MICROPROPAGATION OF ACACIA SEYAL VAR. SEYAL DEL. USING SINGLE NODLE EXPLANTS.

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DEDICATION

This thesis is dedicated to my parent’s soul
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Abstract

*A.seyal var. seyal* (Talh tree) is considered as an important multipurpose tree species in Sudan, and there is a great need for developing *in vitro* propagation methods for clonal propagation of elite cultivars. The aim of this study is to develop a direct micropropagation of *A. seyal* plantlets and test the effect of basal salt mix of MS medium and some growth regulators on shoot growth and root formation.

Single node explants was obtained from mature tree of *A. seyal* and were cultured on Murasige and Skoog medium (1962). MS strengths, sucrose and glucose as source of carbohydrates, inositol, MS vitamins and modified White vitamins (1939), adenine sulfate, activated charcoal and silver nitrate are tested as experiments of basal MS media. The growth regulators, cytokinins and auxins are tested singly.

The results show that 1.0x and 0.5x (full and half) strength of MS salt mix medium are the best basal salts for *A. seyal*, 2-3% w/v of sucrose; 100mg/l inositol, asingle dose (1.0x) of MS vitamins; 40.0mg/l adenine sulfate; 2.0g/l of active charcoal and 1.0 and 5.0mg/l silver nitrate are quite suitable for propagation of Talh trees.

The addition of 1.0 or 1.5mg/l BA, 1.5 or 2.0mg/l kinetin, 1.5 or 2.0mg/l IBA, 1.5 or 2.0mg/l NAA are suitable concentrations for culture of single node explants of Talh trees.

Excised shoot from *in vitro* explants were used for rooting. Different types of MS strength were tested with different concentration of IBA. Rooting ability was achieved on ¼ MS medium with 0.5mg/l IBA (rooting 30%).
ملخص الاطروحة

1. نمو الأشجار | أبحث عن التفسير الصحيح لتصنيف الأشجار في الناحية الفسيولوجية. نجد أن النمو الأشجري يعتمد بشكل كبير على بيئة التغذية. النمو الأدنى كان في النباتات التي تم إعطاؤها كمية قليلة من الزراعة العضوية. النمو الأعلى كان في النباتات التي تم إعطاؤها كمية كافية من الزراعة العضوية ونسبة من 100. نحن نستنتج أن النمو الأشجري يمكن أن يتم تعديله من خلال تغيير النسب المكونة للزراعة العضوية.

2. تأثير الرطوبة | أبحث عن تأثير الرطوبة على النمو الأشجري. النمو الأدنى كان في النباتات التي تم إعطاؤها نسبة عالية من الرطوبة. النمو الأعلى كان في النباتات التي تم إعطاؤها نسبة منخفضة من الرطوبة ونسبة من 30%. نحن نستنتج أن النمو الأشجري يمكن أن يتم تعديله من خلال تغيير نسبة الرطوبة.

3. تأثير الصوديوم | أبحث عن تأثير الصوديوم على النمو الأشجري. النمو الأدنى كان في النباتات التي تم إعطاؤها نسبة عالية من الصوديوم. النمو الأعلى كان في النباتات التي تم إعطاؤها نسبة منخفضة من الصوديوم ونسبة من 1.5. نحن نستنتج أن النمو الأشجري يمكن أن يتم تعديله من خلال تغيير نسبة الصوديوم.

4. تأثير البوتاسيوم | أبحث عن تأثير البوتاسيوم على النمو الأشجري. النمو الأدنى كان في النباتات التي تم إعطاؤها نسبة عالية من البوتاسيوم. النمو الأعلى كان في النباتات التي تم إعطاؤها نسبة منخفضة من البوتاسيوم ونسبة من 2.0. نحن نستنتج أن النمو الأشجري يمكن أن يتم تعديله من خلال تغيير نسبة البوتاسيوم.
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Chapter one
Introduction

1-1 Background

The biodiversity of world’s forests is declining at a rapid rate; however, the demand for wood is predicated to double in this century (Gupta et al., 1993). Global problems, such as increasing populations and diminishing resources, necessitate improvement in the strategies for breeding of woody perennials. It is necessary to protect current resources against genetic erosion, diseases, fire, and other natural disasters. Forests need to be replenished as they are continually being harvested. Reforestation requires tree improvement and emphasis has shifted to the production of genetically superior trees with the aim of producing faster-growing trees with improved dimensions and increment, better wood quality, disease resistance and environmental tolerance (Durzan, 1988).

Vegetative propagation is a pivotal technology, where large trees with long, complex life cycles are brought into the laboratory for improving productivity and for clonal propagation. Genetic gains can be maintained by clonal propagation and new variations can be introduced through biotechnological techniques (Durzan, 1988).

*Acacia seyal* variety *seyal Del.* is one of few strongly gregarious of Sahelian tree species. It combines tolerance of periodically inundate Sahelian heavy clays with major roles in fuel and fodder production in countries at the southern edge of the Sahara desert, especially Mali, Chad and Sudan (Hall and Allan, 1993). It is considered as one of the most important multipurpose tree species in Sudan, and listed as a top priority species in the forestation programs (Warrag et al., 2002). A gum
(gum talha) is collected from the tree and a proportion enter of it international grade (Hall and Allan, 1993).

It is widely distributed and covers most of the Sudan, extending from the semi – arid lands in the North, to the Savannah woodland in the south (El Sheikh, 1987). The tree is mainly used in Sudan for firewood, charcoal, and fodder (Jackson, 1959). It was first discovered in Sudan by European explorer Delile, east of the Blue Nile drainage basin, specifically at Jebel Guli in Gedaref region (Ross, 1979).

El Amin (1973) reported two infraspecific variants of \textit{A. seyal} Del., widely distributed in Sudan, separated on the basis of the presence and absence of the ant-galls and the colour of the bark. He reported that ant-gall and white colored bark are the characteristics of variety \textit{fistula}, while the bark of variety of \textit{seyal} is green or red. Economically, \textit{Acacia seyal} forests have many economic and environmental values.

They constitute about 8-10% of the plantation programs of the Forest National Corporation (Mahgoub and Dafalla, 1996). The economic values include the production of fuel wood, gum, tannins and forage. Talh gum, second to hashab (\textit{A. senegal}), is traded by the Sudan under the trade name (talha). It makes up to 10% of the annually exported Gum Arabic (3000-6000 ton/year) (Von Maydell, 1986). Domestic and wild animals graze the leaves, young branches, flowers and pods especially in dry seasons. Pods contain more than 20% protein and are very nourishing (El Sheikh, 1987). The tree is good in soil stabilization and soil fertility as it fixes atmospheric nitrogen. It is widely used as a medicinal plant in Sudan the bark, leaves, and gum are used for the treatment of many diseases such as haemorrhage, cold, and diarrhoea (El Ghazali \textit{et al.}, 1998).
1-2 Vegetative propagation

Vegetative propagation is an asexual reproduction of plants that shows the ability of plant parts to regenerate roots and shoots and grows into a new plant that is genetically similar to the parent plant (Hartman and kester, 1975). Rooted stem cuttings for operational uses, has some problems like; ageing, early selection and testing, interaction with environment where are grown and genetic variation existence (Kleinschmit, 1977). Young trees were found to root easily, while older trees that are difficult to root. Physiological mature tissues have lower rooting percentage and it takes along time to initiate roots than juvenile tissue (Ducci and Locci, 1978, Franclet, 1979). Other problematic factors including, tropic seasonality and microclimate such as (humidity, and temperature) and rooting media (Talbert et al., 1982; Ali, 1986, Barnes and Burley, 1987; Zobel et al., 1987; Leakey, 1987; Rhodes and Felker, 1988; Longman, 1993). Rooted stem cuttings as a method for vegetative propagation of most woody trees species, was reported to be difficult in Acacia species. Abu Elgasim (2000) failed to propagate A. seyal by stem cuttings of old or mature tree. Clonal propagation by tissue culture offers an alternative to cuttings and has the potential to provide high multiplication rates of uniform genotypes, resulting in short term gains.
(Durzan, 1988) and providing an alternative for species, which are difficult to root (Gupta et al., 1993).

