KARYOTYPE ANALYSIS OF *ACACIA SENEGAL* VAR. SENEGAL

By

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DEDICATION

To my parents, father Mohammed and mother Fawthia

To my brothers, Nizar and Ahmed

To my sisters, Eglal, Safa and wafa

To my beloved friends

Lubna

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Praise and thanks be to Almighty Allah (WAT), who granted me health and patience to complete this work.

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Abstract

Hashab tree (*Acacia senega*l) is an important species for its environmental and economical benefits like Gum Arabic production. Gum Arabic yield varies considerably between individual trees, which may be due to variation in karyotype. The aim of this study was to karyotype Hashab trees in terms of chromosomes number, length, symmetry index and centromere position. Seeds were obtained from Kordofan States. They were treated with hot water and placed in damp filter paper at temperature between 22 to 25 Cº for germination. Roots tips were collected after three days in the morning and midday and two pretreatments of colchicine and potassium chloride applied before fixation. They were prepared for microscopic examination by squash and permanent methods. The results showed that roots collected at midday showed active mitotic divisions compared to those collected in the morning. Also, pretreatment with potassium Chloride resulted in full spread of the chromosomes better than colchicines. The karyotype showed the number of chromosomes was 26 chromosomes (2n = 2x =26) while only one cell showed possible tetraploidy (52 chromosomes). The chromosomes were ranked from 1 to 13 according to the total length that varies from 2.80 µm to 5.41µm. According to the centromere position, the haploid set of the chromosomes was classified as one median, three slightly sub median, seven sub median and two highly sub median. The symmetry index and total formula were 0.51 and 43.75% respectively, indicating the symmetry of *A. senegal* genome.

الخلاصة

تعتبر شجرة الهشاب من الأشجار الهامة جداً في السودان وذلك لتعدد فوائدها البيئية والاقتصادية مثل إنتاج الصمغ العربي.

يختلف إنتاج الصمغ العربي بين أشجار الهشاب فربما يكون النمط النووي للصبغيات (karyotype) للأشجار المستقلة هو المسؤول عن تلك الاختلافات.

الهدف من هذه الدراسة هو تحديد النمط النووي للصبغيات والذي يعنى بدراسة عدد الصبغيات ،أطوالها، معامل التماثل وموقع السنترومير وحجمها.

كان مصدر البذور من محافظة كردفان ، و عوملت البذور بماء دافئ وتمت زراعة البذور في أطباق زجاجية فيها أوراق ترشيح مبلله تحت درجة حرارة تتراوح بين 22- 25 م لتشجيع الإنبات، وتم جمع القمم النامية للجذور بعد ثلاثة ايام في أوقات مختلفة من اليوم (الصباح ومنتصف النهار) عوملت القمم النامية بمعاملتين الاولى محلول كلوريد البوتاسيوم والثانية محلول الكولشيسين وذلك قبل التثبيت، و جهزت الشرائح باستخدام طريقتى الهرس والشرائح الدائمة وتم فحصها باستخدام المجهر الضوئى.

أثبتت نتائج هذه الدراسة أن الوقت المناسب لجمع القمم النامية للجذور هو منتصف النهار (11:30-11) والذى يعطي انقسام ميتوزي نشط للخلايا مقارنة بالقمم النامية للجذور التي جمعت في الصباح.

وأن استخدام محلول كلوريد البوتاسيوم لمدة ساعة ساعد على انتشار الصبغيات بصورة افضل من الكولشيسين.

اوضح النمط النووي (karyotype) ان عدد الصبغيات هو 26 صبغي ، وخلية واحدة العدد الرباعي (52 صبغي). ورتبت اطوال الصبغيات من 1الي 13 بناءا علي الطول الكلي لها من 5.41 مايكرون الي 2.80 مايكرون .

بناءا علي موقع السنترومير قسم العدد الأحادي للصبغيات الى صبغي وسطي السنترومير، 3 صبغيات قريب شبه وسطي السنترومير، 7 صبغيات شبه وسطي السنترومير وكذلك صبغيين عالي شبه وسطي السنترومير.

وكان معامل تماثل الصبغيات 0.51 والصيغة الكلية 43.75 % مما دل على تماثل النمط النووي لصبغيات أشجار الهشاب.

CHAPTER ONE

INTRODUCTION

1.1 Background

Acacia senegal (L.) willd is a multi-purpose leguminous tree species of high priority in arid and semi-arid parts of Sudan. It has a wide geographical range in the Savannah belt of Africa. In Sudan it is reported between lattitudes 10°N and 4°N, approximately, in an area that varies in climate and soil type (Harrison and Jackson, 1958; Seif Eldin, 1984). Fadel Elmola (2003) reported that the species is found between 9° and 15° N. The trees have wide range of environmental and socioeconomic benefits of which gum Arabic is the most important one. Sudan is a major producer and supplier of Gum Arabic to the international market.

Large variability exists between individual trees in growth rate, morphological characters, survival, gum productivity and seed yield (Ballal, 1991). The sources of this variability need to be understood and quantified for better management and for higher benefits. Some of the variability is attributed to management of natural stands and plantations and climate (Ballal, 2002). Also, the genetic make up of trees contributes to this variability (Elfeel, 1996). Some trials have indicated that gum yield is affected by regeneration methods, tree height, diameter and crown width and age (Hassan, 1998). However, Abdelrahman *et al*., (2003) reported poor correlation between these factors and gum yield. Average yield of gum per tree is 250g (IIED and IES, 1989). The variability between trees is very high, ranging from few grams to 2 kg or up to 10kg (NAS, 1979). Growth and yield seem to increase significantly with age and then decrease with old age (Awouda, 1985; Hassan, 1998).

These studied points to the importance of the genetic components in gum arabic production.

The nucleus is the site of hereditary material in the cell that is present as chromatin, which is DNA and neucleo protein. The chromatin appears as a fine network of chromosomes diffusely distributed in the nucleus and forming the chromatin reticulum in the non dividing cells (Suzukie *et al*. 1981). The word chromosome is derived from two Greek words chromos' meaning color and 'soma' which means 'body' a chromosome is a (colored body). Chromosomes are only seen during cell division. They are colorless in natural living cells. They can be made visible when fixed with certain chemicals such as acetic acid and absolute alcohol and then stained with certain basic dyes such as Carmine, Basic fuchsin (Sharma,1965). The chromosome carries thousands of genes. The process of cell division distributes the genes carried on the chromosomes from cell to cell. Thus the chromosomes are link between one generation and the following generation.

Morphology of chromosomes at mitotic metaphase is described according to the total length and position of the centromere. Careful cytological observation have shown that each chromosome is composed of two major delicate threads which are coiled around each other and known as the chromatids (Mcclintock, 1929). The two chromatids of the chromosome are separated from each other except at a certain point known as the centromere (Levan *et al*. 1964). The number of chromosomes per nucleus is usually similar for all the individuals of any species, but varies from one species to another. The study of chromosome number is useful for answering a variety of taxonomic and evolutionary questions (Lammers, 1987 and 1988). The basic chromosome number of the somatic cell of a higher plant is known as the diploid somatic number

(2n). The diploid number of chromosomes constitutes the chromosome complement of the individual. The number of chromosomes is reduced to the half of that present in the somatic cell after meiotic division to form gametes that possess the haploid number of chromosomes (Niizeki and Oono, 1971). Some plants may contain multiples of the haploid number of the chromosomes and are known as polyploidy (have more basic chromosome sets 3x, 4x, 6x, etc) (Lewis, 1980).

1.2 Problem statement

Cytogenetics is the correlated study of genetics and cytology (Burnham, 1962). It is a natural out growth of the fact that chromosomes are the vehicles of that portion of heredity that follows Mendelian laws. Chromosome behavior furnishes the mechanism for the observed genetic segregation and breeding behavior. There are striking differences in gross morphology of the chromosomes between and within species (Burnham, 1962). Karyotype analysis, which means studying of the number, size and morphology of chromosomes, is important for systematic and genetic investigation. It is best studied at metaphase of mitotically dividing cells.

