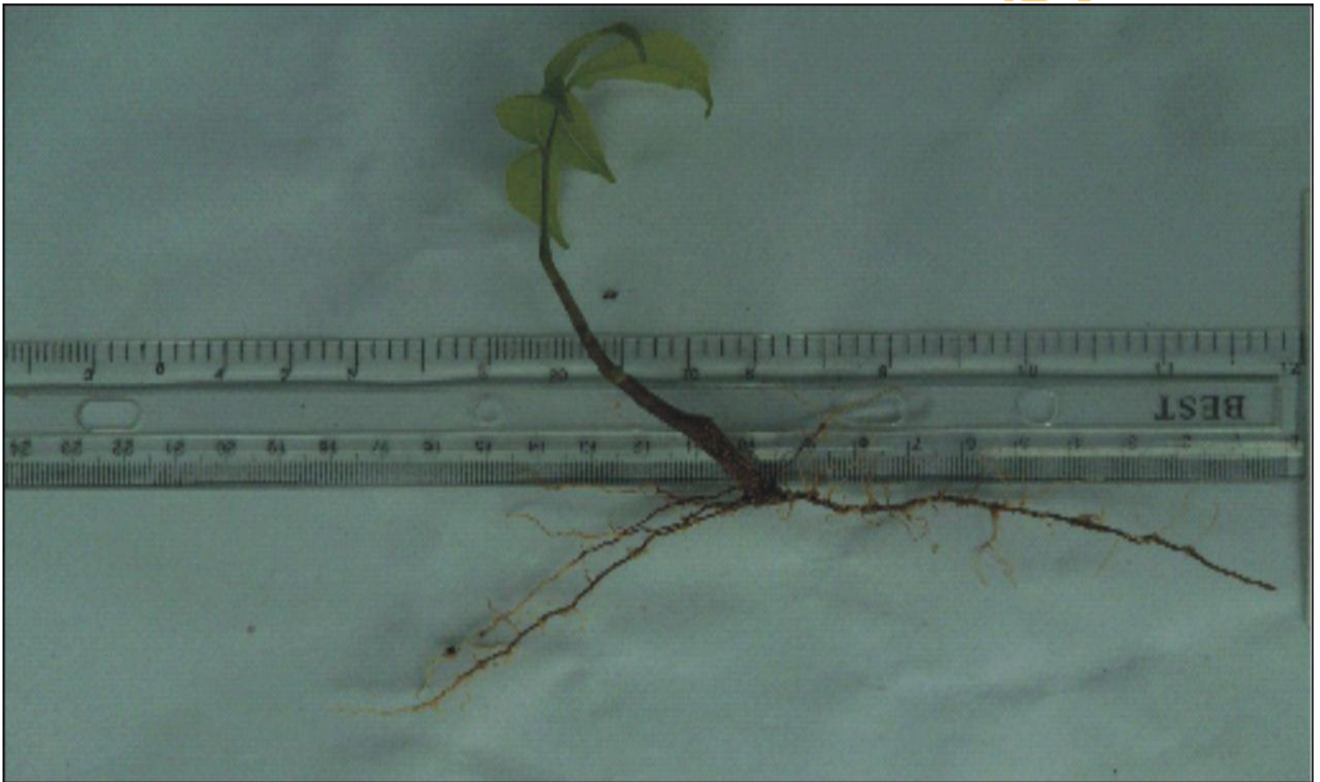

Plate 4.1: Cutting of *K. grandifoliola* with developed root and shoot

Plate 4.2: Rooted cutting of *K. grandifoliola*

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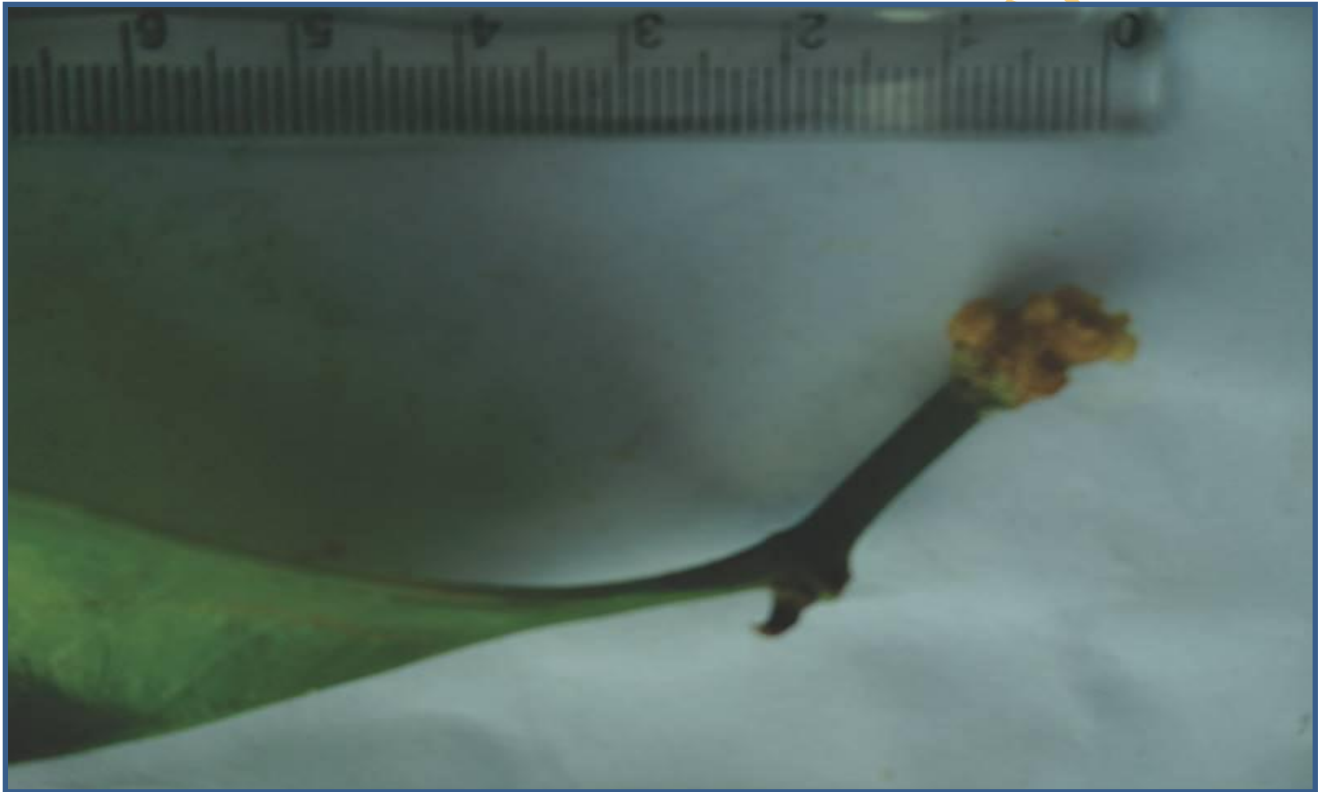

Plate 4.3: Callused cutting of *K. grandifoliola*

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4.7.0 **In vitro *Propagation of* K. grandifoliola**

In vitro propagation of *K. grandifoliola* was achieved with embryos using modified MS medium (Plate 5.1 – 5.4). Despite strict aseptic conditions that were observed in the laminar flow cabinet, high rate of contaminations on the embryo cultures were still observed (75% in some cultures). It was further observed that all embryo cultures contaminated never regenerated.

4.7.1 Shoot length

The highest mean shoot length (7.40cm) was recorded from plantlets cultured on treatment G (MS-medium supplemented with 0.05mg/L (BAP) + 0.01mg/L (NAA) + 10mg/L Adenine Sulphate) which was followed by 5.87cm from cultures on treatment H (MS-medium supplemented with 0.05mg/L (KIN) + 0.01mg/L (NAA) + 10mg/L Adenine Sulphate) while, the lowest mean of 2.70cm was recorded for explants cultured on treatment I (Control: MS Only) (Table 4.12). There were significant differences among the different concentrations of the cytokinin BAP and the auxin NAA on the regeneration of plantlets ($P < 0.05$) (Appendix 9).