1-3 Problem statement

Vegetative propagation of most Acacia species by conventional means as stem cutting and grafting has been difficult and the percentage of rooting was low. Ali (1997) reported rooting percentage of 16.7% for old explants and 10% for younger ones of stem cuttings of A. senegal. Recently limited success has been achieved by production of some Acacia species plantlets through tissue culture. The first reported work that encouraged the continuation and development of tissue and organ cultures on the genus Acacia was on shoot tip of A. koa (Skolmen and mapes, 1976). This was followed using cotyledonary bud of A. albida (Duhoux and Davies, 1985); shoot tips and axillary buds of A. auriculiformis (Rao et al., 1989; Mittal et al., 1989). Regeneration of nodes and internodes of field grown twigs of A. nilotica (Mathur and Chandra, 1983), nodal explants, of in vitro raised shoots from mature nodal explants of 4-year-old tree, juvenile nodes, hypocotyls and axillary buds of A. mangium (Darus, 1991), and apical meristems from A. mearnsii (Beck et al., 1998). Ibrahim (2000) reported that nodal explants obtained from in vitro raised seedlings of A. senegal, produced 6 shoots per nodal segment on MS medium supplemented with 20g/l sucrose, 100mg/l
inositol, 10mg/l silver nitrate, 60mg/l adenine sulfate, 2mg/l kinetin or BA. (90%) rooting was obtained on half MS medium with 2 mg/l IBA with reduced sucrose concentration to 1% w/v.

*Acacia seyal* is normally propagated by seeds. Thus, there are considerable variations in the progeny produced, and no vegetative means has been reported for asexual propagation (Douglas and Hart, 1984). Rooting of stem cuttings was not successful (Abu Elgasim, 2000). Seed production and viability depend on rainfall, and seeds are also vulnerable to insect attack (Mahmoud *et al.*, 1984). Al-Wasel (2000) reported that *in vitro* mass propagation of *A. seyal* was achieved by culturing seedling shoot tips on MS medium supplemented with 2.0mg/l BA with 0.1 or 0.5mg/l NAA. The highest rooting percentage (80%) was promoted by 4.0mg/l IBA. *A.seyal* as an important species is a suitable target for *in vitro* propagation. Micropropagation of *Acacia* species is said to be a viable method for producing numerous plants for reforestation, conventional breeding and mass propagation (Ortiz *et al.*, 2000).

**1-4 Objectives**

**The main objective**

- 1-To test the hypothesis that plantlets of *Acacia seyal* can be produced by direct micro propagation.

The specific objectives were:

- 1-To determines the effect of basal media concentration on shoot growth.
Chapter Two

Literature Review

2-1 The species

The genus Acacia belongs to the largest, widely spread family; the Leguminosae, which is considered to be one of the largest families of the angiosperms. It includes about 570 genera and 17,600 species (Hutchinson, 1964). *A.seyal* Del.belongs to the genus Acacia, family Leguminosae, and sub- family Mimosaceae.

2-1-1 Botanical Descriptions

The tree is about 3-17m high, crown spreading or irregular, bark powdery, smooth or sparsely flaking, whitish to greenish yellow or orange-red sometimes green and red
bark occurs in the same tree. Young branchlets almost glabrous, with numerous reddish glands (El Amin, 1973).

As described by Abu Elgasim (2002) stipules length, 1.3-4.5cm, leaves 1-12cm long, leaflets 7-20 paired, petiole length 0.6-1.2cm, and pinna length 0.9-2.3cm.

Inflorescence capitates, yellow, peduncle 1-4cm long. Pods falcate, fruit length 10-13cm, seed length 0.6cm. Flowering November-April, fruiting January-May.

2-1-2 Habitats

The tree is found in dark cracking clays, found often on higher slopes of rivers and valleys in addition to the land clay plains of central Sudan (El Amin, 1973).

2-1-3 Distributions

*Acacia seyal* is widely distributed in north Africa, in the Sahelian zone from Senegal to Chad, across Sudan in Eastern Africa, from Egypt southward to Somalia, Kenya, Mozambique and Namibia. It has also been recorded in Syria, Jordan and Sinai (Mohamed, 1963). The main belt where *Acacia seyal* occurs on clay plains lies between latitude 10° and 14° N, and has annual rainfall range of 600–800 mm.

2-1-4 Seed Germination

Germination rate is low (10-25%) because of the hard seed coat. It could be increased up to 80% when seeds are treated mechanically or soaked in concentrated sulphuric acid for 15 min (Mahgoub and Dafalla, 1996).

2-1-5 Natural Regeneration

Reproductive stage may be reached rapidly within five years in natural stand, unless the growth is retarded by local events such as intense
browsing or fire. Natural regeneration is excellent from seeds and coppice. Mustafa, (1997) suggested that the regeneration of natural forests of *A. seyal* should rely on seed rather than on coppice. Regeneration can also be enhanced by fire and soil disturbance (Badi *et al.*, 1989).

### 2-2 Vegetative propagation

Vegetative propagation and clonal selection offer means to increase yield and improve quality of forest products of commercial planting in tropics (Leaky *et al.*, 1990). This differs greatly from sexual propagation that retains the potential for diverse genetic variation (Fretz *et al.*, 1979).

Foresters employed vegetative propagation for production of uniform plants of known genotypes (Fielding, 1963), mass multiplication and for provision of genetic and physiological information (Hinds, and Krugman, 1974; Dormaling *et al.*, 1979). Rooting of cuttings is a method of macropropagation in which cuttings can be made from stem, leaf, root, and tissues. Stem cuttings are the most common type used in propagation of forest tree. The ability to root cuttings varies with species, but hard wood species are generally easier to root than conifers (Duryea and Brown 1984). It is not always successful with older material due to the maturation effect that results in poor rooting of most plants. The ability of cuttings to produce root depends on age, species and the genotype of
plant. Also, it varies with position in crown of the donor plant and time of year (Franclet et al., 1987). Abu Elgasim (2000) failed to propagate *A. seyal* by stem cuttings from mature trees.

2-3 Tissue and organ culture concept

The main concept of tissue culture is based upon the ability to culture individual plant cells, tissue and organs in a suitable growth medium, under aseptic conditions, in order to regenerate a functional plant that can be established in the field. This process requires carefully controlled environmental condition like light, temperature and humidity in addition to other basic culture requirements.

Tissue culture methods have greatly increased the scope and potential of propagation by exploiting the regenerative behavior more efficiently than conventional ones (Hussey, 1980). In addition, large-scale clonal multiplication is most widely used as one of the commercially successful application of biotechnology (Vasil, 1990). Plant tissue culture provided means to multiply endangered tree spp, disease - free plants, recalcitrant species (Wang and Hu, 1980; Bunn and Dixon, 1992); over coming of seasonality, safe germplasm exchange, and a research tool for better standing of physiological, cytological, biochemical and pharmaceutical processes. In addition, tissue culture techniques have been applied to a wide range of tree species. Nevertheless, it is evident that there is a strong and intricate interaction between the explants, genotype, plant growth regulators and culture conditions (Nehra and Kartha, 1994).

2-3-1 Stages of Micro propagation:
Murashige (1974) defined three stages and Debergh and Maene (1981) added two more, namely stage zero and stage 4.

**Stage 0:** stock plants selection.

Careful attention should be made that the stock plant is a typical variety or cultivar, vigorous and healthy.

**Stage 1:** Establishment of an aseptic culture.

The goal is to obtain large percentage of explants free from surface pathogens and exudates that blacken the media and affect the whole culture.

**Stage 2:** Multiplication of the tissue.

The stage of rapid multiplication, through somatic cell embryogensis, axillary bud development or adventitious shoot formation.

**Stage 3:**

Murashige (1974) devoted stage 3 for rooting and Debergh and Maene (1981) divided it into 3A and 3B, where the former was devoted for elongation of shoots formed during stage 2 and the latter for rooting of elongated shoots *in vitro* or *ex vitro*.

**Stage 4:** *ex vitro* rooting and acclimatization:

Propagules are transferred from culture vessel to greenhouse or growth chamber for acclimatization to the field condition. For successful accomplishment of this phase; the propagules are thoroughly washed to remove the adherent agar and nutrient media from root. The plantlets are then transferred to small containers; sterilization of the potting media is preferred. Potted propagules are then covered with glass or plastic cover or they are placed under fogy or intermittent mist in the greenhouse. Generally, *in vitro* propagules lose water rapidly when removed *ex vitro*, due to reduced leaf epicuticular wax layer. Gradual reduction of the humidity, and increase of light intensity favor better survival chance, Debergh (1987) was able to achieve higher
number of rooted plants in vivo and Vengadesan et al., (2002) reported that the success rate of establishing the rooted plantlets of A. sinuate in the field was 55%.