Mitosis can be examined easily in meristematic cells in root tips, apical buds and small active axaillary buds. Root tips were reported as the most suitable part for karyotype analysis (Dyer, 1979; Bukhary, 1997; Kalkman, 1984). It is important to determine the age, the length of the root tips as well as the time during the day at which maximum mitotic division of the cell occurs. The preparation of the plant material for microscopic examination is usually carried through squash method, permanent slide technique and c-banding (Dyer, 1979; Sass, 1958; Schwarzacher *et al*., 1980).

The diploid number of *Acacia sengal* is 2n=26 (Bukhary, 1997). Similar number was reported for *Acacia siberana* (Bennett and Leitch, 1995). ,*Acacia albida* (*Faidherbia albida*) *Acacia magium* (Turnbull, 1998), *Acacia dealbata* (Blakesley *et al*., 2002) and *Acacia mearnsii* (Beck *et al*., 2003). However, tetraploid was reported for *Acacia dealbata* (Blakesley *et al*., 2002) and *Acacia mearnsii* (Beck et al., 2003). In addition, *Acacia Miller* range in ploidy from 2n=26 to 2n=104 (Bennett and Leitch, 1995). Bukhari *et al*. (1997) reported hexaploidy and octoploidy in *Acacia* sub genus are geologically more recent than tetraploidy. The presence of tetrapoloid may points to variation in chromosome number within the acacias. This may explain some of the morphological variation and gum yield between *Acacia senegal* trees.

Studies in crops plants revealed variation in length and position of centromere as in *Pennisetum glaucum* and *Pennisetum violaceum* where the length ranged from 3.92 to 6.00µm and from 3.00 to 4.50µm, respectively (Salih *et al*., 1999). Also, Guar lines (*Cyamopsis tetragonoba)* showed differences in length between chromosomes ranged from 4.13µm to 1.40µm (ElAmin, 2000). Accordingly, variation in the chromosomes morphology (length and position of centromere) within and between *Acacia senegal* trees are expected.

Since, detailed karytype of *Acacia senegal* was not reported there is a need for such study for this important species.

1.3 Objective

The main purpose is to investigate the variation in the number and morphology of *A. senegal* chromosomes complement. The specific objective is to test the hypothesis that there is variation in chromosomal number and morphology between the individual trees.

The specific objectives are:-

1-Assess the suitable time for collection of root tips for higher mitotic cell divisions.

2-Investigate the chromosome number.

3- Investigate the chromosome morphology (total length of chromosomes and the position of centromere) between individual trees.

4- Investigate the chromosome size of *Acacia senegal*.

CHAPTER TWO

LITERATURE REVIEW

2.1 The species:

Acacia senegal (Willd). L have four varieties namely variety senegal, Kerensis schwenif, leiorhachis brenan, rostrata brensis (Elfeel, 1996). The variety senegal is the most common one in Sudan and known as Hashab or Alloba.

2.1.1 Description: -

A. senegal is described by (Andrews, 1952) as a small tree or as a shrub ranging from less than 6m high, sometimes up to 12m high with variable or flat crown, spreading or rounded. The bark is yellowish to light brown or grey. It has rough prickles at nodes in three, 2 lateral pointing up wards and one central pointing back wards, dark brown with gray base. Pods are flattened, oblong, pale brown or fawn color, papery, with one to five seeds (Badi *et al*., 1989). Flowering starts from October onwards, and fruiting starts from November with peak period in January (Ballal,1995). The trees put leaves just before rain. Leaf shedding is irregular but most trees shed their leaves in November or December. However, in many areas some trees may retain their leaves considerably longer. Seeds ripe between January and April (Sahni, 1968) and are usually collected within this period. The seed usually develops a long tap root which goes down to considerable depths in sands, while on clay soils it is probably that shallower root system is developed (Badi *et al*., 1989).

2.1.2 Importance of *Acacia senegal*

A. senegal is the most important tree species in Sudan and listed a top priority one (Warrag *et a*l., 2003). It produces the well known Gum

Arabic, stabilizes sand dunes, enrichment of soils through nitrogen fixation and leaf litter mineral enrichment, shelter breaks, fodder for grazing animals and source of fuel wood, fiber, shade and shelter (NAS,1979). Presently, in many afforestration projects hashab is identified as a major contributor in combating desertification problems in the gum belt. More over hashab trees are the main components of the agro forestry system practiced in Western Sudan (Elfeel, 1996). Gum Arabic is produced by two species the hashab gum which is high grade gum that exudes from *Acacia senegal* and that gum which has an inferior quality gum is obtained from *Acacia seyal*. Hashab is economically important for the production of the famous commercial Gum Arabic (Beshi, 1984). Its gum is an international commodity and Sudan is the leading exporter. Sudan used to export about 85% of the world demand of Gum Arabic Company, 1999. Gum Arabic is a source of cash income to the farmer (Ballal, 1991). It is a natural product uses such as food stuffs, pharmaceuticals, cosmetics, postage stamp, ink, adhesives, paints and a wide range of additional industrial products (Ballal, 1991). Paper, metals to prevent corrosion, preserved seeds of *A. senegal* are used by people as vegetables NFTA (1991). The foliage and pods are rich in protein and browsed by sheep, goats and camels (Doran *et al*., 1983). The seeds contain fat that is used in medical and soap making (Scholte, 1992).

2.1.3 Distribution

A. senegal is found in a belt 300km wide along the southern frontier of Sahara desert and is found through out the Sahelian Zone from Senegal to Somalia. The variety senegal occurs in Africa and Asia (Brenan, 1983) in Africa it is recorded in Gambia, Senegal, Mali, Sudan, Ethiopia, Zaire, Cameron, Rwanda, and

Kenya. In Asia it is recorded from Pakistan and India. In Sudan it is distributed from east to west between attitudes 10°N –14°N forming what is called the Gum Belt (Elhadi, 1984) or between (Fadel ElMola, 2003). It has two main areas of natural distribution, the first is on stabilized sand, where it is found under rainfall of 280-450mm, and second is on the dark cracking days under rainfall of 500mm. Thus it is distributed in southern Nuba, from Barber to Mongolia, Blue Nile province, Kassala province and Kordofan (Sahni, 1968) and Darfurs states the species is often found in pure stands giving the Sudan the advantage of being the biggest producer and exporter of the best qualities of Gum Arabic. The term Gum Arabic belt is used to denote a zone of approximately 200.000 square miles, which extends a cross central Sudan between 10°N and 15°N latitude. In the last five years there was a little change in the belt and in the south there are now some productive areas which were not known before (Ballal, 1998). *Acacia senegal* is also found on some other soil types, such as stony ground near Juba in the southern Sudan, with rainfall of about 900mm and on the hard surfaced soil (Badi *et al*., 1989).

2.1.4 Ecology

Rainfall: It is best found in sandy areas of annual rainfall of 300-450mm but can grow under 200mm and up to 800mm (National Academy of Sciences, 1980). In clay soil it grows in sites with optimum annual rainfall of 500 mm above. However the rainfall of 300-450 mm is considered to be suitable for the production of Gum Arabic (NAS, 1979; Doran *et al.,* 1983).

Temperature: The mean annual temperature within the species range is

between 25°C and 27°C and maximum of 45°C (Goor and Barney, 1976).

Soils: The species exists in a number of soil types the two major and most extensive types are the sandy soils and the dark cracking clay. The species is more common in sandy soils (Goz). The sandy soil is characterized by being poor in organic matter and nitrogen, with high and speed of infiltration and is easy to cultivate (Giffard, 1975). The suitable PH is about 7.8 to 8 (Von Maydell. 1986) .In additions, the species is found on other soil types such as the Pedi plain soils locally known as "gardud" a soil in south Kordofan and Darfur (IIED and IES, 1989).

2.1.5 Importance of genetic studies

Genetic variability of the economically important traits is essential to improve forest trees (Zobel and Talbert, 1984). Analysis of the variation can help identify sources of variation and effect on population genetic dynamics (Namkoong, 1983). Variation has genetic and environmental components and genetic analysis are necessary to separate genetic and environmental influences. Estimates of these components are crucial to tree improvement programs as they determine the efficiency of selection and breeding program.

2.1.6 Genetic of Acacia

Acacias have high basic chromosome number that range between 26 to 104 and the reported diploid number for *Acacia senegal* is 2n = 26 and the haploid one is n = 13 (Atchison 1948; Sharma and Bhattacharyya, 1958; Mehra and Bawa, 1968; and Bukhari, 1997). Detailed chromosomal studies on *Acacia senegal* have been pursuit by several investigators and they indicated that the small and numerous chromosomes impaired the detailed cytological investigations (Atchison, 1948; Khan 1951; Sharma and Bhattacharyya, 1958).