4.7.2 Root length

Plantlets cultured on treatment H (MS-medium supplemented with 0.05mg/L (KIN) + 0.01mg/L (NAA) + 10mg/L Adenine Sulphate) recorded the highest mean of 7.53cm, which was followed by 5.5cm from plantlets cultured on treatment G (MS-medium supplemented with 0.05mg/L (BAP) + 0.01mg/L (NAA) + 10mg/L Adenine Sulphate. The lowest mean of 1.47cm was recorded for explants cultured on treatment I (Control: MS Only) (Table 4.12).

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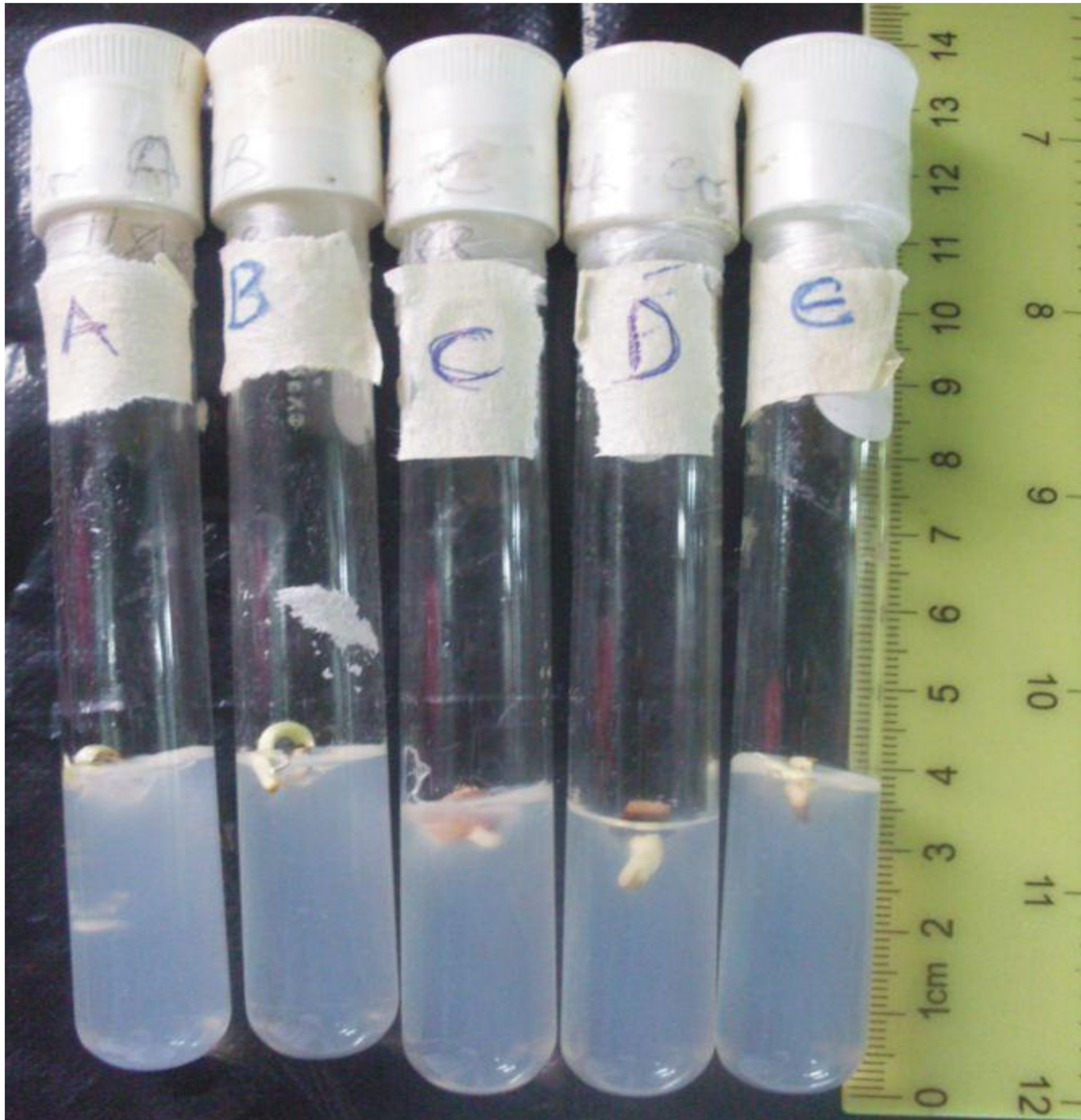

Plate 5.1: One week old *in vitro* embryo culture of *K. grandifoliola*

Plate 5.2: Four weeks old *in vitro* embryo culture of *K. grandifoliola*

Plate 5.3: Twelve weeks old *in vitro* embryo culture of *K. grandifoliola*

Plate 5.4: Twenty-four weeks old *in vitro* embryo culture of *K. grandifoliola*

Table 4.12: Mean values of *In vitro* propagation traits of *K. grandifoliola* plantlets

Treatment	Media	Shoot Length (cm)	Root Length (cm)	Number of node	Number of root
A	0.125 mg/l (BAP) + 0.01 mg/l (NAA)	4.67^{f}	1.53^{g}	2.0^{c}	2.0^{b}
B	0.125 mg/l (KIN) + 0.01 mg/l (NAA)	4.20^{g}	3.50^{f}	3.0^{b}	1.0^{c}
C	0.15 mg/l (BAP) + 0.01 mg/l (NAA)	4.82^{e}	4.53^{c}	2.0^{c}	1.0^{c}
D	0.15 mg/l (KIN) + 0.01 mg/l (NAA)	3.70^{h}	4.20^{d}	3.0^{b}	1.0^{c}
E	1mg/l (BAP) + 0.01 mg/l NAA	5.78^{c}	4.03^{e}	3.0^{b}	1.0^{c}
F	1mg/l (KIN) + 0.01 mg/l NAA	4.92^{d}	4.50^{c}	3.0^{b}	3.0^{a}
G	0.05 mg/l (BAP) + 0.01 mg/l (NAA) + 10 mg/l (Adenine sulphate)	7.40^{a}	5.53^{b}	3.0^{b}	3.0^{a}
H	0.05 mg/l (KIN) + 0.01 mg/l (NAA) + 10 mg/l (Adenine sulphate)	5.87^{b}	7.53^{a}	4.0^{a}	3.0^{a}
I	Control (MS only)	2.70^{i}	1.47^{g}	2.0^{c}	1.0^{c}
LSD		0.14	0.13	00 0.00	0.00

NB: Means with the same letter under each column are not significantly different at P<0.05. IBA – Indole butyric acid, NAA – Napthalene acetic acid, IAA – Indole acetic acid, KIN – Kinetin, BAP – Benzyl amino purine, ADS – Adenine sulphate

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4.7.3 Number of nodes

The highest mean of 4 nodes was recorded from plantlets cultured on treatment H (MS-medium supplemented with 0.05mg/L (KIN) + 0.01mg/L (NAA) + 10mg/L Adenine Sulphate), which was followed by 3 nodes from plantlets cultured on treatments B, D, E, F and G. The lowest mean of 2 nodes were recorded for explants cultured on treatment A [MS-medium supplemented with 0.125mg/L (BAP) + 0.01mg/L (NAA)] and I (Control: MS Only) (Table 4.12). The mean value for number of nodes of the plantlets cultured on treatments A, C and I were not significantly different from each other. However, there were significant difference between the mean numbers of nodes of the plantlets cultured on treatment A and treatments B, D, E, F, G, H.