2-3-2 Requirement of Tissue Culture

2-3-2-1 The Explant

Explants are, the organs, or pieces of tissue from a whole plant that are used for culture initiation. Plant organs are either determinate which are destined to have only a limited shape and size such as leaves, flowers and fruits, or indeterminate where growth potentially unlimited e.g. apical meristem of shoots or roots. Selection of explant materials has been reported to be very important for successful culture establishment (Ben-jacov and Jacobs, 1986).

Shoot tips and nodal segments are excellent explants, for clonal propagation. The shoot tips, from apical or lateral buds of intact plant consist of meristematic cell in the apex with substended rudimentary stem bearing several leaf initials. Single node explant bearing one or more axillary buds is employed and was found to be beneficial for culture initiation as in the case of Eucalyptus (De Fossard et al., 1977). Dewan, et al., (1992) used stems of A. senegal, nodes and internodes of twigs of A. nilotica grown in the field.


The time of the year in which the explant is taken may affect the results of the micropropagation program. Changes in temperature, day – length, light intensity, and
water availability throughout a year will affect the levels of carbohydrates, proteins and growth substances in the stock plant. Best results are generally achieved when the explant is taken during the active phase of growth. Tiwari et al., (2002) reported that explants collected from *Tectona grandis* in May showed a maximum shoots response (76.8%).

The easiness of the propagation declines as the age of the mother plant increases from juvenile to mature phase (Cheng, 1975; Bonga, 1982). According to Boulay (1987), *in vivo* juvenile plant materials compared to mature ones are characterized by high K/Ca ratio, high IAA/ABA ratio, and they exhibit high peroxidase activity. In *Eucalyptus* spp., Roux and Vansaden (1991) found that the number of surviving explants was largely dependent on the condition of the stock plant and young, healthy, vigorously, growing shoots, produced best results. Cloning of mature trees is generally preferred over juvenile ones, because it is not always possible to determine whether the juvenile tree will have the desired characters when they are mature (Bonga, 1987).

Successful *in vitro* establishment of explants from adult *Acacia* trees has been achieved for *A. auriculiformis* (Wantanabe et al., 1994; Reddy et al., 1995; Toda et al., 1995; Zhang et al., 1995), *A. catechu* (Kaur et al., 1997), *A. mangium* (Bhaskar and Subhash, 1995; Toda et al., 1995; Zhang et al., 1995), *A. meransii* (Beck et al., 1998) and *Robinia pseudo acacia* (Han et al., 1997). In these species, axillary buds were used as explants and the results varied from 28% shoot production of 2-years-old *A. auriculiformis* (Toda et al., 1995) to 71% of 2-years-old *A. mangium* (Toda et al., 1995). Rejuvenation and characterization of juvenile plant material prior to *in vitro* culture have proved to be important (Jones and Van Staden, 1997). Rejuvenation occurs in two specific areas in a tree, namely the meristematic structures and at the base of the trunk (Franclet et al. 1987). Thus, the use of stump shoots and the practice
of coppice are common methods for rejuvenation (Thorpe et al., 1991). Stump sprout explants exhibit better stem elongation and a greater rooting ability than that regenerated from crown explants (Bonga and Aderkas, 1992).

2-3-2-2 Genotypic effects of explant
Pierik, (1986) reported that the genotype of the explant has its effects on in vitro culture response, due to differences in cell division and regenerative capacity between plants within a single species.

Variation in genotypic response within species can be enormous in cultures derived from 17-year-old *Pseudotsuga menziesii*. The rate of shoots formation varied greatly between provenances and even between trees within provenances (Dunstan et al., 1989). The rooting percentage of shoots of mature *Pinus radiata* was found to vary with genotype (Horgan and Holland, 1989). The ease of micro propagation of mature *Quercus robur* varied with genotype within provenance (Juncker and Favre, 1989), and provenance also affects somatic embryogensis in *Picea glauca* (Tremblay, 1990).

For example in a test of 16 clones of *Populus deltoids*, carried out by Coleman and Ernst, (1989) they found that four clones rooted significantly better than the others and six clones did not respond at all.

2-3-2-3 Explant sterilization
Shoot tips and nodal explants 0.5-2cm in length after excision, and before their transfer to culture medium, should be freed from contaminant. Contamination with micro-organisms is considered to be the most important reason for losses during in vitro culture of explants (Boxus and Terzi, 1988). Surface sterilization of juvenile material, which
was grown in greenhouse, is generally not difficult. However, explants from old trees are difficult to sterilize, unless the tree produces juvenile sprouts. Explants 5- years – old *Eucalyptus grandis* were all contaminated after surface sterilization while more than half of those taken from its juvenile sprouts were clean (Warrag *et al.*, 1990).

Enjalric *et al.* (1988) reported that explants taken from current growth are easier to be surface sterilized than explant from older section of branch. Apical meristem of mature *Juglands nigra x regia trees* has been reported to be free of contaminants whereas stem sections were severely contaminated (Meynier and Arnould, 1989). Proper plantlets were produced in spite of the fact that at least two bacterial contaminants survived surface sterilization with sodium hypochlorite (Monette, 1986). Enjalric *et al.*, (1988) found that sodium hypochlorite was more effective than mercuric chloride in surface sterilization of stem sections of *Hevea brasilienses*. However, mercuric chloride is effectively surface sterilized lateral shoots of *sequoiadendron giganteum* (Bon *et al.*, 1988) and it was more effective in hard- wood species (Chalupa, 1987a). Normand and Fortin (1982) found that hydrogen peroxide was the most popular sterilant for seed coats of *Alnus, crispa, and azomin* and when treated with 30% hydrogen peroxide resulted in the highest percentage of clean cultures and morphologically normal seedlings.
Traditional sterilization is sometimes ineffective and improved sterilization was obtained with fungicides and bactericides such as benomyl and rifampicin. Haldeman et al., (1987) working with *Camellia sinensis* and *Japonca* shoot tips explants, used benomyl and rifampicin respectively for 24 hours after sterilization in sodium hypochlorite. They found that this treatment reduced contamination rates and had no phytotoxic effect.

2-3-2-4 Nutrition of plant tissue culture

Ozias- Akin and Vasil (1985) stated that isolated explant lack the ability to synthesize their own supply of carbohydrates, vitamins and plant growth substances. Consequently (George et al., 1987) regarded that plant tissue culture medium is a mix of nutritional and regulatory factors. Adequate medium is crucial for growth and various genotypes have different nutritional requirements.

2-3-2-4-1 Media Composition

Media formulations were proposed by many authors (White, 1943; Murashige and Skoog, 1962; Gamborge et al., 1968; Lloyd and Mc Crown, 1981; Schenk and Hilderebrandt, 1972; Bonner and Deverian, 1939). White (1943) outlined a formula with low mineral salts compared to others. Although it is employed extensively in research work, it is also used for cultures of excised roots and anthers. The formula forwarded by Murashige and Skoog (1962) designated MS- in order to optimize the growth of tobacco pith callus was characterized by high levels of mineral salts.
Nitrogen supply comes from potassium nitrate along with ammonium nitrate in high concentration. In addition to salts, it also included sucrose, inositol, and a mixture of vitamins.

Zhang et al., (1995) used MS medium for culturing explant buds from 4-years’ old trees of *A. auriculiformis*, and obtained 66.7% rooting, and Galiana et al., (1991) used it for culturing nodes excised from *A. mangium*. In cultures of many woody species full strength MS was reported to be inhibitory to organized growth, and that has been diluted by lowering the mount of ammonium or total nitrogen (Tsogas and Bouriquet 1982, Vieitez et al., 1983, Perez- Bermudez and Sommer, 1987). Shoot formation occurred in embryo explant of *Pinus strobes* on half strength MS medium while shoot formation failed to occur on full strength MS due to high ammonium content (Flinn et al., 1986). Han et al., (1997) used half strength MS medium for culturing shoot explants from *Robinia pseudo acacia*; Ide et al., (1994) used half strength MS medium for culturing shoot explants from *A. auriculiformis* and Nandwani (1995) used half strength MS medium for culturing shoot from *A. tortilis*. Ahee and Duhoux (1994) used 1/5 strength MS medium for culturing root explants from *A. albida*.