(Bukhari, 1997) reported a good prefixing treatment that could give well spread of chromosome for detailed cytological investigation in *A. senegal* and opened the way for karyotyping of the species.

2.2 karyotype of *Acacia senegal*

Karyotype analysis as described by (Battaglia, 1952) includes the study of the number, size and morphology of chromosomes. Total length and arms ratios of chromosomes constitute important parameters for karyotype study of different taxa and are helpful in systematic and phylogenetic investigations; cytological features can be used as an aid in establishing taxonomic and phylogenetic relationships among species and genera (Anderson, 1937). Basic number, size and morphology of chromosome can be very useful in taxonomic classification, but these should be subsidiary to morphological characters in any taxonomic treatments (Pilger, 1954). Jackson (1971) reviewed the earlier literature on the use of karyotype characters in systematic studies where chromosomes are generally measured at somatic metaphase after pretreatment which condense and spread them. The inherent drawbacks of these studies are related to the high error in the measurement due to pretreatments.

2.2.1Chromosomes

The nucleus is the site of hereditary material of the cell. The chromatin of the nucleus is the carrier of the genetic factors. The chromatin takes different shapes according to the stage of cell division. When the cell is not dividing or in the interphase stage, the chromatin material appears as a fine network diffusely distributed in the nucleus forming the chromatin

reticulum (Suzukie *et al*., 1981). During preparation for cell division the chromatin material coils up tightly and forms thread like structures called the chromosomes. Watson (1976) reported that the main chromosome component is deoxyribonucleic acid (DNA) and the protein component accounts up to 50% of the chromosome of higher plant. The word chromosome is derived from two Greek words chromos' meaning color and 'soma' which means 'body' a chromosome is a (colored body). Chromosomes are only seen during cell division. They are colorless in natural living cells. They can be only made visible when fixed with certain chemical such as acetic acid and absolute alcohol in the ratio of 1:3 and then stained with certain basic dyes such as carmine, Basic fuchsin (Sharma, 1965). Chromosomes carry the genes in a linear form (Suzuki *et al*., 1981) with each chromosome may carry thousands of genes. The chromosomes are links between generations by the process of cell divisions and gamete formation.

2.3 karyotype variability

2.3.1 Chromosome Morphology

The morphology of a chromosome in mitotic metaphase is described by two major factors; it is total length and the position of the centromere. The classical studies of the morphology of the individual chromosomes of plant species was reviewed by (Lewitsky, 1931) and continued by (Taylor, 1925). The external form of the chromosome is dependent on the stage of the cell division. Careful cytological observation have shown that each chromosome is composed of two major delicate threads which are coiled around each other and known as the chromatids (Mcclintock, 1929). Chromatids are the fundamental units during cell division (Suzuki et al., 1981). The two chromatids are separated from each other except at a certain point known as the centromere (Levan *et al*., 1964). Electron

microscope studies have indicated that the chromosome is composed of a bundle of fibers at the interphase and prophase stages.

2.3.2 Chromosome number

The number of chromosome per nucleus is always similar for all the individuals of any species, but varies from one species to another. The study of chromosome number is useful for answering a variety of taxonomic and evolutionary questions (Lammers, 1987 and 1988). Of primary interest in studies of cytological evolution is the basic chromosome number (X) the ancestral number from which other numbers have been derived (Grant, 1981; Stuessy, 1990). The pair of homologous chromosomes is similar chromosome in morphological features like size, length, position of the centromere. Because of the presence of the chromosomes in pairs, the chromosome number of the somatic cell of higher plant is known as the diploid somatic number (2n). The number is reduced to the half of that in the diploid resulting in haploid number of chromosomes (n) (Niizeki and Oono, 1971). The diploid number of chromosomes constitutes the chromosome complement of the individual. Some plants may contain multiples of the haploid number of the chromosomes and are known as polyploidy (3x, 4x, 6x, etc). Chromosome number is some multiple of (n) with 2n as diploid cells, 3n as triploid, and 4n is tetraploid. Polyploidy had played an important role in the evolution of many plant species (Lewis, 1980). Polyploidy is common in plants, approximately half of all Angiosperms may be polyploidy (Grant, 1981).

The chromosome number of some of the *Acacia* species was investigated. The diploid number of *Acacia sengal* is 2n=26 (Bukhary, 1997). Similar number was reported for *Acacia siberana* (Bennett and Leitch, 1995). ,*Acacia albida* (*Faidherbia albida*) *Acacia magium* (Turnbull, 1998),

Acacia dealbata (Blakesley *et al*., 2002) and *Acacia mearnsii* (Beck *et al*., 2003). However, tetraploid was reported for *Acacia dealbata* (Blakesley *et al*., 2002) and *Acacia mearnsii* (Beck *et al*., 2003). In addition, *Acacia Miller* range in ploidy from 2n=26 to 2n=104 (Bennett and Leitch, 1995). Bukhari et al. (1998) reported that hexaploidy and octoploidy in *Acacia* sub genus were geologically more recent than tetraploidy.

2.3.3 Chromosome size

The size of the chromosome varies widely according to species. Variation in chromosome size between many diploid species was reported (Coates, 1979). Differences in size among homologues chromosomes of the same species could largely be attributed to differences in the phase of the cell cycle (Khan, 1951; Barlow, 1978). The chromosome size is either presented in length measurements or classified into groups according to length (Edwards, 1979). Ranganath *et al*., (1990) classified the chromosome into four classes, A= long (more than 4µm), B= medium sized (4-2.5µm), C= short (2.5-1µm) and D= very short (less than 1µm).

2.4 Chromosome structure

2.4.1Centromere

The centromere is a point on the chromosome where the two daughter chromatids are joined together forming two arms. It is also the position at which the chromosome attach to the microtubules that orient the chromatids in to their respective poles during cell division (Levan *et al*., 1964).

The chromosome is classified as metacentric, sub metacentric, acrocentric telocentric if the centromere is located in the middle, a little of the center, towards one end and close to one end, respectively (Levan *et al*., 1964).

Edwards (1979) established four classes of centromere positions according to the ratio of the long (L) and short (S) arms: (i) median (L/S ratio between 1.00 and 1.10), (ii) slightly sub median (L/S ratio 1.31 and 1.70) and (iv) highly sub median (L/S ratio greater than 1.71). Also, Ranganath *et al*. (1990) classified the chromosomes to median, sub median and sub terminal. The position of the centromere plays an important role in the studies of evolution and speciation of taxa that include centromere index, arm ratio and total formula of the chromosome complement (Edwards, 1979). Consequently the centromere is considered as one of the features that distinguish the chromosomes of a species. The total formula percent gives an estimate of mean position of the centromeres while the percent of symmetry estimates the symmetry within the units of the complement (Ramesh and Salimuddin, 1992).

2.4.2 Secondary construction

The secondary constriction (nucleolar organize region (NOR)) or satellite chromosome exists in chromosomes of variable taxa and is widely used as marker in cytogenetic studies (Payne *et al*., 1984). Sidhu *et al*., (1984) attributed the absence of satellite chromosome in some of the varieties to the difficulty of their detection. Koul and Gohil (1990) referred to the absence neither of NOR in their material, to the fact that either these regions are not clearly differentiated or have escaped notice. Also, Koul and Gohil (1990) indicated that sometimes NOR lie very close to the centromere and as such it is difficult to differentiate the two.

2.4.3 Heterochromatin

The chromatin is the entire complex of DNA and protein. There are two types of chromatin the euchromatin and the heterochromatin. The euchromatin is the major type of chromatin; it is genetically active and

contains the larger part of genes (Ray and Keteswaran, 1979). The heterochromatin is composed of the following two types: (i) constitutive heterochromatin that is highly condensed (darkly stained) at all the stages of cell division cycle and can not be transcribed and it does not appear to contain genes and replicates after the euchromatin (Ray and Keteswaran 1979; Csink *et al*.1997), (ii) facultative heterochromatin that has potential to be constitutive heterochromatin, but all time it is fully extended like eu chromatin. It contains structural genes, which are only active when the chromatin is in euchromatin state. This Facultative heterochromatin is a mechanism of turning off the activity of whole chromosome (Sass and Henikoff, 1998).