4.7.4 Number of roots

Plantlets cultured on treatments F, G and H recorded the highest mean of 3 roots which was closely followed by 2 roots from plantlets cultured on treatment A (table 4.12). The lowest mean 1 root was recorded for explants cultured on treatments B, C, D, E, and I. The mean number of roots of the plantlets cultured on treatments B, C, D, E and I were not significantly different from each other. However, there was significant difference between the mean number of roots of the plantlets cultured on treatment A and other treatments. Number of root was significantly different among the different concentrations of the cytokinin BAP and the auxin NAA on the regeneration of plantlets ($P < 0.05$) (Appendix 9).

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4.8.0 Molecular Characterization *of K. grandifoliola*

4.8.1 Extraction of DNA

DNA extracted from young leaves of *Khaya grandifoliola* using a modified Dellaporta protocol (Dellaporta *et. al.,* 1983) yielded good quality. Results from the 1.2% agarose gel electrophoresis showed a clear and clean band intensity of DNA (Plate 6.1). The quality was further estimated by measuring the optical density (OD) at wavelength 260 and 280 and the absorbance ratio (OD 260/OD 280), which varied between 1.528 and 1.662 (Table 4.13).

4.8.2 RAPD analysis

The amplification of DNA with the aid of six RAPD primers was clear as the PCR analysis carried out on the six collections sources produced a large number of distinct fragments for each primer. Six selected arbitrary primers generated a total of 109 scorable bands of which 94 were polymorphic, with an average of 18.17 amplicons per primer (Table 4.14). The RAPD profile for the 6 collections yielded by OPD 08 and OPD 11 are shown in Plates 7.1 *a* and *b.* The numbers of amplified products obtained were in the range 4–27 with the primer OPD 13 producing the minimum number of (4) bands while primers OPA 18 and OPD 08 produced the maximum number of bands (26 and 27) respectively.

4.8.3 Dendrogram analysis

Genetic similarity among sources of the 6 collections was scaled between 0.4 and 0.9 (Jaccard's similarity coefficient). The unweighted pair group method with arithmetic average (UPGMA) dendrogram produced using neighbour joining method of cluster analysis separated all the 6 collections into one cluster and one ungrouped at 0.53 similarity coefficient (Fig. 4.5).

Cluster 1 consisted of collections from Kutia, Owo, Boki, Oba-Akoko and Ayegunle-Akoko while collection from NACGRAB was ungrouped. Cluster 1 showed high genetic resemblance at 0.53 similarity coefficient even though their collection sources were distant apart. However, within this Cluster, there was a sub-cluster which contained collections from Kutia and Owo. At 0.65 coefficients of similarity one distinct cluster which comprises collections from Kutia, Owo, Boki and Oba-Akoko; two ungrouped, which were collections from Ayegunle-Akoko and NACGRAB. The higher the coefficient of similarity, the more the collections were resolved into distinct clusters (Fig. 4.6).

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Table 4.13: DNA spectophotometric readings of the six *K. grandifoliola* seed sources from Southern Nigeria.

S/n	Tube No.	Conc (nm). At OD 260	OD260 $^{0}A_1$	OD280 $^{0}A_2$	$^{0}A_1/^{0}A_2$
1	A	179	1.798	1.102	1.632
2	B	178	1.774	1.161	1.528
3	C	41	0.41	0.250	1.637
4	D	7	1.075	0.647	1.662
5	E	178	1.778	1.118	1.590
6	F	179	1.788	1.143	1.564

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Table 4.14: Six RAPD primers sequence and amplification characteristics of the six sources of *Khaya grandifoliola* seeds from Southern Nigeria

No	RAPD Primers	Sequence 5’ - 3’	Total (scorable) bands	Polymorphic bands (%)
1	OPD-08	>GTG.TGC.CCC.A<	27	22 (77.8)
2	OPD-11	>AGC.GCC.ATT.G<	18	18 (100)
3	OPD-13	>GGG.GTG.ACG.A<	4	1 (25)
4	OPA-18	>AGG.TGA.CCG.T<	26	20 (76.9)
5	OPD-18	>GAG.AGC.CAA.C<	17	17 (100)
6	OPD-20	>ACC.CGG.TCA.C<	17	17 (100)

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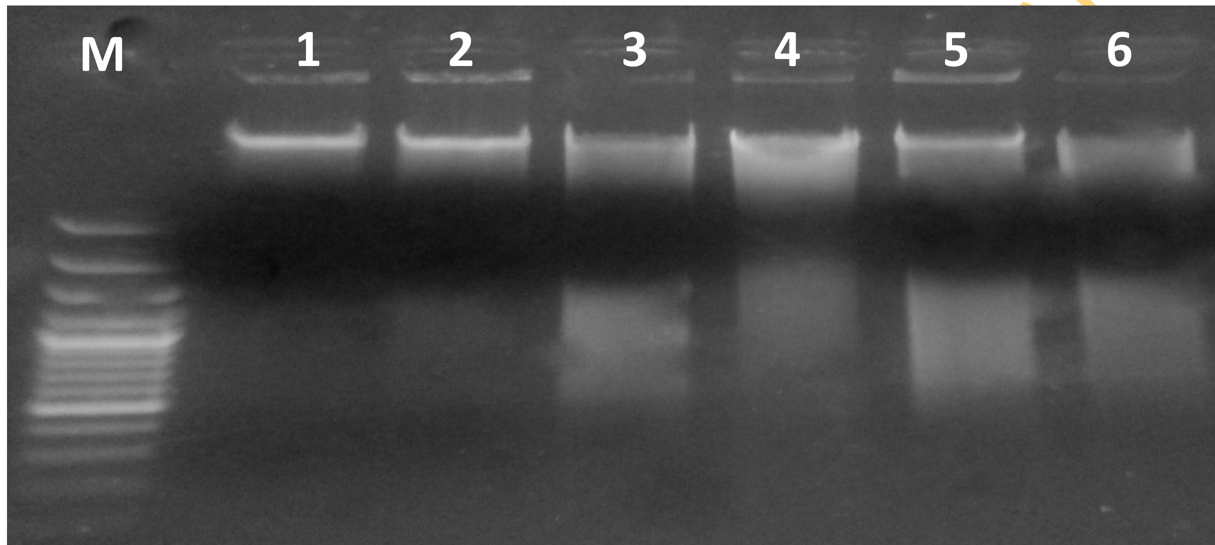

Plate 6.1: Electrophorogram of extracted *K. grandifoliola* Genomic DNA leave samples from the six sources.