Lloyd and MC Crown (1981) formulated a lower level salt base medium commonly known as Woody Plant Medium (WPM) used by Rout et al., (1995) for culturing immature explant of *A. Catechu* and by Palma et al.,
(1996) culturing one nodal explants from *A. senegal*. However, Beck *et al.*, (2000) used MS, half MS and WPM medium for culturing meristem explant from 30- day old *in vitro* grown plantlets from adult and coppice trees of *A. mearnsii*.

Dewan *et al.*, (1992) used Gamborge *et al.*, 1968, medium (B₃) used for culturing cotyledons, nodes and shoot explants from *A. nilotica*; Skolmen and Mapes (1976) used Schenk and Hilderebrandt, 1972(SH) medium for culturing shoot tip explants from *A. koa*. Ahee and Duhoux (1994) used Bonner and Deverian, 1939(BD) medium for culturing root explants from *A. albid* and Semsuntud and Nitiwattanachai (1991) used white (1943) medium for culturing shoot explants from *A. auriuliforneis*.

2-3-2-4-2. *Carbohydrates*

Carbohydrate functions in tissue culture have been reviewed by several authors (Thorpe 1982; Grey *et al.*, 1987; Thompson and Thorpe, 1987). They are found to be obligatory for cell growth when the cells are grown in the non-photosynthetic light; serve as an energy source, an osmoticum and have distinct morphogentic effects.

According to Ozias- Akin and Vasil (1985) the most commonly used carbohydrate in plant tissue culture media is the disaccharide, sucrose, at concentration of 2-3%. High carbohydrate concentration (more than 10%) was required in cultures of immature zygotic embryo and during
induction of somatic embryogenesis in *Carica* species suspension cultures (Litz, 1986). In contrast, high sucrose concentration in the medium has possible negative consequences in stimulating the formation of the phenolic compounds (Zaprometov, 1978). In addition, exposure of cells of *Vitis vinifera* to high concentrations of sucrose or mannitol resulted in water stress and anthocyanin formation (Do and Cormier, 1990).

Other carbohydrates such as glucose, fructose have also been used for example Chauvin and Salesses (1988) reported that micropropagation of mature *Castanea* sp. proceeded at a higher rate, with less necrosis, and with improved quality on medium containing glucose or fructose than on sucrose containing medium.

Chee and Pool (1988) tested various sucrose concentrations for rooting of *Vitis* explants. The percentage of rooting decreased with the increase of sucrose concentration more than 1% whereas shoots rooted in 3% sucrose were of low quality and unsuitable for transfer to the soil.

Skolmen (1986) added 20g/l sucrose for culturing *A. Koa*; Badji *et al.*, (1991) added 40g/l sucrose for culturing *A. senegal*; Mittal *et al.*, (1989), added 20g/l sucrose in MS medium for rooted shoots of *A. melanoxylon* and Kaur *et al.*, (1997), added 30-g/l for shooting and 15-g/l for rooting of *A. catechu*. From the previous mention, therefore the different stages of culture may exhibit different sugar needs.
2-3-2-4-3 Myo- inositol

Myo- inositol was reported to be one of the active ingredients in coconut milk (Street, 1979) and was involved in the synthesis of phospholipids and cell wall pectin’s (Anderson and Wolter, 1966; Street, 1979; Grey et al., 1987). Kyte (1983) described inositol as a sugar alcohol, and in its phosphate form, it is a part of various membranes of certain organelles such as chloroplasts.

Linsmaier and Skoog (1965) encountered 50% growth decrease in tobacco callus when inositol was omitted from the medium. Zimmerman and Cobb (1989) found that some explants do not become vitrified, when inositol at a sufficiently high rate to satisfy their chemical requirement. Gupta et al., 1988 and Attree, et al., (1989) reported that inositol in the medium for development of cell colonies from Pseudotsuga menziesii and Picea glauca, protoplast induction was stimulated by high amounts of insitol. Ahee and Duhoux (1994) used BD medium supplemented with 9-mg/l myo-inositol for culturing explants from A. albida and Mohamed (1994) used MS medium supplemented with 100mg/l inositol for culturing shoot tips excised from Psidium guajava.

2-3-2-4-4 Vitamin

Vitamins are needed in small quantities by plant cells to perform catalytic roles in metabolism. Thiamine (Vit. B1) is regarded to be the only essential vitamin in most tissue culture media whereas others such as
biotin and niacin are involved in electron transfer dehydrogenises, lipid catabolism and photosynthesis (Kutsky, 1973, Hagen et al., 1991). Moreover, pyridoxine regulates protein, carbohydrates and lipid metabolism. All these vitamins are rapidly removed from the culture medium and utilized by growing cells (Hagen et al., 1991). Murashige and Skoog (1962) included thiamine- HCl, pyridoxine- HCl, niacin, and glycine beside inositol in their culture medium. Calcium panthothenate and riboflavin are occasionally added to culture media (Bhojwani and Razdan, 1983). For micro propagation of *Eucalyptus* spp, the multiplication medium was further enriched with 0.1-mg/l calcium panthothenate (a member of B vitamins) and 0.1 mg/ biotin (Vit. H) (Roux and Vansaden, 1991) and Beck et al., (2000) added 0.1mg/l calcium panthothenate for culturing *Acacia mearnsii*.

In an experiment with isolated embryos of *Pinus contorta*, Arnold and Eriksson (1981) tested several vitamins with various strengths and they found that 1/4strength was optimal for adventitious shoot formation. Ibrahim (2000) used a modified Whites vitamin according to Mahdi (1985) for *Acacia senegal*. Mohamed (2003) used MS vitamins for culturing nodes of *Silvadora persica*.

2-3-2-4-5 Adenine sulfate

George and Sherrington (1984) indicated that adenine sulfate brings about or reinforces responses normally attributed to cytokinin action. However Bonner and
Deverian (1939) reported that it effect on leaf area expansion. It can promote direct adventitious shoot formation on the explants in the presence of a recognized cytokinin (Ziv et al., 1970). Pack et al., (1987) found it to be the only beneficial factor for growth and multiplication of Chinese cabbage, compared to auxins, cytokinins, and other tested factors. In Acacia species adenine sulfate used mostly in the concentration of 7.8-274.4µM (Vengadesan et al., 2002). Vengadesan et al., (2003) added 135.7µM adenine for A.sinuate and Ibrahim (2000) added 60.0mg/l for A. senegal.

2-3-2-4-6 Growth regulators

Phytohormones occur naturally in plant tissue in very low quantities and regulate growth and development. Five principal classes of plant growth hormones (auxins, cytokinins, gibberellins, Abscisic acid and ethylene) have been recognized. However, growth process is controlled by interaction of two or more of the growth hormones (Leopold, 1987).

2-3-2-4-6-1 The cytokinins

The cytokinins commonly applied are 6-benzylaminopurine (BAP), 6-furfurylamine purine (kinetin), 2-isopentenyladenine (2ip) and zeatin where the latter two are natural ones. Cytokinins are required for callus formation and other processes involving cell division (Minocha, 1987a) and uptake of potassium (Green and Muir, 1979). BAP is the most active, cheapest, cytokinin and it can be autoclaved. It is the one most used; particularly in commercial micro propagation where cost and ease of
handling are of major considerations (Zaerr and Mapes, 1982; Thomas and Blakesley, 1987).

Lights have been reported to affect the action of cytokinins. Baraldi et al., (1988) found that BAP promoted shoot formation in *Prunus institia* shoot cultures exposed to light, whereas, shoot elongation was inhibited by BAP in cultures kept in light or dark.