2-5 Preparation of root tips

The commonly used methods to prepare plant material for karyotype analysis are squash method, permanent technique and the c-banding.

2.5.1 Squash method

The common squash method used is as described by Dyer (1979) for the examination of freshly prepared plant material such as root tips, apical buds and small active axially buds. Root tips are the most suitable part for karyotype analysis. The method is usually used to determine the age, the length of the root tip as well as the time during the day at which maximum mitotic division of the cell occur. Rattenbury (1956) proposed a simple and rapid method to make a squashed temporary preparation into a permanent one by replacing 45% acetic acid with a mixture of 10 parts of 45% acetic acid and 1 part of glycerine.

2.5.2 Permanent slide technique

The technique described by Sass (1958), and followed with some modification by Schwarzacher *et al.,* (1980) and Kalkman (1984). Was used for the study of chromosomes complement

2.5.3 C-band technique

The method adapted by (Salih 1997) and (Schwarzacher *et.al*, 1980) with some modifications was used for the study of chromosomes complement.The method is similar with permanent at all steps expect the final step (staining).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Material

3.1.1 Seeds

Seed were collected from *Acacia senegal* trees in western Kordofan from Elsaata area (Elsaata Om Gamina and Elsaata Tagaano and Elsaata Bukhary) in March 2003. The area is as major site of gum production and characterized by sandy soil and rainfall between 300 and 450 mm. Additional seeds were collected as bulk seeds in March 2004 from Kordofan states.

3.1.2 Chemicals

Chemical reagents, paraffin wax and stain used were laboratory products from international chemical product companies. They were prepared as described by Creedy (1987) as follows:

(a) Basic Fuchsion

It was prepared by dissolving 0.1g of basic fuchsion powder in 160 ml of distilled water with 1ml of absolute ethanol.

(b) Aceto Carmine

It was prepared by dissolving one gram of carmine powder in 45ml of glacial acetic acid by heating. It was then diluted to 100ml with distilled water.

(c) Colchicines

For the preparation of 0.1% colchicines solution, 0.1g of colchicines powder was dissolved in 100 ml of distilled water.

(d) Aceto alcohol

It was prepared by mixing absolute ethanol with glacial acetic acid (3:1 v/v) for the use in the squash method. The one used in microtome permanent slides was prepared from formaldehyde, glacial acetic acid and 70% ethanol (5: 5: 90 v/v).

3.2 Method

3.2.1 Root tips

Seeds were immersed in hot water and left to cool in water for 24 hrs. The treated seeds were placed in damp filter paper at temperature of about 22 to 25 Cº to germinate for three days. Root tips were collected when the length of the emerged root tips were 1- 1.5cm. Two collection times were tested: in the morning (7 am) and midday (11-11.30 am) to know the suitable time for collection of root tips at which mitotic division occur in the cells.

3.2.2 Chromosome morphology

Chromosome morphology was studied at the metaphase of the mitotic cell division in the root tips using the following techniques:

1- Squash method.

2- Permanent slides technique.

3.2.3 Squash method

The squash method was used for the examination of the prepared root tips similar to Dyer (1979) with slight modifications as follows:

1- Root tips were collected when the roots of germinating seeds were 1-1.5 cm long at two times of the day: at 7 am (will be referred to as early morning collection) and between 11 -11.30 am (will be referred to as mid collection).

2- Fixation: - Root tips were then fixed in aceto-alcohol (70% ethanol glacial acetic acid in a ratio of 3:1 v/v) and kept in the fixative over night.

3- Dehydration: The root tips were then washed in distilled water at room temperature, transferred in to I N hydrochloric acid (HCL) at 60ºC in a water bath for 3-5 minutes. The root tips were then washed with distilled water in the same test tube at room temperature.

4- Staining: Basic fuchsin stain was added to the root tips for three minutes. The root tips were then placed on filter papers for drying, and then transferred to slides. They were then cut with a razorblade and just the very tips (first 2-3 mm from the tip) were kept and the rest parts were discarded. The tips were then squashed in aceto Carmine.

6- The slides were then covered with a cover slip and a glass rod was used to press gently over the cover before examining under the light microscope.

3.2.4 Permanent technique

The technique described by Sass (1958) with some modifications as suggested by Bukhary (1997) and according to the results obtained in the squash method was used as follows:

1- Root tips from recently germinating seedlings were collected after three days from preparation of seeds for germination. The length of the root tips was 1 to 1.5 cm at midday.

2- Prefixing treatments was applied to root tips to examine the spread of the chromosomes. The treatments were immersion of root tips in:

A- Solution of 75Mm potassium Chloride (KCl) for 1 hour.

B- Solution of 0.1% colchicine for 2 hours.

3- Fixation: Root tips were fixed in acetoalcohol (70% ethanol glacial acetic acid in a ratio of 3:1 v/v) and kept in the fixative over night

4-Dehydration: it was carried out by passing the root tips through series of ethanol concentrations (50%, 70%, 90%, 95% and absolute ethanol, respectively) each for 6 hours. The root tips were then placed in 95% alcohol +1% eosin stain. The eosin gives root tips a red color to make them observable for orientation in wax and for sectioning.

5- Clearing: It was carried by transferring the root tips into a mixture of absolute ethanol: cedar wood oil (1:1 v, v) and kept for 12 hours, then in to cedar wood oil: xylene (1:1, v/v) for 12 hours followed by pure xylene for 12 hours.

6- Wax embedding: the material was transferred to a mixture of xylene and melted wax then into pure melted wax for two consecutive changes, each change for about 30 minutes.

7- Sectioning: Two L shaped moulds and a rectangular metal sheet were used to form the melted wax containing the plant material into blocks. The roots were oriented to give longitudinal sections. The wax was stuck to the wooden blocks before sectioning with a rotary microtome (Adjusted at 6µm micrometers) into long ribbons. The ribbons were then cut to small sections, transferred to wet and clear slides by tail brush. The slides were placed over a hot plate adjusted at a temperature just below 60ºC, the melting point of the wax. The hot plate gave full stretch of the ribbons and good adherence of the material to the slides. The slides were left overnight to ensure complete dryness before staining.

8- Staining: The process of staining required de-waxing, re-hydration, staining and dehydration of the material. These were performed by passing the slides through a series of coupling jars containing the

following chemicals in the order mentioned: xylene, xylene absolute ethanol, absolute ethanol, 95% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, sefranin stain, 50% ethanol, 70% ethanol 90% ethanol, 95% ethanol absolute ethanol, fast green stain and xylene.

At the end of this process, a drop of D. P.X was then added before placing the cover slip. The slides were kept for 3 days in an oven, adjusted at 60ºC before microscopic examination.

3.3 Microscopic examining

3.3.1 Squash method slides

Light microscope was used for investigation of 50 slides for the suitable time of the collection of root tips to evaluate the presence of mitotic stages. Eye glasses with 10X and 40X magnification were used first to focus on the mitotic stages. The magnification of 100 X was then used for evaluation of the mitotic phases. A drop of emersion oil was applied to condense light on the slides before setting of the 100 X magnification.

3.3.2 Permanent slides

Light microscope was used for investigation of the permanent slides for

A- Spread of the chromosomes according to the pretreatments of colchicine and KCl with 50 slides to each.

B- Counting the chromosomes number and measure their long and short arms length and accordingly deciding the centromere position. First, About 100 slides that were pretreated with KCl were surveyed for metaphase stages. Second, 25 cells were then taken randomly for counting the number of chromosomes per cell and the length of long and short arms of the chromosomes.

The magnification of 40 X was used to determine the spread of the chromosomes to focus on the mitotic phases and then the magnification

of 100 X was used for the evaluation of the mitotic phases. A drop of emersion oil was applied to condense light on the slides before setting of the 100 X magnification. Photographs were taken at 100 X magnification. Eye glasses 10x were used with scale under 10x10 magnification and parted into micron setting with the chromosomes longitude, and identifying the total longitude of it. And with determining the centromere point, the long arm can be measured and writing its longitude and also short arm and writing its longitude.