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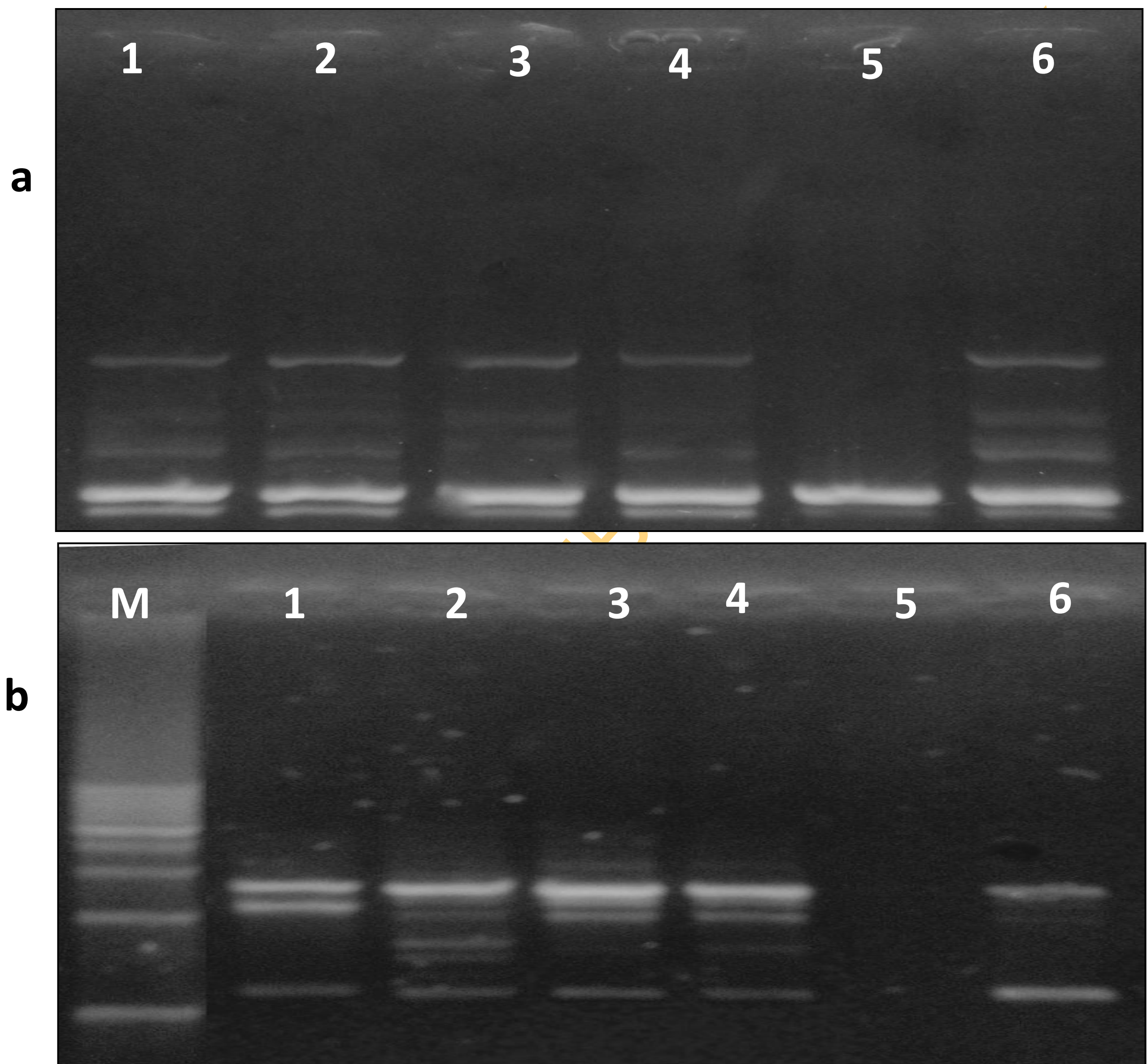

Plate 7.1: ***a*, *b*.** RAPD profiles for *Khaya grandifoliola* generated by primer OPD 8 (***a***) and primer OPD 11 (***b***) Lane 1: Kutia, Lane 2: Ayegunle-Akoko, Lane 3: Oba-Akoko, Lane 4: Owo, Lane 5: NACGRAB, Lane 6:Boki, Lane M = 1-kb ladder.

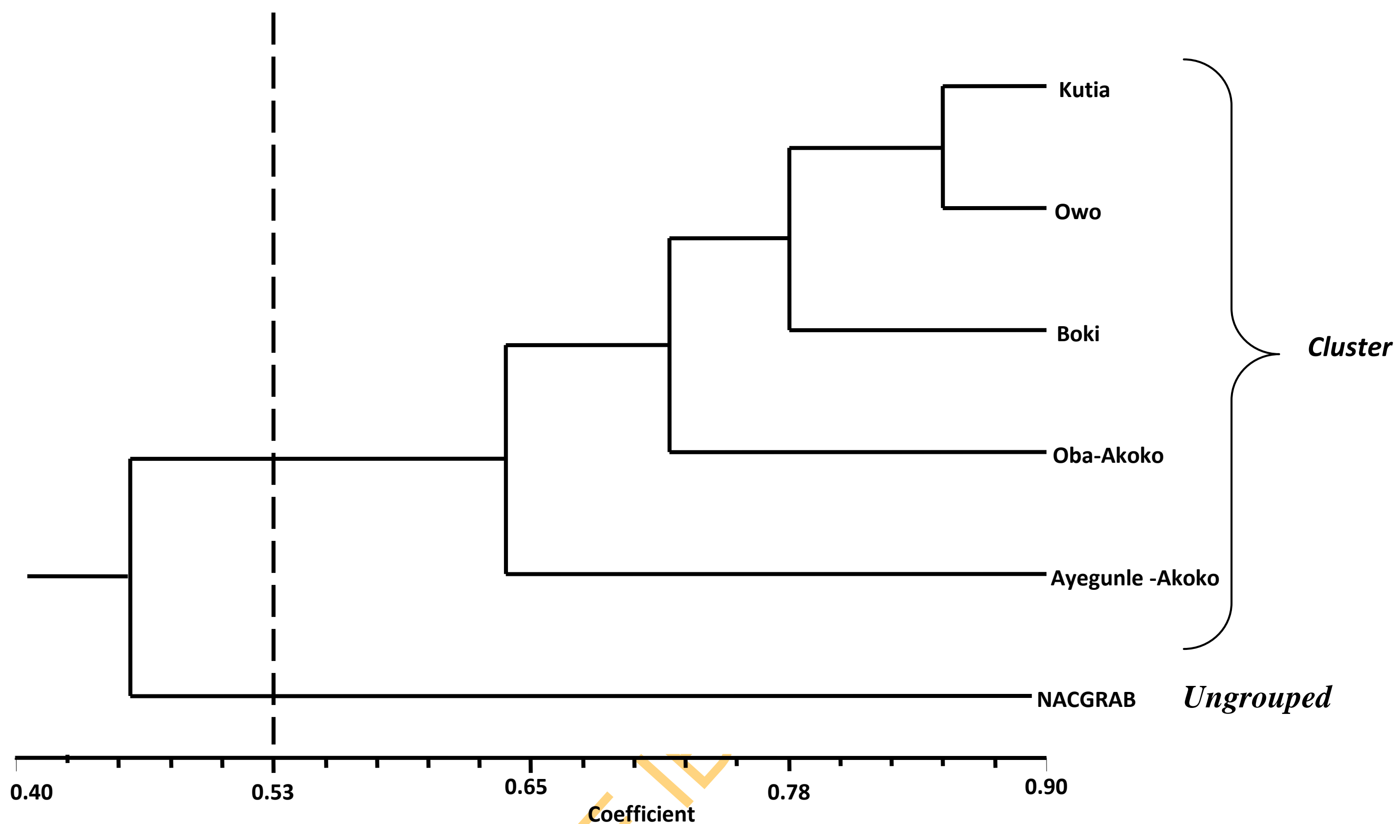

Fig.4.5: Dendrogram of the six *Khaya* genotype collections from Southern Nigeria based on the similarity index by RAPD markers at Jaccard's similarity coefficient of 0.53