Flinn et al., (1986), using embryonic explants of *Pinus strobes*, found that adventitious shoots were induced 10-20 times more effectively by BAP than by 2ip. Banko and Stefani (1989), using nodal explants from mature *Oxydendrum arboreum*, reported that the rates of shoot formation and shoot elongation were higher with zeatin than with either BAP or 2ip. Beck et al., (1998) obtained shoots from nodes of coppice of *A. mearnsii* on MS medium supplemented with 2.0 mg/l BAP. Barakat and EL-Lakany (1992) obtained shoot elongation from buds of *A. saligna* on MS medium supplemented with 0.3 mg/ l BA+ 0.2 mg/ l IAA and Jones et al., (1990) obtained callus and axillary shoots from nodes, internodes, and phyllode explants of *A. salicina, A. saligna* and *A. sclerospera* on MS medium supplemented of IAA or IBA + BAP. Moreover, Macrae (1994) obtained shoot bud formation from cotyledon nodes of *A. tortilis* on MS medium supplemented with 0.1-mg/l NAA+5 mg/l BAP. Deguchi et al., (1994), obtained shoots from buds of *Robinia hispida* on half strength
MS medium supplemented with 0.1mg/l BAP. Dewan et al., (1992) obtained shoots from cotyledonary nodes of *A. nilotica* on B5 medium supplemented with 1.5 mg/l BAP and Toda et al., (1995) obtained 71.4% shoots production from axillary buds of 2-years old seedling of *A. mangium* on MS medium supplemented with 1.0 mg/l BA. Bhaskar and Subhash (1995) obtained shoot production and subsequent rooting from nodes of 8-years old adult tree of *A. mangium* on MS medium supplemented with 3.0mg/l BA +0.1mg/l NAA +100gm/l ascorbic acid.

### 2-3-2-4-6-2 Auxins

Auxins such as indole acetic acid (IAA) are involved in expansion, elongation and in cell wall synthesis. IAA is an unstable compound, easily oxidized and may be destroyed by light (Nissen and Sutter 1988). Thorpe (1978) showed that, IAA is involved in morphogenic processes like root formation, and callus formation of woody trees. IAA induces shoots in cultures of *Pseudotsug amenziesii* (Cheng, 1975); *Tsuga heterophylla* (Cheng, 1976); and shoots and roots in cultures of *Eucalyptus alba* (Kitahara and Caldas1975).

Indole 3-butyric acid (IBA) is similar to IAA in molecular structure but differs in the length of the side chains and is more stable. It is effective as a rooting agent and it has been used in cultures at higher concentration (1-10mg/l) (Coleman and Thorpe, 1977).
Naphthalene acetic acid (NAA) is another auxins widely used in cultures of gymnosperms (Straus and Epp, 1960) and angiosperms (Chalupa, 1974). The mode of action of NAA is probably similar to that of IAA, but the former is much more stable than the latter. As it is a synthetic chemical not subjected to enzymic oxidation. For these reasons, NAA has been used extensively as a substitute for IAA by tissue culturists. Rooting is commonly induced on calli as well as on embryos or explants by NAA (Wolter, 1968; Coleman and Thorpe, 1977). In addition, NAA is effective in initiating adventitious buds in culture over a relatively wide range of concentrations (Cheng, 1977).

2, 4-dichlorophenoxy acetic acid (2, 4-D) is an effective and strong auxin in callus induction. It is avoided when direct organogensis or enhancement of axillary shoots is an objective. It is use should be limited since it can induce mutation (Pierik, 1986). However, it is necessary for inducing somatic embryogensis (Guerra and Handro, 1988) and callus formations in shoot cultures of Eucalyptus ficifolia (Gorst et al., 1981). Darus (1991) noticed that excised micro-propagated shoots of Acacia mangium treated with a hormone rooting powder (Seradix No.3), produced more roots than shoots treated with auxins NAA or IAA. Das et al., (1993) induced roots on shoots of A. auriculiformis using MS medium supplemented with 1.0 or 1.5 mg/l IBA. Toda et al., (1995)
rooted shootlets of *A. mangium* on MS medium supplemented with IBA and / or NAA; Bhaskar and Subhash (1995) rooted shoots of *A. mangium* on MS medium supplemented with 1.0 mg/l IBA and 0.5 mg/l NAA and Huang *et al.* (1994) rooted shoots of *A. mearnsii* regenerated *in vitro* on half – strength MS medium supplemented with 0.6 mg/l NAA.

In some explants the production of endogenous auxin is sufficient for shoot induction, and addition of low concentration of auxin to the medium is inhibitory such as in *larix decidua* cultures (Bonga and Aderkas, 1988) and *Picea abies* (Born man, 1983).

There are many physiological factors that control endogenous auxin level such as IAA- oxidase activity, which was influenced by micronutrient phenolics compound (Webb *et al.*, 1988), and environmental factors such as temperature. In addition endogenous auxin levels are also determined by the rate of release of IAA from inactive IAA- conjugates in the plant cells (Zaerr and Mapes, 1982).

**2-3-2-4-6-3 Gibberellins (GA₃)**

Gibberellins such as *g*ibberellic acid are growth hormones it can control cell elongation, bud break, seed dormancy and flowering and they were either promotors or inhibitors (Zaerr and Mapes, 1982). Multiplication of *Thuja occidentalis* shoots was promoted by *GA₃* (Harry *et al.*, 1987).

Addition of 5.0 mg/l of *GA₃* enhanced elongation of main shoot of *A.*
*auriculiformis* (Ide *et al*., 1994). In cultures of some *Morus* genotypes, GA₃, stimulated shoots and root formation (Jain *et al*., 1990).

**2-3-2-4-6-4 Abscisic acid (ABA)**

Abscisic acid is the most prominent among the natural inhibitors and it produces a respond when trees are exposed to environmental stress (Johnson, 1988). ABA should not be considered merely as a cell division inhibitor and many in cases it has a morphogenic role. It stimulates the induction of somatic embryos of several conifer e.g. *Picea abies* (Arnold and Hakman, 1988, Boulay *et al*., 1988), *Picea glauca* (Dunstan *et al*., 1988); promotes the accumulation of storage protein, mRNA and oil-body protein during zygotic and somatic embryogensis in oil seed species (Taylor *et al*., 1990); plays a major role in the control of leaf form heterophylly (Goliber and Feldman, 1990), and may promote the transition of plants from the juvenile to the flowering stage (Podolnyi *et al*., 1989). Barghchi, (1987) reported that ABA inhibited axillary shoot multiplication but not shoot elongation in cultures of *Robinia Pseudoacacia*

**2-3-2-4-6-5 Ethylene**

Ethylene production by tissues is stimulated by auxins, kinetin, CO₂, calcium, and copper (Lau and Yang, 1976; Yang and Hoffman, 1984; Romani, 1987), and its biosynthesis is counteracted by cobalt and nickel
In green leaf tissues ethylene production is inhibited by light (Yang and Hoffman, 1984). In addition, it is stimulated or inhibited by a variety of chemicals added to the nutrient medium, such as silver nitrate. Some cultures failed to form shoots on media supplied with various auxins/ cytokinin combination unless silver nitrate is added to the medium (Purnhauser et al., 1987; Roy and Mangat, 1989). Low concentrations of silver nitrate can cause cell necrosis and the amount of ethylene produced as a result of silver nitrate induced necrosis can be greater than the lost one. This was found to be due to the assumption that silver nitrate induced inhibition of ethylene biosynthesis in non-necrotic cells in the same tissue (Liu et al., 1990). Chalutz et al., (1980) found that inorganic phosphate at high concentrations is an inhibitor of ethylene biosynthesis. Ethylene has been reported to induce inhibition of embryogensis that is counteracted by addition of polyamines (Bradley etal, 1984) or salicylic acid (Roustan et al., 1989). Hackett et al., (1988) reported that Ethylene inhibited root elongation but not root initiation in cuttings of Hedera helix.

Other important aspect of ethylene in tree physiology it is effects on phenylalanine ammonia- lyase (PAI) (an acidic peroxidase) that regulates lignin formation (Gaspar et al., 1985). In addition, ethylene has a profound effect on the regulation of growth and morphogenes (Yang, 1980), and rooting (liu et al., 1990).
Ibrahim (2000) added 10mg/l of silver nitrate for culturing *A. senegal* and Mohamed (1994) added 1mg/l of silver nitrate for culturing guava explants.