3.4 Chromosomes morphology

Karyotype morphology was analyzed as described by Levan *et al*., (1964) as follows:-

a) Total chromosome length which is equal to the addition of the length of the long arm (L) to the short arm (S) (L+S).

b) Calculation of the arms ratio that is equal the L divided by S in every homologous pair of chromosomes. The grouping according to centromere position (arms ratio) followed Edwards, 1979 classification.

c) Symmetry index was determined by dividing the total length of the shortest chromosome in the complement by the longest chromosome in the complement per cell (Salih, 1997).

d) Total formula percent was determined as a ratio of the sum of short arms to the total length of the complement (Huziwara, 1962).

F) Chromosomes size were divided in a group according to (Ranganath and Krishnappa, 1990) classification in four, A=long (more than 4 μm), B=medium sized (4-2.5 μm), C =short= (2.5-1 μm) and D very short (less than 1 μm)

3.5 Statistical analysis

The statistical analysis system (SAS) was used for data analysis. Analysis of variance procedure was used to determine significance of variables tested. Significant differences between means were separated using Duncan's multiple range tests.

CHAPTER FOUR

RESULTS AND DISCUSSION

Knowledge about the karyotype of a plant species is important for the understanding of its genetics and consequently the formation of breeding programs as well as the comparative description of related species. Karyotype formula, calculated from the means of the evaluated *Acacia senegal*, are presented in figures, photographs and tables. The study confirmed that diploid chromosome number for *Acacia senegal* is 2n = 26 and the haploid one is n =13. The result is in accordance with reported by Bukhari (1997) for other *Acacia* species. The numerous chromosomes continue to impair detailed cytological investigations similar to that reported by other investigators (Atchison1948; Kahn 1951; Sharma and Bhattacharyya, 1958; Hunziker *et al.,* 1975; and Coulaud *et al*., 1995).

4.1 Squash method

The preliminary observation of this study indicated that the suitable age to collect root tips is after 3 days from seed treatment for germination under temperature of 22 to 25°C. Also, the suitable root is when the first root reaches 1 to 1.5 cm in length and before the emergence of adventitious roots. Collection of root tips at midday (11-11.30 am) had more mitotic dividing cells as compared to the morning one (7 am) (**plate 1 a and b). M**itotic phases of prophase, metaphase, anaphase and

telophase were observed in the slides of root tips collected in midday while only interphase and prophase were observed in the other periods.

4.2 Permanent slide technique

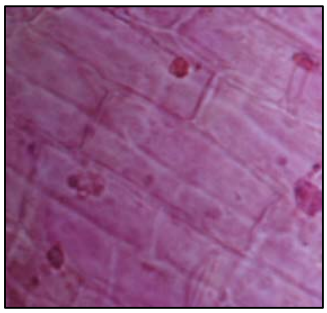
Different mitotic phases, prophase, metaphase, anaphase and telophase were observed in the prepared slides from root tips collected at midday **(plate 2).** Since the slides prepared by the squash method last for not more than three hours, therefore permanent slide preparation is important for karyotype analysis and chromosome measurement.

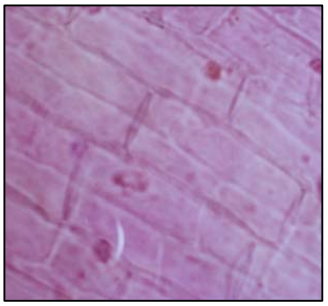
4.2.1 Effect of prefixation treatment:

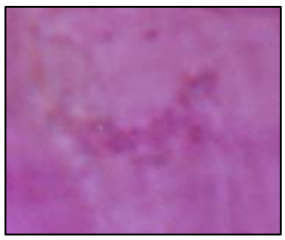
Treatment of root tips with Potassium Chloride (KCl) before fixation resulted in better spread of the chromosomes as compared to colchicines using the permanent methods (**Plate 3 a and b**). The full spread of the chromosomes was obtained with the treatment of 75 Mm KCl for one hour (Plate 3a). As Bukhari (1997) explained this may be due to effect of potassium ions that are pumped across the plasma membrane into the cell. This would make the cytoplasm under more hypertonic condition that forces water to diffuse into the cell so that the over swelled cells would give better chromosome spread.

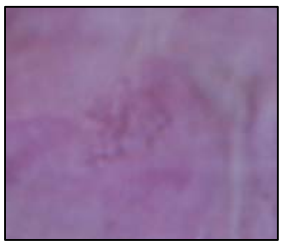
Colchicine gave condensed mitotically dividing cells but failed to improve the spreading of chromosomes, which agrees with other reports (Sangowawa, 1994; coulaud *et al*. 1995) (**Plate 3 b**)

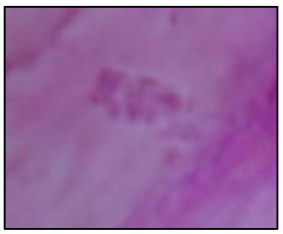
A)

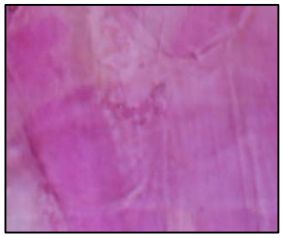


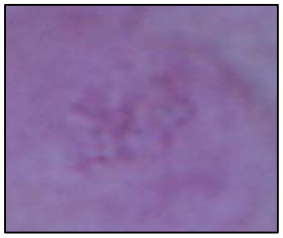












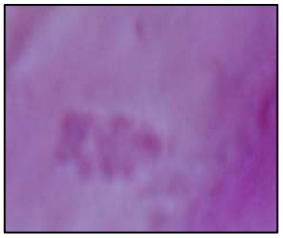


Plate 1: Effect of time of collection of *Acacia senegal* root tips in the appearance of mitotic phases (a) collection at 7 am and (b) collection at 11.30 am.

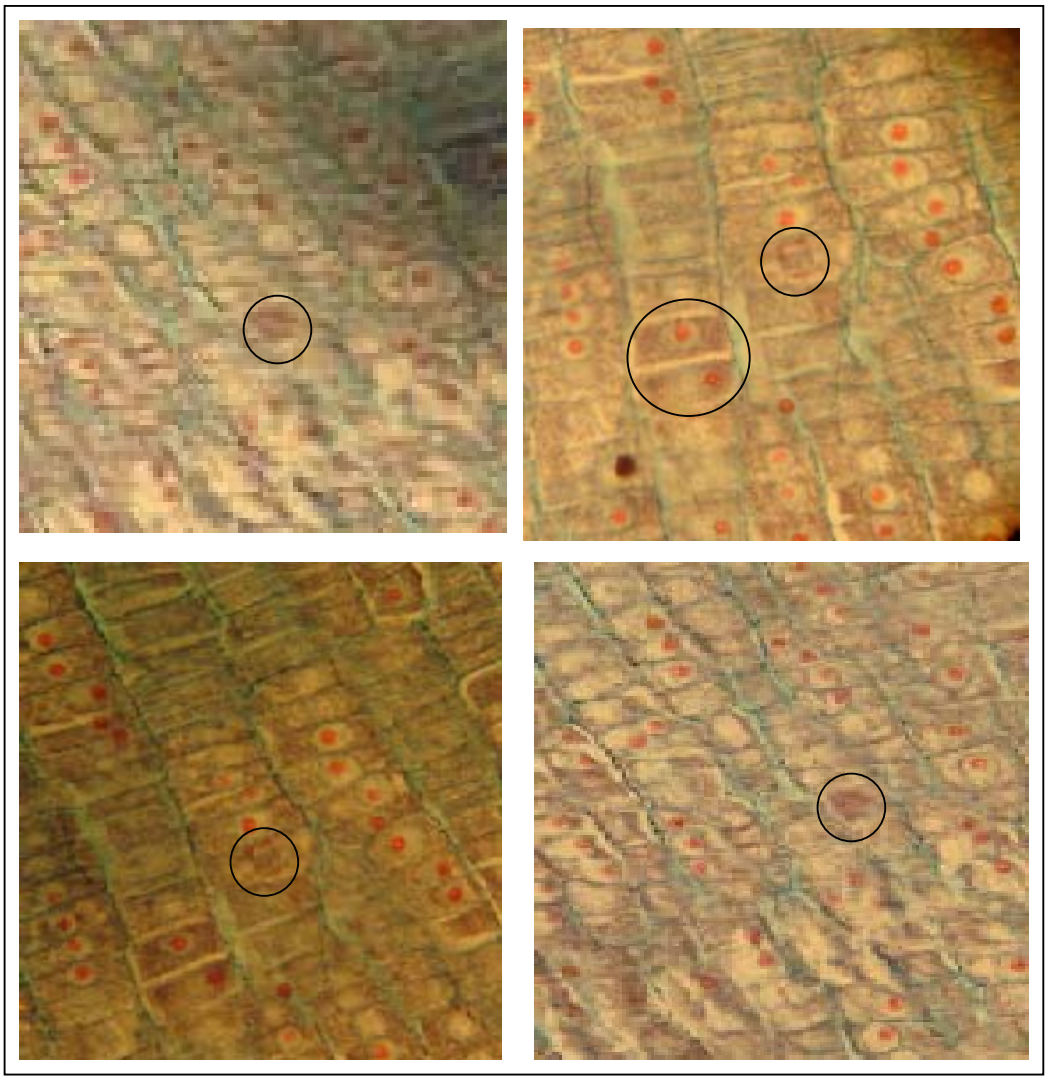
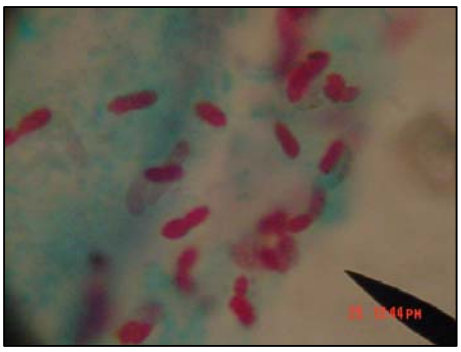