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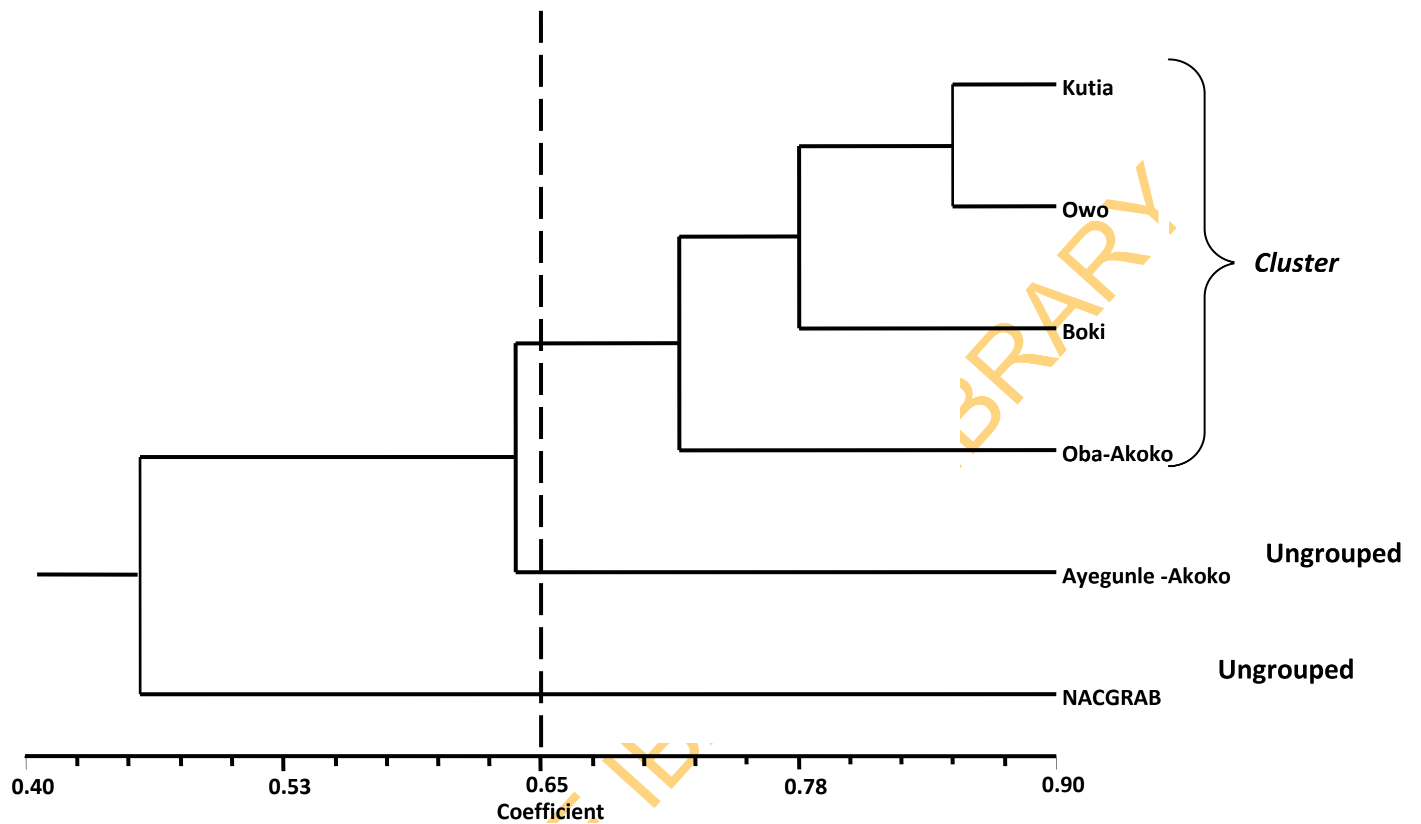

Fig.4.6: Dendrogram of the six *Khaya* genotype collections from Southern Nigeria showing three distinct Clusters at Jaccard's similarity coefficient of 0.65.

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CHAPTER FIVE

5.0 DISCUSSION

5.1 Morphological Variation in Seeds of *K. grandifoliola*

Variation in traits among seed sources are usually influenced by different intensities of natural constraints prevailing in the geographical range of a particular species. This variation is advantageous for wide range of adaptability. In this study, seeds of *K. grandifoliola* from six different sources studied exhibited a strong variation in all the metric characters (seed weight, seed length and seed width) investigated. Variation in the seed metric character may be an expression of possible genetic or environmental variations between the sources. NACGRAB recorded the highest seed weight and this could be attributed to large food reserves in the seeds. This might also have accounted for the early comparative growth advantage in the resultant seedlings. The finding in this study is in tandem with those of Micheal *et al.,*(1988) who observed that variation in seed metric character among species of *Zostera marina* L. (Zosteraceae) was linked with the environmental gradients of soil moisture and light intensity. Probert (2000) also observed variations among and between populations of different tree species in different traits studied. Different seed sources of *Dalbergia sissoo* also exhibited such significant variations in seed traits (Singh and Sofi, 2011). Mukherjee *et al.,*(2004) reported significant variations in cone traits on 63 seed sources of *Pinus roxburghii*. Significant variability in seed characters was also observed by Annapurna *et al.,* (2005) in seed from clonal seed orchard of *Santalum album* in southern India. Rawat and Bakshi (2011) in their study on provenance variation in cone, seed and seedlings of *Pinus wallichianana* also reported significant variability in the seed and seedling traits. Since collection sources had a great influence on the seed variations; in seed collection for orchard or plantation establishment, seed weight should be considered very important so as to select suitable seed sources with desirable traits.

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5.2 Morphological variation in seed germination and seedling growth of *Khaya grandifoliola*

Trees are predominantly outcrossing, thus resulting in progeny that segregate with respect to parental traits thereby affording the opportunity for selection. Since most seed collections for orchard or plantation establishment are obtained from naturally occurring trees, the evaluation of the superiority of seed growth from different sources is of paramount importance (Akinyele, 2007). In this study, variations were observed in seed germination percentage and seedling growth (seedling height, height of branching, number of branches, internode length and number of leaves) among the seed sources. The seed characters expressed in terms of germination and seedling growth differed during the study period indicating genotype-environment-interaction. This might be due to the differences in the genetic makeup of various seed collection sources and environmental factors during seed development. Germination and growth of seedlings are influenced largely by the food reserve in seeds, which increases with seed weight (Khan and Shankar, 2001, Benard and Toft, 2007). Seeds collected from NACGRAB were heavier than seeds from other sources and this might have explained why these seeds had higher germination percentage and better seedling growth. This implies that seed weight had a great influence on nursery germination and this agrees with the observations of Gunaga *et al.,* (2007) who noted that bigger sized seeds in *Pongamia pinnata and Vateria indica* showed higher seed germination and seedling vigour. Singh and Pokhriyal (2001) also reported significant variations in seed traits and seedling growth in *Dalbergia sissoo* collected from different sources. However, seed weight may have had some influence on nursery germination as revealed by this study but Indira *et al.,* (2000) in their study on *Tectona grandis* revealed that fruit size (except for very small seeds) did not have influence on seed germination, seedling survival or seedling growth in the species. Seedling collar diameter did not differ significantly during the study. This suggested that seedlings collar diameter at nursery stage were uniform. This seems to negate the findings of Maku *et al.,* 2014, which reported different hormones at different concentration had a positive effect on the seedling collar diameter. Diameter growth reduced when height growth increased which suggested that appearance of inherited characters for collar diameter growth and development was species oriented (Md. Salim Azad *et al*., 2014). Besides these, crowding of seedlings at nursery bed may have a big consequence on collar diameter growth. Collar diameter growth and development appeared to be more susceptible to climatic factors than height. Loha *et*

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al., (2006) suggested height growth can be much more important at the early stage than diameter growth.

5.3.0 Biomass estimation

5.3.1 Relative growth rate (RGR)

The RGR of a plant is the reflection of the increase in dry weight by unit biomass per unit time. Data on RGR had similar trend with NAR in this study. Seedlings from Ayegunle-Akoko, Oba-Akoko and NACGRAB recorded positive values for RGR throughout the experiment period. Similarly, seedlings from Kutia, Owo and Boki recorded positive NAR values throughout except for weeks: 4-6 (Kutia), 2-4 (Owo) and 4-6 and 8-10 (Boki) that had negative values. According to Atkin *et al*., (1998), the RGR of plant species from contrasting environment is highly variable and this is true in Ayegunle-Akoko, Oba-Akoko and NACGRAB. Although, Raddad (2007) and Abdelbasit *et al.,*(2012) in their RGR studies on *Acasia senegal* and *Acasia tortilis* respectively, from different provenance revealed no significant difference in RGR. In this study, NACGRAB, Oba-Akoko and Ayegunle-Akoko recorded high leaf area values as well as positive RGR throughout the study period. It was also recorded in this study that most provenances that recorded positive RGR equally observed the same in NAR. This was clearly evident in NACGRAB, Oba-Akoko and Ayegunle-Akoko that had high NAR values and also had positive RGR values throughout the study period.