2-3-2-5 Additives

2-3-2-5-1 Gelling Agent

Agar and gelrite are the most common gelling agent used in plant tissue culture (Pierik, 1986). Agar contains high quantities of sodium and copper whereas gelrite contains potassium and magnesium at high concentration (Debergh, 1983; Pasqualetto *et al.*, 1988). Currently, gelrite (a highly purified gelling agent) is increasingly used at 0.2% W/V. Arnold, (1987) found that somatic embryogenesis of *Picea abies*, is similar response on media with 0.7-1.3% agar or with 0.2-0.5% gelrite. Ladyman and Girard (1991) observed that the type of the gelling agent has no effect on the multiplication phase. Various starches have been used to solidify nutrient media; e.g. barely, corn, potato, rice, and wheat starch (Henderson and Kinnersley, 1988). Shoots of *Betula pendula* have been cultured on medium solidified with apple pulp, and rooting was reported to be better than on medium solidified with agar (Titel *et al.*, 1987).

2-3-2-5-2 Charcoal

Activated charcoal is added to the medium primarily to adsorb and/or absorbs exudates such as phenolics compound, 1, 5 hydroxymethylfurfural (a toxic produced as a result of the break down of sucrose during autoclaving), iron in complex form and zinc (Misson *et al.*, 1983, Nissen and Sutter, 1988, Lange, 1989). Micro-
propagation was improved in many cases by activated charcoal treatment (Curir et al., 1986; Kim and Lee, 1988; Lee et al., 1988; Bach 1988; and Chen et al., 1988). It was found to stimulate embryogensis in some cases by removing abscisic acid from plant tissues cultured on medium (Johansson, 1983) and promoted elongation and rooting of adventitious shoots of Picea glauca (Rumary and Thorpe, 1984).

During micro-propagation of Phoenix dactylifera, activated charcoal fastened growth by reducing browning of the tissue (Tisserat, 1979). Chee and Tricoli (1988) washed cell suspensions with activated charcoal, and then cultured them on an extract devoid of activated charcoal and this resulted in 60-70 % of the embryo developing into normal plantlets.

Presumably, the charcoal absorbs unwanted phenolic oxidizing products and some ingredients essential to proper growth (Marks and Simpson, 1990) and thiamine and nicotine acid (Weatherhead et al., 1979). Nissen and Sutter (1990) reported that activated charcoal at concentration ranging 0.1-5%, reduced initial concentration of 10µm of IAA and IBA in liquid MS medium by 97%. Biondi et al., (1984) reported that cytokinin levels in Ulmus compestris shoots in vitro remained unaffected when transferred to medium with charcoal. Ibrahim (2000) added 2.0g/l of activated charcoal on medium for culturing A. senegal.

2-3-2-6 pH of media:

Cultures are sensitive to the pH of the medium (Minocha 1987b, Parfitt et al., 1988; George and Sherrington, 1988), and it is generally adjusted to 4.6 to 5.8. Embryogenic cultures of Picea abies were obtained at pH 6.5-7.5 than at pH 5.0-6.0 (Arnold, 1987). Pierik (1986) reported that low pH causes precipitation of iron and phosphate salts and retards
ammonium uptake, make agar sloppy, and reduces the stability of thiamine, GA$_3$, IAA, and calcium pantothenate.

**2-3-2-7 Incubation environments**

The most important factors in the physical environment of the cultures are temperature and light and have been studied by many authors (Chalupa, 1987b; Corbineau *et al.*, 1990; Nagl and Popp 1983; Hart, 1988; Mancinelli, 1989; Evers, 1982ab).

Hussey (1980) indicated that cultures grow within the range of 10-25°C, and the specific requirement of each species should be taken into consideration. For micro-propagation of shoots of hard wood species, the optimal temperature for rooting is higher (20-25°C) (Chalupa, 1987b).

Light is involved in a variety of functions in plant development where it serves as an energy source in photosynthesis and furthermore, affects cell differentiation and morphogenesis (Nagl and Popp, 1988; Mancielli, 1989). Green and Muir (1979) reported that light produces a response similar to that obtained with cytokinins and stimulates the formation of nitrate reductase resulting in reduction of leaves. Consequently, plants grown permanently under low light intensity tend to accumulate nitrate (Marschner, 1986). Fluorescent tubes and cool white fluorescents light promote IAA and IBA degradation in both liquid and agar media. However, the optimum light intensity for shoot initiation in several herbaceous genera is 1000 lux with a daily exposure to 16 hours. Evers (1981) reported that shoots of *Pseudotsuga menziesii* grew less when exposed to 8 hours light per day than 16 hours. However, the photoperiod is not a critical determinant for *in vitro* response (Murashige, 1977).
2-3-2-8 Problems Limiting Micro-propagation

2-3-2-8-1 Browning

Browning can occur in response to excision of some explants or later in culture and generally results in reducing growth rate and eventual death of cultured tissues. *In vitro* exudates have rarely been characterized chemically and scientists refer to them as tannins or oxidized polyphenols (Compton and Preece, 1986). *In vivo* phenolic compounds are evolved in compartmentalization. That is a process in woody plants in which plant seal-off damaged tissues by establishing chemical barriers or walls to guard against infection and decay (Harris, 1983). On the other hand, the role of phenolics in *in vitro* culture is not very clear and it may be protective against pathogens (Compton and Preece, 1986). It has been reported that the accumulation of phenolics is more in older stock plants than in young juvenile ones (Welsh *et al.*, 1979; Chevre *et al.*, 1983; Muhitch and Fletcher, 1984).

Marks and Simpson (1990) reported that keeping explants of *Quercus robur* under reduced light intensity for 25 days during the rapid spring growth phase. Compton and Preece (1986) inhibited browning of explants by soaking in water. Also Bonga (1981) kept *Abies balsamea* and *Picea glauca* explants submerged in water for some time before sterilization and culture. Durand- Cresswell *et al.*, (1982) soaked *Eucalyptus grandis*
explants for 3 hours in distilled water followed by 8 days of culture in darkness. These treatments were effective in reducing browning and increasing their subsequent survival in vitro. Other workers treated explants during excision by antioxidants such as Polyvinylpolyrrolidone (PVP) (Hohtola, 1988; Gupta et al., 1980 and Gharyal and Maheshwari, 1990).

To avoid browning antioxidants are used in the nutrient medium where browning of Fascicles of Pinus and brutia was reduced by adding sodium diethyldithio carbamate (SDD) to the initiation medium and PVP to later media (Ablullah et al., 1987).

Vitamin E reduced browning and stimulated rooting in cultures of Eucalyptus rudis and quercus borealis (Nkanka, 1982), as well as ascorbic acid, which prevented browning during secondary, shoot formation in Picea mariana (Rumary and Thorpe, 1984). Toxic exudates can also be removed by absorption by 3.0g/l activated charcoal (Wang and Huang, 1976).

2-3-2-8-2 Vitrification

Many authors reported vitrification as a common problem in micro-propagation that appears in stem and foliage of plantlets. Vitrified leaves often lack proper palisade tissue and they have mesophyll tissues with large intercellular spaces (Gaspar et al., 1987). Vitrified stems are often
swollen and translucent. The waxy cuticle is generally very thin and there are often fewer stomata, and further more many of the stomates are not functioning (Hutchinson and Zimmerman, 1987).

It has been reported that lowering of water vapor and ethylene levels in the atmosphere above the nutrient medium vessel often reduces vitrification, and it is also minimized by reducing chloride, ammonium, and growth regulator levels in the culture medium (Boulay 1984, Aitken-christie and Jones 1985, Gaspar et al., 1987).

McLaughlin and karnosky (1989) reported that vitrified shoots of Larix decidua reverted to normal ones when transferred to a medium with half the total nitrogen. The percentage of vitrified shoots of Pseudotsuga menziesii was found to reach a maximum after a few subcultures and declined in subsequent subcultures (Bekkaoui and Franclet, 1986).

Arnold and Eriksson (1984) reported that increasing the agar or gelrite concentrations reduced the level of vitrification in an adventitious shoots of Picea abies. In addition Rugini et al., (1986) reported that replacing sucrose with fructose in shoot cultures of Prunus dulcis reduced vitrification and shoot dry weight increased with fructose.

2-3-2-9 Tissue Culture in Forestry

The majority of forest trees have been propagated from seeds and through vegetative propagation. Superior stocks have been propagated vegetatively by rooted cuttings and grafting (Franclet et al., 1987). Currently, most of improved germplasm comes
from long-term breeding programs, which are normally costly. Some important trees can now be selected, rooted, micro propagated, grafted, rejuvenated, and cloned by somatic embryogenesis and polyembryogensis (Durzan, 1988). Tissue culture is a very useful tool and in forest tree improvement programs for improving efficiency and quality. Tissue culture techniques were incorporated into clonal programs for *Eucalyptus* and *Pinus* species (Jones and Van Staden, 1997). Clonal forestry through tissue culture is receiving increased recognition as an alternative to conventional vegetative practices as it allows for immediate and total capture of genetic gains (Han *et al.*, 1997). In addition it provides an alternative for species, which are difficult to root (Gupta *et al.*, 1993). This method may be expensive, but it can provide stock plants for further multiplication (Jones, 1986).