Plate 2: Mitotic phases of metaphase, anaphase and telophase in root tips of *Acacia senegal* collected at midday

a



b

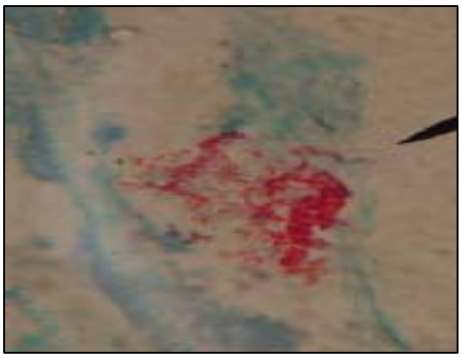


Plate 3: Effect of prefixation treatment of *Acacia senegal* root tips with a- K Cl (Potassium chloride) and b- colchicines on spread of chromosomes

4.2.2 Karyotype analysis

Mitotic phases were examined in 25 cells randomly taken at the metaphase stage to count chromosome number, length, and position of centromere. The chromosome took the red color of the safranin stain, whereas the cytoplasm took the Fast green stain. Thus the chromosome appeared as red structures over a green background, which made it easier for the study.

4.2.2.1 Chromosomes number

The study has showed that the chromosomes number in the examined cells was 26. This result is similar to that reported for a number of Acacia species like, *Acacia albida* (*Faidherbia albida*) *Acacia magium* (Turnbull, 1998), *Acacia dealbata* (Blakesley *et al*., 2002) and *Acacia mearnsii* (Beck *et al*., 2003) and *A. sieberana* (Bennett and Leitch, 1995) and *A. senegal* and *Prosopis* (Bukhari, 1997). However, No natural polyploidy has been found in the species; however in one slide a cell had showed 52 chromosomes **(plate 4)** This result is similar to that reported for tetraploid of *Acacia dealbata* (Blakesley *et a*l., 2002).

4.2.2.2 Chromosomes lengths:

The results of the measurement on the haploid set of the chromosomes (13 chromosomes) are presented in Table 1. The results include the length of the short and long arms, calculation of ratios of long arm to short arm ratio (L/S) and total chromosome length. The arrangement of chromosomes according to their length in a descending order as suggested by Salih (1997) is shown in **plate 5**. Chromosome one was the longest and number 13 was the shortest one. Chromosomes varied in length from 2.80 µm for the shortest to 5.41µm for the longest (**Table1 and plate 5**).

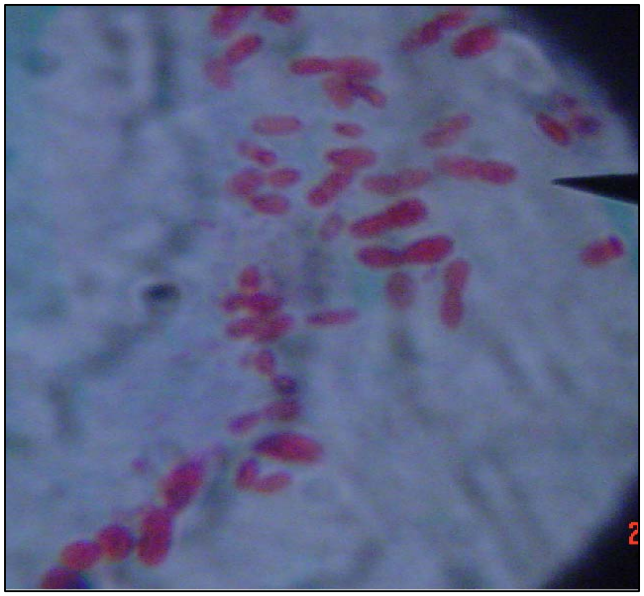


Plate 4: Tetraploid of *Acacia senegal* (2n = 4x= 52).

Table 1: Mean length of long arm (L), short arm (S), total chromosome and arm ratio (L/S) of the haploid chromosome complement of *Acacia senegal*

Chromosome number	Long arm (L) µm ±SE	Short arm (S) µm ±SE	L/S ratio ±SE	Total length µm ±SE	Chromosome class
1	3.12 0.04	2.29 0.04	1.36 0.04	5.41 0.02	S.M
2	2.91 0.03	2.02 0.05	1.44 0.06	4.93 0.02	S.M
3	2.75 0.07	1.84 0.07	1.49 0.1	4.59 0.03	S.M
4	2.61 0.06	1.71 0.08	1.52 0.1	4.32 0.04	S.M
5	2.20 0.06	1.91 0.04	1.15 0.07	4.11 0.03	S.S.M
6	2.33 0.06	1.57 0.06	1.48 0.1	3.9 0.01	S.M
7	2.25 0.05	1.50 0.06	1.5 0.1	3.75 0.04	M
8	2.24 0.05	1.26 0.04	1.77 0.09	3.5 0.04	H.S.M
9	2.03 0.02	1.29 0.03	1.70 0.06	3.49 0.03	S.M
10	2.01 0.01	1.17 0.04	1.70 0.05	3.72 0.03	S.M
11	1.94 0.04	1.08 0.04	1.79 0.06	3.02 0.01	H.S.M
12	1.59 0.03	1.44 0.03	1.11 0	3.03 0	S.S.M
13	1.56 0.04	1.24 0.03	1.25 0.05	2.8 0.02	S.S.M

The abbreviations M, S.S.M, S.M. and H.S.M. indicate median, slightly submedian, submedian and highly submedian centromere position, respectively.