Summarily, seedlings from NACGRAB had the best overall performance in biomass estimation followed by Ayegunle-Akoko while Boki had least performance. This is observed in their RGR and NAR recordings which clearly showed that throughout the study period NACGRAB seedlings maintained positive results while seedlings from Boki recorded more negative results than other seedlings.

5.3.2 Net Assimilation Rate (NAR)

The Net Assimilation Rate (NAR) of plants is a reflection of the photosynthetic efficiency of the leaves. In this study, seedlings from Ayegunle-Akoko and NACGRAB recorded positive values for NAR throughout the experiment. Also seedlings from Kutia, Oba-Akoko, Owo and Boki recorded positive NAR values throughout except for weeks: 4-6 (Kutia), 6-8 (Oba-Akoko), 2-4 (Owo) and 4-6 (Boki) that had negative values. The study further revealed that seedlings from Ayegunle-Akoko and NACGRAB had a high leave area values compared to

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those from other sources and this may not be unconnected with a highly efficient photosynthetic ability of its leaves that resulted in overall positive NAR values recorded by Ayegunle-Akoko and NACGRAB. In biomass study, seed mass is a very important component because it represents the reserves available for growth in the first stages of plant establishment. Variation in seed mass is an important trait which may have consequences for growth and survival of seedlings. Seedlings derived from higher seed mass often have greater biomass allocation than seedlings derived from lower seed mass (Martinez-Vilalta *et al.,* 2007) and this is very true for seed collected from NACGRAB and Ayegunle-Akoko. In the same vein seedlings from bigger seeds allocated photosynthates more to the roots than the leaves (Benard and Toft, 2007) and this was found to have been exhibited by the seedlings from NACGRAB seed source. However, the equally heavy seeds of Ayegunle-Akoko allocated dry matter more to the leaves than the roots in the seedlings (Vaughton and Ramsey, 2001).

5.4 Morphological and Molecular Characterization

In this study, the RAPD technique was very useful, thus providing important information about the genetic pattern of *K. grandifoliola* populations. The genetic similarity among the six collection sources of *K. grandifoliola* was relatively low at 0.45 similarity coefficient, however, the collection from NACGRAB was distinct from the other *K. grandifoliola* collections. Low genetic diversity estimates have also been reported in cotton using different DNA marker systems (Lacape *et al.,*2007; Wang *et al.,*2007; Rahman *et al.,*2008). Although collections from Kutia, Owo, Boki, Oba-Akoko and Ayegunle-Akoko have great resemblance at 0.53 similarity coefficient but collection from Boki was more closely related to Kutia and Owo than Ayegunle-Akoko and Oba-Akoko. Meanwhile, morphologically all the six collections were distinct from one another except for collections from Ayegunle-Akoko and Oba-Akoko that showed high genetic relationship at 0.08 similarity coefficient. This finding is in agreement with those of Ullah *et al.,*2012 who reported a very high similarity among 19 Cotton collections studied. The present study clearly showed that all the six were from different ancestors except for Ayegunle-Akoko and Oba-Akoko. This may be true because the two collections were close to each other in location. Furthermore, from the morphological characterization in this study, it was also clear that if the six *Khaya grandifoliola* sources were to be classified, five different collection groups with Ayegunle-Akoko and Oba-Akoko grouped as one would be seen while, molecular

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characterization would group them into three clusters at the most with NACGRAB and Ayegunle-Akoko distinct; and Kutia, Owo, Boki and Oba-Akoko would be grouped as one.

5.5 Storage Temperature and Duration of Storage

Most tropical forest tree seeds are short lived under natural condition due to their recalcitrant nature, apart from their irregular and erratic flowering and fruiting patterns which combine and hamper the availability of seeds for regeneration purposes. Some tropical species have alternate heavy and light seeds years (Okoro, 1983, Oni, 1992). Therefore, seeds produced during a buoyant fruiting year needed to be stored in order to secure the supply of good quality seeds for a planting programme whenever needed.

In this study *K. grandifoliola* seeds maintained their viability for five (5) months in 24°C Short Term (ShT) storage facility. However, the viability of seeds of *K. grandifoliola* decreased with increasing storage period under the different temperature regimes. This is in agreement with the findings of Yilmaz and Aksoy (2007) and Zaman (2013) who reported gradual declines in the germination percentage and/or seed viability with increase in storage period irrespective of different storing conditions of *Swertia chirayita* seeds and reduction in seed germination percentage of *Zygophyllum qatarense* seeds with storage respectively. However, Wang *et al.,* (2010) reported an increase in seed germination with the increase in duration of seed storage time in some sub-alpine species. The study further revealed that the viability of *K. grandifoliola* seeds under cold storage condition lost viability more rapidly compared to a warmer temperature. The poor storability in cold conditions may have resulted from the damp nature in these facilities which might have instigated mild imbibition of moisture by the dry and crispy seeds. The result suggests that there is no need to store *K. grandifoliola* seeds at a cool temperature if planting is to be done within six months of harvest.

5.6 Vegetative Propagation

Vegetative propagation provides an alternative source of planting stock for a wide range of useful indigenous species especially those that are threatened or at the verge of extinction. There were variability in the number of rooted leafy stem cuttings and number of roots per cuttings in response to rooting media types, auxin types and auxin concentrations.

The importance of rooting medium for rooting of cuttings is widely recognized (Tchoundjeu *et al.,*2004, Atangana *et al.,*2006). Evidence from this study indicates that rooting

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medium strongly affects rooting percentages of *K. grandifoliola* cuttings, sawdust being the best compared to washed river sand and 50:50 mixtures of sand and sawdust. This agrees with the high rooting percentages observed in the water retentive sawdust by Mialoundama *et al.,* (2002) and Tchoundjeu *et al*. (2002, 2004) for *Dacryodes edulis*, *Prunus africana* and *Pausynistalia johimbe*, respectively. It also concurs with the observations Akinyele (2010) made on the effect of rooting media on the cuttings of *Buchholzia coriacea.* Soil naturally gives mechanical support to plant and also provides them with the necessary nutrients which they need to grow and develop by aiding the anchorage of the roots to the numerous organic matters locked in them. River sand was too porous and could not keep enough humidity required by the cuttings. The presence of sufficient nutrients in cured sawdust gave it an edge over washed river sand (that did not contain any nutrient) and the mixture of sand and sawdust. However, it contrasts the high rooting percentages identified in sand media in *Irvingia garbonesis* (Okere, 1999), *Garcinia kola* leafy stem cuttings (Nyansi, 2004), *Vitellaria paradoxa* (Yeboah and Amoah, 2009). An appropriate rooting medium for a particular species would generally have an optimal volume of gas-filled pore space and an oxygen diffusion rate adequate for the needs of respiration (Andersen, 1986). This condition was perhaps adequately provided by sawdust in the study of this species.

The ability of stem cuttings to form adventitious roots depends on interaction of many exogenous and endogenous factors, including hormone. Most reports of adventitious root induction of woody species have involved treatments with exogenous auxins such as IBA, NAA or IAA (Ainsley *et al.,* 2001). Two main effects of applying auxin to cuttings are to increase the supply of carbohydrates at the cutting base for root formation (Dick and Dewar 1992) and also to increase percentage of rooting (Ullah *et al.,* 2005). The exogenous auxins are only required at an early stage to stimulate emergence of new formed roots (Dobranszki and Silva, 2010).