Mohamed (2003) showed that *in vitro* propagation of *Silvadora persica* (Arak), single nodal explants placed on double strength MS medium supplemented with 20.0g/l sucrose, with a combination of IBA and BA at 0.5mg/l gave the best result for shoots formation, while 2.0mg/l NAA gave good rooting. Tiwari *et al.*, (2002) reported that the maximum culture establishment was 76.8% when nodal explants from *Tectona Grandis* (teak) were cultured MS medium supplemented with 22.2µM BA and 0.57µM IAA. *In vitro* shoots were rooted *ex vitro* by dipping in 9.8µM, IBA for 2 min followed by planting in polyethylene containing pots soil: vermiculite (1:1 v/v). This resulted in 77.9% survival of the plantlets.

### 2-3-2-9-1 Tissue culture of Acacias

Ali (1991) investigated *in vitro* propagation of *A.tortilis sp sprocarba* using apical shoots and epicotyl explants from aseptic grown seedling (3
weeks old) cultured on MS medium supplemented with 0.25mg/l BAP and 0.2mg/l NAA. The number of shoots developed on apical shoots was 3 per explants and one shoot obtained from epicotyl explant. Rooting was obtained on Whites medium containing 0.1mg/l IBA.

Aziz et al. (2002), investigated the micro propagation of *Acacia tortilis* sub sp., *raddiana* and *A. nilotica*. Culture of *A. nilotica* from nodal segment was achieved on MS medium. The highest number of shoots per explants (3.58) was obtained on medium containing 2.5 mg/l BA. Shoots transferred to half strength MS medium with 4.0 mg1-1IBA, formed healthy roots in 62.5% of the cultures. *A.tortilis* failed to produce more than one shoot per explant and did not develop roots in any medium.

Nanda and Rout (2003) reported that *in vitro* somatic embryogensis were achieved in callus cultures from immature zygotic embryos of *A. nilotica*. These somatic embryos proliferated rapidly by transfer to MS medium supplemented with 6.66µM BA and 6.78µM 2,4-D. The maximum number of somatic embryos per callus was 72.6% after 8 weeks.

Vengadesan et al., (2003) used on nodal explants excised from mature elite trees of *A. sinuata*, and found that *in vitro* tissue browning was circumvented by soaking explants in a solution of 238µM citric acid. Maximum shoot proliferation (75.2%) was achieved on MS medium supplemented with 8.9µM BA, 2.5µM (TDZ) and 135.7µM adenine sulfate. Rooting was achieved on half MS medium supplemented with 7.4µM IBA.
3-1 Plant material

*Acacia seyal.* Del. (Talh) mature trees are found in Shambat area, at the Faculty of Agriculture, University of Khartoum, it is a mature tree. Plant material was excised from new twigs of a selected mature tree. Single nodes with an axillary bud were used as explants for all experiments.

3-2 Preparation of explants

New twigs were removed from the mother trees, and were washed under running tap water for 30 min to remove surface dust and to reduce
contamination, and then the explants were soaked in a solution of antioxidant (distilled water + charcoal 2.0 g/l) for one hour.

3-3 Surface- Sterilization

The explants were immersed in 70% ethanol for 30 seconds, washed by several changes of sterilized distilled water, and then the explants were immersed in 100 ml solution of 30% (v/v) sodium hyprochlorite (1.5% available chorine) with 2 drops of Tween-20 for 20 min with continuous shaking and then rinsed three times with sterilized distilled water under laminar airflow hood.

3-4 Preparation of Basal Nutrient media

The basal salt mix of the nutrient medium of Murashige and Skoog (1962) was used. It was supplemented with the following compounds (per liter): 3%w/v sucrose, 100mg/l inositol, and MS vitamins. The culture medium was solidified by the addition of 0.8% w/v agar, the pH was adjusted to 5.7±1 with 0.1N HCl or 0.1N NaOH prior to the addition of the agar. The medium was dispensed in 12-15 ml aliquots in 25 X150mm cultures tubes and each tube was capped with a Bellco kaput. The medium was sterilized by autoclaving at 15 psi and 121C° for 15 min.

3-5 Equipments

All glassware were sterilized by autoclaving at 15 psi and 121C° for 15 min, whereas the forceps, scalpels, and blades were sterilized by dipping in 100% ethanol and then flamed before use.
3-6 **Incubation conditions:**

All cultures were incubated in an incubation room where the temperature was adjusted to (25±2 °C), under a light intensity of 1000 lux and a 16 hours photoperiod using (Gro- Lux light) white cool fluorescent lamps.

3-7 **Experimentation**

In all experiments MS basal salt mix of the nutrient medium supplemented with sucrose 30% w/v, 100mg/l inositol. Solidified by 0.8% w/v agar was used. The pH of the media was adjusted to 5.7± 1

3-7-1 **Experiments of the basal medium**

3-7-1-1 **Exp.1:** - Effect of basal MS medium at different salt mix strengths on growth parameters of *A. seyal*.

**MS strengths at:** -0.25x, 0.5x, 1.0x, 2.0x, and 3.0x

3-7-1-2 **Exp.2:** - Carbohydrates source

Two different sugars (sucrose and glucose) were tested as carbohydrate source incorporated in MS salts medium; the medium is devoid of
sucrose or with sucrose. Different levels of concentrations were tested for both sucrose and glucose: - 0.0; 10; 20; 30; 40 and 60 g/l.

3-7-1-3 Exp.3: - Effect of Myo-inositol

Myo-inositol at different concentration: - 0.0; 25.0; 50.0; 75.0; 100.0; and 150.0 mg/l

3-7-1-4 Exp.4: - Effect of vitamins

Two vitamin mixtures were tested. MS vitamins (thiamine-HCl, 0.1, nicotinic acid, 0.5mg/l, pyridoxine-HCl, 0.1mg/l, and glycine 2.0mg/l) and modified Whites vitamins (1939) (thiamine–HCl 0.1mg/l, pyridoxine-HCl, 0.5mg/l, nicotinic acid, 0.5mg/l, and glycine 3.0 mg/l.). Added to basic salts of MS medium used in the study experiments prepared as describe above. The two vitamins were tested singly at different strengths: - 0.0x; 0.5x; 1.0x; 1.5x; and 2.0x

3-7-1-5 Exp.5: -Effect of adenine Sulfate

Adenine sulfate was tested at concentrations: - 0.0; 40.0; 80.0; 120.0, and 160.0 mg/l

3-7-1-6 Exp.6: - Effect of charcoal
Charcoal at concentration: -

0.0; 1.0; 2.0; 3.0; 4.0 and 5.0g/l

3-7-1-7 Exp.7: -Effect of AgNO₃

Silver nitrate salt solutions were examined at concentration: -

0.0; 0.5; 1.0; 5.0 and 15.0mg/l

3-7-2 Experiments of growth regulators

3-7-2-1 Cytokinins

3-7-2-1-1 Effect of BAP

6-benzyl amino purine (BAP) was tested singly on basic salts of MS medium prepared as described above. BAP at concentrations used are: - 0.0; 0.5; 1.0; 1.5; 2.0 and 3.0mg/l.

3-7-2-1-2 Effect of Kinetin

Different concentrations of 6-furfurylamine purine (kinetin) singly were tested on basic salts of MS medium prepared as describe. The Kinetins at concentrations used are: -

0.0; 0.5; 1.0; 1.5; 2.0 and 6.0mg/l.

3-7-2-2 Auxins

3-7-2-2-1 Effect of IBA

IBA was tested singly on MS basal medium as described above. IBA at different concentrations: - 0.0; 0.5; 1.0; 1.5; 2.0; 3.0mg/l

3-7-2-2-2 Effect of NAA
NAA was tested singly on MS basal medium as described above. NAA at different concentrations: -
0.0; 0.1; 0.5; 1.0; 1.5; and 2.0mg/l.