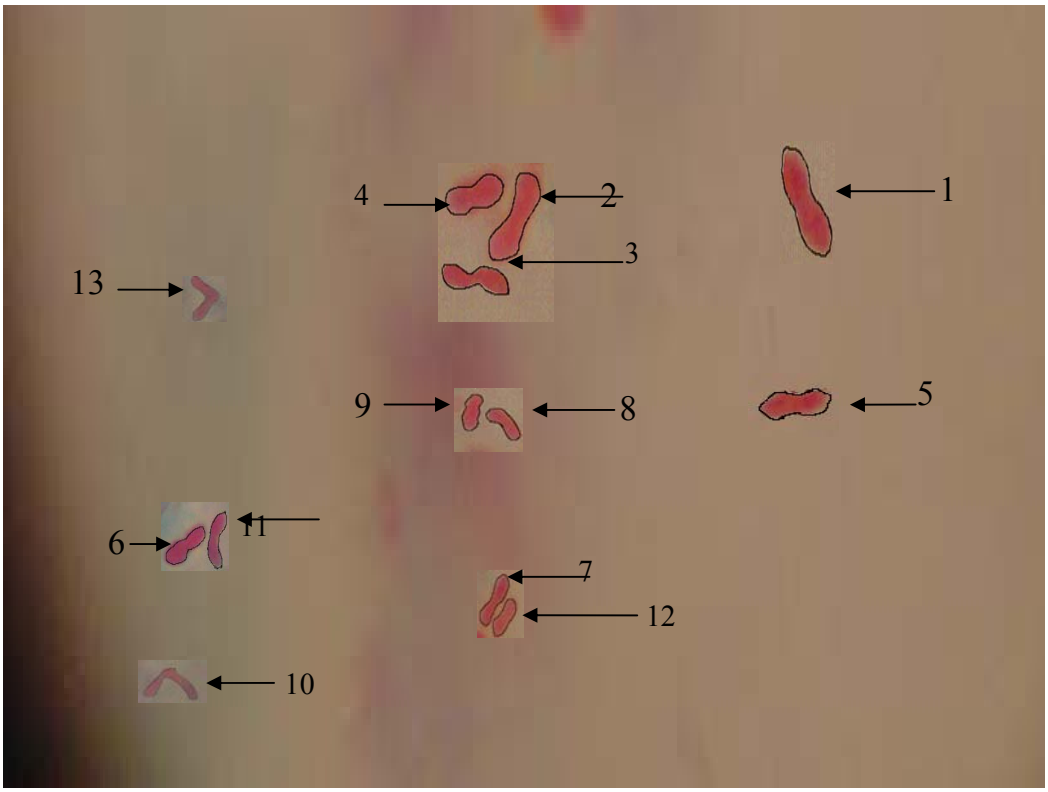


Figure 1: chromosomes complement of the *Acacia senegal*

Table 2 lists the result obtained for the variation between the mean length of the chromosome complement of the *A. senegal* species. There is significant variation between the length of the total length of the chromosomes complement (p = 0.0001). Chromosome **1** is significantly longer than the others in term of the length of the long arm, short arm and total length. Also chromosome **2** is longer than the rest. The ranking of the chromosome according to their length is depicted in figure 1 and 2.

Table 3 lists the result obtained for the total length of the long arm, short arm and total length of the chromosome complement of the *Acacia senegal* species. The total length of the long arms of the haploid complement cell ranged between 28 and 31 μm while that of the short arms ranged between 18 and 22 μm, and that of the total length of the chromosomes ranged between 48 and 52 μm. No significant variations were observed between the cells for the long arms, short arms and total length.

4.2.2.3 Symmetry index and total formula:

The values of symmetry index and total formula were 0.51 and 43.75%, respectively. These results indicate the symmetry of chromosome complement for *A. senegal* species. According to Salih (1997) the values of symmetry index and total formula were 0.55 and 44.29%, respectively for the chromosome complement in cultivated pear millet and these two values further confirmed the suggestion of the symmetry of the chromosome complement in species. Also (Elamin, 2000) found the values of symmetry index and total formula for chromosome complement in *Cyamopsis tetragonoloba* (guar) 0.23 and 42.21% respectively, indicating the symmetry of guar.

Table2: Variation between the mean length of the long arm, short arm, total and the long arm and short arm ratio of the haploid chromosome complement of *Acacia senegal*

CH NO	Long arm	Short arm	Total	Ratio
	Mean	Mean	Mean	mean
1	3.13 A	2.29 A	5.43 A	1.38 C,D,E
2	2.91 B	2.02B	4.97B	1.47 B,C,D
3	2.76 C	1.85D,C	4.60 C	1.62 A,B,C
4	2.61 D	1.72 D,E	4.33 D	1.69 A,B
5	2.21 E	1.91 B,C	4.09 E	1.19 E
6	2.33 E	1.57 F,E	3.98F	1.57 A,B,C,D
7	2.26E	1.50 F	3.76 G	1.60A,B,C
8	2.25 E	1.27G	3.57 H	1.86 A
9	2.04 F	1.29 G	3.36 I	1.62 A,B,C
10	2.01F	1.18G,H	3.17 J	1.75 A,B
11	1.94F	1.08H	3.03 K	1.86 A
12	1.56 G	1.44F	3.00K	1.12E
13	1.59G	1.24 G	2.76L	1.31 D,E

Means with same letter in the same column are not significantly difference at 0.05 level using Duncan multiple test.

Table 3: Total length of the long arm, short arm, total and ratio of the haploid chromosomes complement of *Acacia senegal* for 25 cells

Cell no	Long arm µ m	Short arm µ m	Total µ m
1	28.45	19.75	48.70
2	30.35	19.95	49.95
3	31.15	21.40	52.95
4	30.20	20.05	50.25
5	28.25	21.55	50.50
6	29.85	19.20	49.05
7	30.55	20.85	51.20
8	29.45	19.60	49.05
9	29.15	22.35	52.00
10	29.30	21.40	50.70
11	28.60	21.20	49.8
12	30.35	19.90	50.25
13	29.65	21.65	51.30
14	30.30	20.25	50.55
15	28.95	19.90	49.35
16	31.50	18.25	48.75
17	29.10	20.30	49.40
18	28.36	22.25	50.61
19	30.20	20.15	49.55
20	29.35	19.95	49.30
21	29.35	19.70	49.05
22	29.80	20.55	50.35
23	29.75	19.05	49.95
24	28.8	20.85	49.75
25	29.05	19.35	49.30

4.2.2.4 Position of centromere:

As shown in **table 1** and **figure3**, the results obtained classified the chromosomes into four groups according to the centromere position as suggested by (Edward, 1979).

These were:

(a) Median (L/S) ratio between (1.00 - 1.10).

(b) Slightly sub median (L/S) ratio between (1.11 - 1.30).

(c) Sub median (L/S) ratio between (1.31 - 1.70).

(d) Highly sub median (L/S) ratio greater than 1.71).

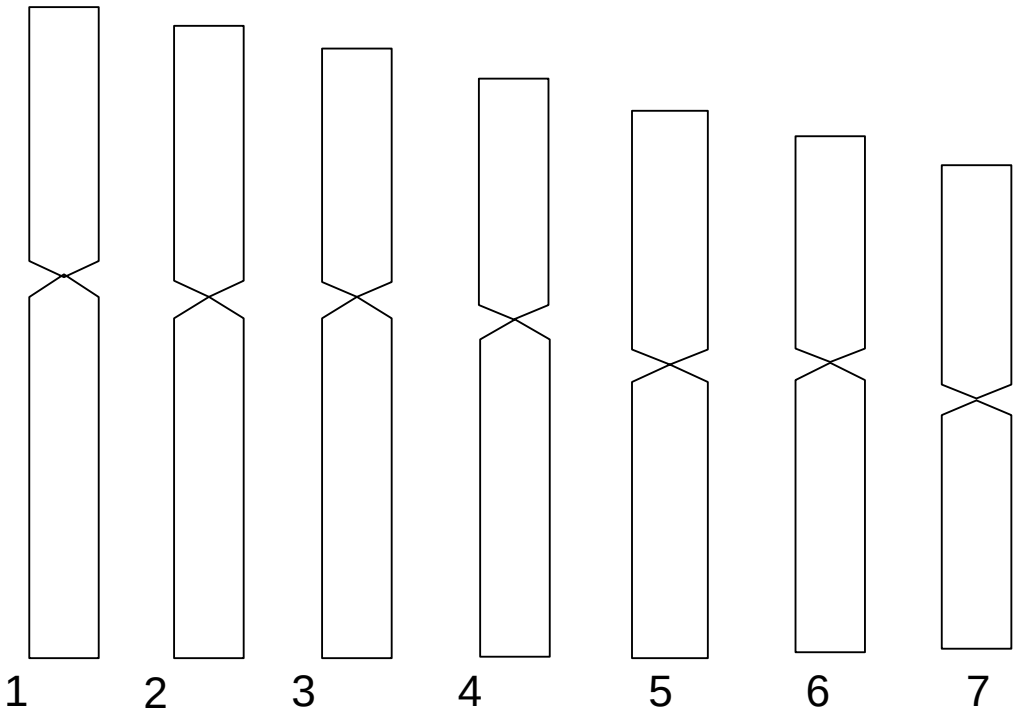
The chromosomes of this species were grouped into three classes:-

Class (1) contained the chromosomes that had median and slightly sub median centromeres these were chromosomes 5-7- 12-13

Class (2) Chromosomes with sub median centromeres, these were chromosomes 1 - 2 -3 - 4 – 6 - 9 -10

Class (3) Chromosomes with Highly sub median centromeres, these were chromosome 8 – 11.