Auxins are reported to play a significant role in stimulating root initiation in stem cuttings of woody plants (Tchoundjeu *et al.,* 2002, 2004). Differences in rooting frequency depending on exogenous auxins or a combination of auxins used have been reported (Klass *et al.,* 1987; Poupard *et al.,* 1994). Among the three auxins (IAA, IBA & NAA) used in this study, the application of IBA to the base of this mahogany cuttings had the most promotive effect on rooting of the single node leafy stem cuttings of *K. grandifoliola.* This agrees with Okere (1999), Abdullah *et al.,* (2005), Akwatulira *et al., (*2011), Rana and Soods (2012) who observed rooting in *Irvingia gabonensis, Baccaurea sapida, Warburgia ugandensis* and *Ficus roxburghii,* leafy

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stem cuttings treated with IBA respectively. Out of the six auxin concentration levels investigated in this study, 150 mg/l seems to favour rooting of single node stem cuttings of *K. grandifoliola*, with 150 mg/l IBA surpassing all other concentration levels in rooting performance. Cuttings with half leaf (50% of the original size) also promoted rooting the most. This finding agrees with Akinyele (2010) who reported best rooting in cuttings of *Buchholzia coriacea* treated with IBA 150 mg/l but contradicts the high root length values recorded for cuttings with full leaf (100% of the original leaf size). Among the *Khaya* species, *K. grandifoliola* is characterized by its large leaf and this could have been the reason why the cuttings with half leaf recorded better rooting results. Besides, the high evapotranspiration rate in the leafy stem cuttings will be higher in full-leaf than in half-leaf coupled with the low reserves in the cuttings.

5.7 *In vitro* Propagation

Cytokinin stimulates the initiation and activity of axillary meristems which result in shoot formation (Dobranszki and Silva, 2010); hence cell division, shoot multiplication and axillary bud formation can be promoted by the cytokinin BAP (Sutter, 1996). Meanwhile, roots formation in tissue culture can be induced by exogenous auxins such as IBA, NAA and IAA and their interaction with endogenous auxins which cannot be sufficiently synthesized by many tissues and small organs isolated *in vitro* (Thorpe *et al.,*2008). It is also reported that for optimum shoot growth and development BAP is required at low concentrations ranging from 0.5 to 2.5 mg/l (Thorpe *et al.,*2008).

The optimization of MS medium by supplementing with the right auxin and cytokinins combination and concentration is a sure way of prompt regeneration of plantlets in culture. This study revealed that the optimum growth medium that will support the culture of *K. grandifoliola* embryo in growth media at different concentrations of benzylaminopurine (BAP), Kinetin (KIN) and a constant concentration of naphthalene acetic acid (NAA) and Adenine sulphate (ADS) with the Murashige and Skoog media as the basic media is 0.05mg/l (BAP) + 0.1mg/l (NAA) +10mg/l Adenine sulphate. This could be due to the interactive effects of BAP and Adenine sulphate on cell division and its ability to increase stem thickness thereby elongating the shoot length. This agrees with Sivanesan and Jeong (2009) and Punyarani and Sharma (2010) who observed simultaneous shoots and roots in the nodal cultures of *Plumbago zeylanica* and *Costus speciosus* respectively. Other observation made in this study revealed that, increase in the

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concentration between kinetin and auxin (NAA) tends to increase root length better than BAP and NAA interaction. This findings are in agreement with those reported by Bustamante and Heras (1990) on Cacti (*Pelecyphora aselliformis)* and *Nealolydial aphophoroides;* Feng *et al.,* (2000) on *Aloe barbebsis* and Mata-Rosas *et al*., (2001) on *Turbini capuslaui* that using a high concentration of BAP and NAA in different concentrations was a limiting factor for shoot formation but increases root formation.

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CHAPTER SIX

6.0 SUMMARY AND CONCLUSION

6.1 Implication for conservation

The results of this study portray a broad morphological and molecular diversity among the sources of *K. grandifoliola.* The highly significant differences in quantitative characters of the seeds and seedlings growth from the study indicate that a good selection can be made from the seed sources. The results of the study also offer an optimization of RAPD primers screening for the evaluation of genetic relationships among the six collection sources of *K. grandifoliola* from some parts in southern Nigeria. RAPD has all the advantages of PCR based marker with added benefits: (a) Primers are commercially available and do not require any prior knowledge of the target DNA sequence; (b) Simplicity (c) Broad applicability and low cost (Baig *et al.,* 2008). Gonzalez *et al*., (2005) reported that accurate and fast molecular marker based assessment of the levels of genetic diversity and degree of genetic relatedness are in demand for the efficient management of the existing genetic resources either for conservation purposes or for breeding programme utilization. For instance, molecular markers are needed to generate information about the extent and distribution of genetic diversity which is essential for the conservation of genetic resources. In this context, one of the objectives of the study was to assess the genetic diversity in the natural populations of the species to facilitate the development of appropriate conservation strategies for *K. grandifoliola.* This study provided information on the genetic variation in the natural population of the species and helped in identifying seed sources for conservation programmes. Programmes of *in situ* conservation should be implemented to create strategies for maintaining wild populations' genetic diversity by appropriate forest management since diverse populations were observed in the course of the study. This will allow the dynamic evolutionary events to continue in the original sites. However, Berthud and Charrier (1988) suggested that genetic diversity can also be increased by establishing field gene bank as in the case with NACGRAB where collections of the *K. grandifoliola* genetic resources are conventionally maintained as living trees.

Although the results indicated that the species is amenable to both vegetative and *in vitro* propagation as an alternative means of regeneration, the results also offer an optimum storage

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condition for *K. grandifoliola* since tropical tree species have irregular fruiting pattern besides being a recalcitrant species. From all the seeds and seedlings growth parameters assessed, NACGRAB source had the best overall performance.

6.2 Implication for breeding

The present investigation provided information not only to design appropriate conservation strategies, but also genetic diversity information needed in breeding programs. Forest geneticist and breeders would be interested in genetic diversity information in order to focus on the populations with high genetic variability to select parental *K. grandifoliola* for crossing. By crossing genetically more diverse parent, the level of variation present in the segregating populations can be maximized. The collection from NACGRAB and Kutia were observed to have the most diverse traits, hence, special attention or priority should be given to them. For instance, in Coffee, Ameha (1990) reported breeding as an appropriate mechanism to significantly and rapidly increase productivity in hybrid varieties which is attributed to presence of diverse genotype. However, the importance of vegetative propagation cannot be overemphasised in tree improvement and breeding, as well as in conservation programmes, because it avails the forest geneticist the possibility of capturing and transferring to a new tree all the genetic potential from the ortet (i.e the original individual tree) for certain traits. The narrow-sense heritabilities for selection of traits in tree breeding have demonstrated that genetic gains can be doubled by using vegetative propagation (Hettasch *et al.,* 2009). Another valuable advantage of vegetative propagation is that in some situations, it can contribute to accelerating the realization of results from tree improvement programmes since the desired genetic qualities of selected trees can be utilized rapidly without having to wait for the complexities of flowering, pollination and seed production before producing propagules for planting operations.

6.3 Conclusion

In the study area, *Khaya grandifoliola* has a broad morphological and molecular diversity and as such a very good selection can be made for conservation and improvement programmes. The species is easily propagated through seeds or cuttings. Due to erratic flowering and fruiting plus the recalcitrant nature of the species, the study has shown that with storage at short term (24°C), seeds could remain viable for five months thus granting genebank managers base line information on seed storage. *K. grandifoliola* amenability to both *in vitro* and stem cuttings

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propagation will make possible the establishment of large scale plantation. The study has also provided geneticists and breeders basic scientific information necessary for molecular breeding and improvement of the species.

6.4 Recommendations

Upon the results of this study the following recommendations could be suggested:-

- Propagules from NACGRAB could be considered in initiating improvement programme as well as establishing orchards for seed collection activities for *K. grandifoliola.*
- Storage of *K. grandifoliola* seeds in Short term (ShT) storage facility (24°C) could sustain at least 70% viability for 20 weeks.
- When seeds are not readily available, propagules for plantation establishment could be raised through juvenile single node leafy stem cuttings treated with IBA 150mg/l in cured sawdust or *in vitro* culture using embryo on a culture medium containing Murashige and Skoog (MS) basal medium supplemented with 0.05mg/l BAP + 0.1mg/l NAA +10mg/l Adenine sulphate.
- *In vitro* propagation of single node cuttings should be subjected to further research to solve the challenges of endophytic microbes and associated phenolic exudation.
- Breeding programme for improved morphological traits in this species should be through the crossing of NACGRAB and Kutia genotypes.
- More RAPD and microsatellite markers based genetic diversity studies should be carried out to confirm the high diversity observed in this study.

6.5 Contribution to Knowledge

1. Genetic variations in the natural population of the species in some part of Southern Nigeria were determined.
2. *K. grandifoliola* is amenable to both *ex vitro* (cuttings) and *In vitro* propagation techniques.
3. An optimum storage condition of 24°C was most suitable for *K. grandifoliola* seeds. However, seeds viability starts to drop after five (5) months.
4. *K. grandifoliola* from NACGRAB was best overall in seed traits and seedling growth parameters.

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5. *K. grandifoliola* from NACGRAB and Kutia were the most diverse in traits among the six sources. This indicates they have most distant relationship.
6. The study provided baseline information for further research on the morphological and molecular characterization on *K. grandifoliola.*

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APPENDICES

Appendix 1: Analysis of Variance for seed metric characters of *K. grandifoliola* from six sources in Southern Nigeria

Seed variables	Df	MS	F
Seed weight			
Source	5	0.23	30.09*
Error	174	0.01	
Seed length			
Source	5	4.41	21.61*
Error	174	0.20	
Seed width			
Source	5	1.76	21.79*
Error	174	0.08	

* = significant at $P < 0.05$

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Appendix 2: Analysis of Variance for seedlings metric character in *K. grandifoliola* from six sources in Southern Nigeria

Variable	Df	MS	F
Height (cm)			
Source	5	202.61	5.98*
Error	102	33.89	
Collar Diameter (cm)			
Source	5	17.88	0.97
Error	102	4.06	
Height of Branch(cm)			
Source	5	15.15	4.41*
Error	102	15.65	
No of Branch			
Source	5	0.30	5.92*
Error	102	0.32	
Internode Length(cm)			
Source	5	0.70	2.54*
Error	102	0.28	
No of leaves			
Source	5	635.35	6.86*
Error	102	92.61	

* = significant at $P < 0.05$

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Appendix 3: Analysis of variance for effect of seed source on seedling biomass

Variable	df	Ms	F
Leaf Area			
Source (S)	5	597737.82	2.71*
Harvesting period (H)	5	8435921.0	51.71*
S X H	25	213881.0	1.31*
Error	114	580476.70	
Leaf Dry Weight			
Source (S)	5	422.20	5.20*
Harvesting period (H)	5	102.71	18.40*
S X H	25	4.36	0.78*
Error	114	127.21	
Root Dry Weight			
Source (S)	5	499.56	4.59*
Harvesting period (H)	5	131.97	15.46*
S X H	25	8.42	0.99*
Error	114	108.76	
Stem Dry Weight			
Source (S)	5	129.94	2.53*
Harvesting period (H)	5	65.09	16.78*
S X H	25	3.70	0.95*
Error	114	51.25	

* = significant at $P < 0.05$

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Appendix 4: Analysis of variance for germination percentage of *K. grandifoliola* seeds stored in different storage temperature and for different durations.

Variable (Germination %)	Df	MS	F
Storage condition	3	862.69	21.60*
Storage duration	4	1187.75	297351*
Error	5		
Sum	12		

* = significant at P < 0.05

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Appendix 5: Analysis of variance for effect of growth hormone, rooting media and leaf area on rooting of juvenile stem cuttings of *K. grandifoliola*

Variable	Degree of freedom	Mean of square	F-Value
Rooting media (M)	2	36.00	5.60
Leaf Area (A)	1	305.00	46.14*
Growth Hormone (H)	2	44.70	4.10*
Auxin Concentration(C)	5	32.60	2.14*
M × A	2	21.30	0.94
M × H	4	15.44	1.40
M × C	10	9.21	1.34*
A × H	2	7.11	0.67
A × C	5	8.50	2.16
H ×C	10	11.21	0.72
M × A × H	4	14.06	1.02
M × A × C	10	3.64	0.67
M × H ×C	20	7.02	0.34
A × H × C	10	9.41	0.51
M × A × H × C	20	12.42	0.92*
Error	108		
Sum	215		

* = significant at $P < 0.05$

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Appendix 6: Analysis of variance for effect of growth hormone, rooting media and leaf area on rooting of juvenile stem cuttings of *K. grandifoliola*

Variable	Degree of freedom	Mean of square	F-Value
Rooting media (M)	2	132.10	27.04*
Leaf Area (A)	1	27.30	4.6*
Growth Hormone (H)	2	17.02	2.09*
Auxin Concentration(C)	5	4.57	0.26*
M × A	2	4.32	0.51
M × H	4	4.11	1.26
M × C	10	3.01	0.89
A × H	2	9.13	0.67
A × C	5	3.70	0.48
H ×C	10	5.20	1.57
M × A × H	4	3.90	0.82
M × A × C	10	4.81	0.76
M × H ×C	20	2.10	0.51
A × H × C	10	3.40	0.14
M × A × H × C	20	3.02	0.71*
Error	108		
Sum	215		

* = significant at $P < 0.05$

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Appendix 7: Analysis of variance for effect of growth hormone, rooting media and leaf area on rooting of juvenile stem cuttings of *K. grandifoliola*

Variable	Degree of freedom	Mean of square	F-Value
Rooting media (M)	2	125.20	22.07*
Leaf Area (A)	1	23.40	3.70*
Growth Hormone (H)	2	15.17	1.02*
Auxin Concentration(C)	5	5.02	0.76*
M × A	2	6.72	1.41*
M × H	4	5.20	1.47
M × C	10	4.02	1.02*
A × H	2	7.45	0.96*
A × C	5	4.20	0.57*
H ×C	10	5.92	0.81*
M × A × H	4	4.10	0.46
M × A × C	10	3.20	1.78
M × H ×C	20	3.10	0.63*
A × H × C	10	4.03	1.51
M × A × H × C	20	5.06	2.51*
Error	108		
Sum	215		

* = significant at $P < 0.05$

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Appendix 8: Analysis of variance for effect of growth hormone, rooting media and leaf area on rooting of juvenile stem cuttings of *K. grandifoliola*

Variable	Degree of freedom	Mean of square	F-Value
Rooting media (M)	2	41.24	7.20*
Leaf Area (A)	1	256.72	52.17*
Growth Hormone (H)	2	56.20	6.02*
Auxin Concentration(C)	5	34.74	3.71*
M × A	2	29.40	1.04*
M × H	4	17.20	0.56*
M × C	10	10.11	1.12*
A × H	2	9.14	0.51*
A × C	5	7.20	1.14*
H ×C	10	12.01	0.56*
M × A × H	4	13.06	0.94*
M × A × C	10	4.02	1.02*
M × H ×C	20	6.14	0.57*
A × H × C	10	8.06	0.69*
M × A × H × C	20	10.64	0.62*
Error	108		
Sum	215		

* = significant at $P < 0.05$

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Appendix 9: Analysis of variance for *In vitro* embryo propagation of *K. grandifoliola*

Source of Variation	Df	Ms	F
Shoot length			
Medium	8	43.390	778.240
Error	18	0.130	
Root length			
Medium	8	112.210	2524.820
Error	18	0.100	
No of Node			
Medium	8	1.333	133.333*
Error	18	0.010	
No of Root			
Medium	8	2.912	413.82*
Error	18	0.007	

* = significant at $P < 0.05$

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