In this study, no satellite or secondary constrictions were detected on any of the chromosomes.



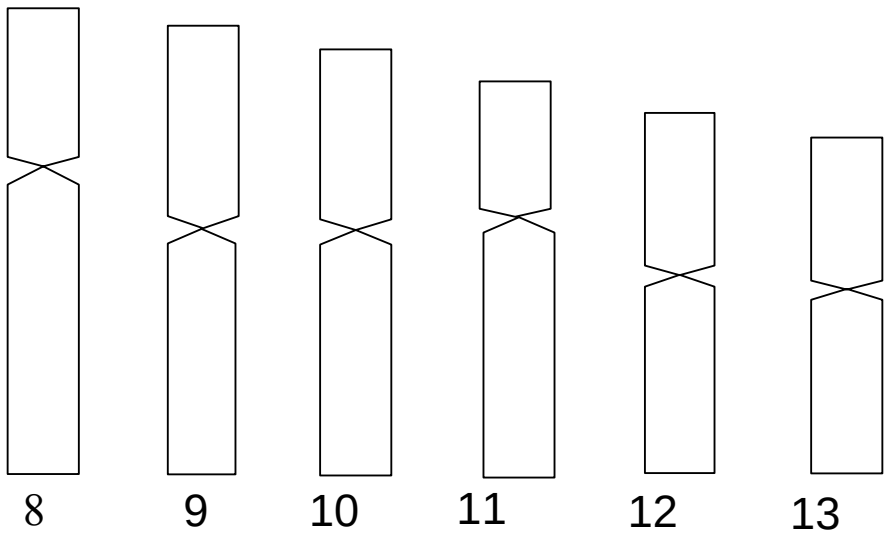


Figure 2: Ideograms showing Karyotypes of the *Acacia senegal*

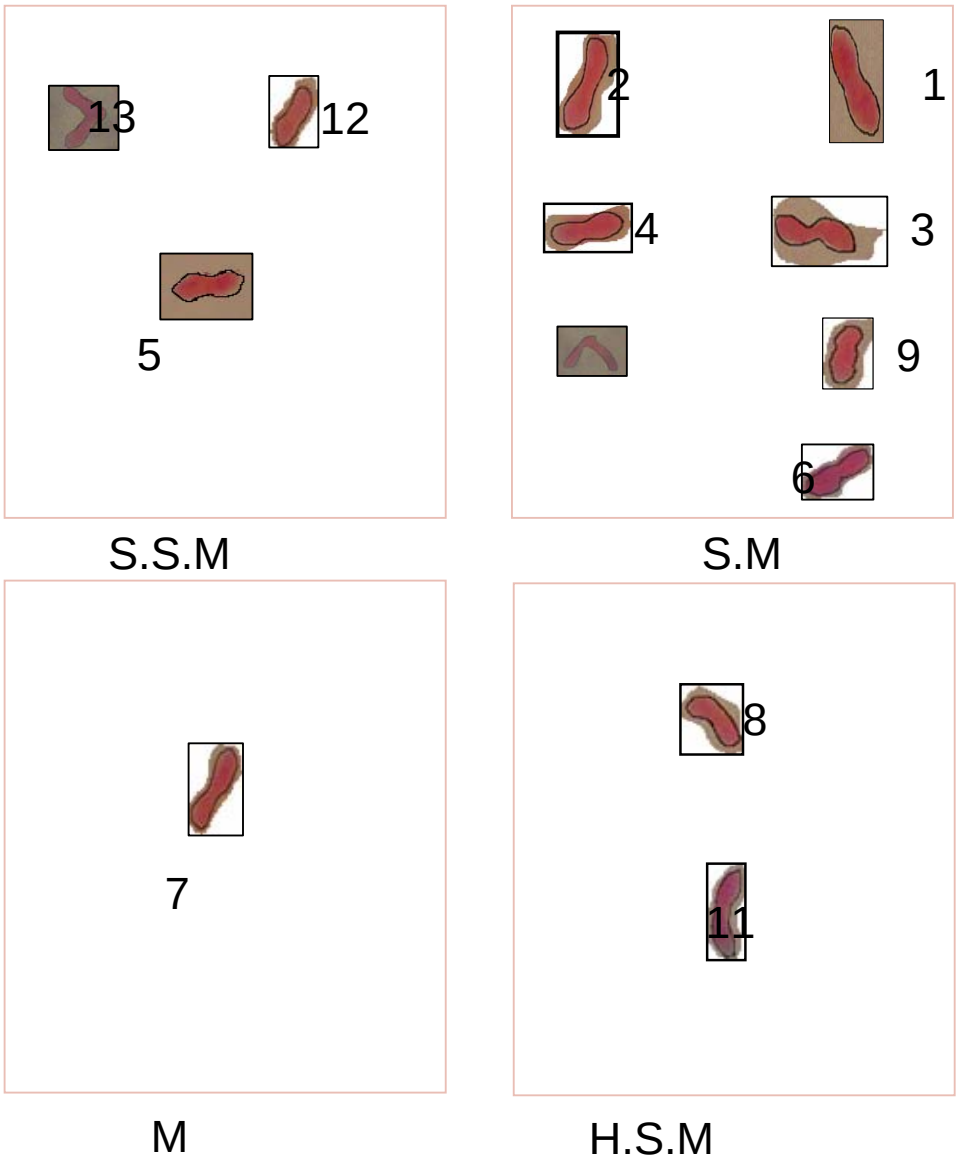


Figure 3: Position of centromere of the haploid chromosomes of *Acacia senegal*

4.2.2.5 Chromosome size

Table 4 shows the size of the chromosomes of *A. senegal* and their classification according to Ranganath and Krishnappa (1990). Chromsomes 1 to 5 are classified as long while the rest were medium. The average chromosome length of the haploid complement was 3.85 µm and classified as median size. The results are in contrast with (Bukhari, 1997).who reported the size of *Acacia senegal* chromosomes as small

Table 4 Mean size of the haploid chromosome complement of *Acacia senegal.* The abbreviations A.B indicate the long and median size of chromosomes respectively.

Chromosome No	Mean / Total	Chromosome class
1	5.43	A
2	4.97	A
3	4.60	A
4	4.33	A
5	4.09	A
6	3.98	B
7	3.76	B
8	3.57	B
9	3.36	B
10	3.17	B
11	3.03	B
12	3.00	B
13	2.76	B

CHAPTERFIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Karyotype features of *Acacia senegal* var. *senegal* were assessed using root tips that were collected after three days from seed treatments. The results showed that:-

1- The suitable collection time of root tips for cytological studies is at mid day (11-11:30) and the length of the roots is between 1 and 1.5 cm.

2- Pretreatment of root tips with 75 Mm KCl for 1 hour gave better spread of chromosomes than colchicine.

3- The haploid number of chromosome complement in *Acacia senegal* was 13 and the diploid one is 26 (2n).

4- There is an indication of tetraploid as one cell showed 52 chromosomes.

5- The chromosomes of the haploid were arranged and numbered according to their total length in a descending order and their ideogram was developed (Chromosomes one was 5.41µm and chromosome 13 2.80µm).

6- Mean length of the long and short arms of the chromosomes was 2.27 µm and 1.56 µm, respectively.

7- No secondary constriction in any chromosome.

8- The centromere position of the chromosomes was sub median to slightly sub median. Further more, symmetry indices and TF% were found 0.51 and 43.75% respectively of the species.

9- The chromosomes are of median size. The mean chromosome length ranged from 2.80µm to 5,41µm. Chromosomes 1- 5 were classified as long while chromosomes 6-13 were of median size.

5.2 Recommendations

From the results of this study and the observations during the experiment the following is recommended:-

1- More investigation is needed to study the presence of tetraploidy.

2- Detailed study for chromosomal abnormality using the identified ideogram.

3- The methods of this study can be used for karyotype analysis of other *Acacia* species.

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