3-7-3 Rooting Experiments
Two experiments are carried out, shoots produced in vitro from single node explants were excised and placed on half strength medium, supplemented with 20g/l sucrose and IBA at different concentrations, 0.0, 1.0, 2.0, 3.0, and 4.0mg/l.
Also ¼ MS medium was used supplemented with 15g/l sucrose, 100 mg/l ascorbic acid + 150mg/l citric acid and IBA at 0.0, 0.5, 2.0, 4.0, and 8.0mg/l

3-7-4 Experimental analysis

The data analysed for the following parameters collected during 8 weeks of incubation.

1. Length of shoot.
2. Number of nodes.
3. Number of leaves
4. Number of shoots
5. Vigor (1=poor, 2=moderate, 3 =good, 4 =V.good, and 5 = excellant)

6. Callus formation (observed)

7. Root formation

The data was analysed by JMP statistical package (1995) by SAS Corporation using the analysis of variance procedure. Means were compared by Tukey- Kramer tests.

Chapter Four

Results

4-1 Effect of MS salts mix strength

The concentration of MS salt had significant effect on shoot length, number of nodes, number of leaves and vigor of A. seyal explants (p=0.0001). 0.5x and 1.0x (half and full) MS strengths gave significantly higher shoot length, number of nodes, leaves, and vigor compared to the others. (Table 1, figure 1, and plate 1). The effects of 0.25x MS and 2.0x MS are similar on node number and leaves number but different effect on growth vigor and length of shoot.
The number of shoots showed no significant difference in all concentration of MS and only one shoot was observed. (Table 1)

4-2 Carbohydrates

4-2-1 Effect of sucrose concentrations

The tested sucrose concentration (0.0, 10, 20, 30, 40 and 60 g/l) (Table 2, Figure 2, and Plate 2) showed, significant difference in shoot length, number of nodes, leaves and growth vigor (p=0.0001). The highest values were obtained on 30g/l and 20g/l, which was significantly higher than others. (Table 2, and figure 2).

4-2-2 Effect of glucose concentrations

The effects of glucose at concentration (0.0; 10.0, 20.0, 30.0, 40.0, and 60.0 g/l) were highly significant on shoot length, number of nodes, number of leaves, and growth vigor (Table 3). The concentration of 20.0 and 30.0 g/l gave significantly higher values compared to the others (Table 3).

4-3 Effect of myo-inositol

The effect of the addition myo-inositol at 0.0, 25, 50, 75, 100, and 150 mg/l have significant effect on shoot length (p=0.0001), number of nodes (p=0.001), number of leaves (p=0.0001) and growth vigor (p=0.001) as show in Table (4). The concentration of 100 mg/l gave the highest shoot length, number of leaves and growth vigor and followed by 50 and 75 mg/l (Table 4). Medium devoid of inositol (0.0) gave the shortest shoot and the lowest growth vigor.

Table (1) Effect of MS strength salts mix on the mean of shoot growth parameters of *A. seyal* nodal explants during 8 weeks of incubation.
Mean values with the same letter in the same column are not significantly different according to Tukey-Kramer test.

<table>
<thead>
<tr>
<th>MS strength (x)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoot</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25x</td>
<td>3.10b</td>
<td>2.87b</td>
<td>3.859a</td>
<td>1.0</td>
<td>2.68b</td>
</tr>
<tr>
<td>0.5x</td>
<td>4.09a</td>
<td>3.60a</td>
<td>4.390a</td>
<td>1.0</td>
<td>3.34a</td>
</tr>
<tr>
<td>1.0x</td>
<td>3.70a</td>
<td>3.46ab</td>
<td>4.062a</td>
<td>1.0</td>
<td>3.34a</td>
</tr>
<tr>
<td>2.0x</td>
<td>2.70bc</td>
<td>2.34b</td>
<td>2.968b</td>
<td>1.0</td>
<td>2.07c</td>
</tr>
<tr>
<td>3.0x</td>
<td>2.39c</td>
<td>2.31b</td>
<td>2.921b</td>
<td>1.0</td>
<td>1.82c</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Figure(1). Effect of MS strength of some growth parameters of A.seyal nodal segment ex plants after 8 weeks incubation.

A) Shoot length

B) Number of nodes

C) Number of leaves

D) Shoot Vigor
Table (2) Effect of sucrose concentration on the mean of shoot growth parameters of *A. seyal* nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>Sucrose concentrations (g/l)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoot</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.76d</td>
<td>1.625d</td>
<td>1.767c</td>
<td>1.0</td>
<td>1.357c</td>
</tr>
<tr>
<td>10.0</td>
<td>3.06bc</td>
<td>2.875bc</td>
<td>3.035b</td>
<td>1.0</td>
<td>2.071b</td>
</tr>
<tr>
<td>20.0</td>
<td>3.78a</td>
<td>3.875a</td>
<td>4.428a</td>
<td>1.0</td>
<td>3.142a</td>
</tr>
<tr>
<td>30.0</td>
<td>4.19a</td>
<td>4.035a</td>
<td>4.482a</td>
<td>1.0</td>
<td>3.250a</td>
</tr>
<tr>
<td>40.0</td>
<td>3.27b</td>
<td>3.107b</td>
<td>3.910a</td>
<td>1.0</td>
<td>2.321b</td>
</tr>
<tr>
<td>60.0</td>
<td>2.72c</td>
<td>2.464c</td>
<td>2.821b</td>
<td>1.0</td>
<td>2.428b</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.
Figure (2). Effects of sucrose concentration of some growth parameters of A. seyal nodal segments ex plants after 8 weeks incubation.

A) shoot length

B) Number of nodes

C) Number of leaves

D) Vigor
## Table (3) Effect of glucose concentration on the mean of shoot growth parameters of *A. seyal* nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>Glucose concentration (g/l)</th>
<th>Length of shoots (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoot</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.42c</td>
<td>1.42c</td>
<td>2.12d</td>
<td>1.0</td>
<td>1.56f</td>
</tr>
<tr>
<td>10.0</td>
<td>2.01bc</td>
<td>2.82b</td>
<td>3.48bc</td>
<td>1.0</td>
<td>2.23c</td>
</tr>
<tr>
<td>20.0</td>
<td>2.46a</td>
<td>3.29ab</td>
<td>4.25a</td>
<td>1.0</td>
<td>2.79b</td>
</tr>
<tr>
<td>30.0</td>
<td>2.55a</td>
<td>3.59a</td>
<td>4.35a</td>
<td>1.0</td>
<td>3.04a</td>
</tr>
<tr>
<td>40.0</td>
<td>1.98bc</td>
<td>2.82b</td>
<td>3.67ab</td>
<td>1.0</td>
<td>2.20d</td>
</tr>
<tr>
<td>60.0</td>
<td>1.72bc</td>
<td>2.25cb</td>
<td>2.95c</td>
<td>1.0</td>
<td>1.81f</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.
Table (4) Effect of myo-inositol concentration on the mean of shoot growth parameters of *A. seyal* nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>Myo-inositol concentration (mg/l)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoots</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.41d</td>
<td>2.94b</td>
<td>2.92c</td>
<td>1.0</td>
<td>2.14c</td>
</tr>
<tr>
<td>25</td>
<td>3.25bc</td>
<td>3.05b</td>
<td>3.48abc</td>
<td>1.0</td>
<td>3.01abc</td>
</tr>
<tr>
<td>50</td>
<td>3.74ab</td>
<td>3.35ab</td>
<td>3.75ab</td>
<td>1.0</td>
<td>3.78a</td>
</tr>
<tr>
<td>75</td>
<td>3.50abc</td>
<td>3.07b</td>
<td>4.10a</td>
<td>1.0</td>
<td>3.05abc</td>
</tr>
<tr>
<td>100</td>
<td>3.96a</td>
<td>3.71a</td>
<td>4.03a</td>
<td>1.0</td>
<td>3.64ab</td>
</tr>
<tr>
<td>150</td>
<td>3.20c</td>
<td>2.96b</td>
<td>3.32bc</td>
<td>1.0</td>
<td>2.44bc</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.001</td>
<td>0.0001</td>
<td></td>
<td>0.001</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.

4-4 Vitamins

4-4-1 Effect of MS vitamins

The MS vitamins tested in 0.0, 0.5x, 1.0x, 1.5x, and 2.0x strengths showed significant variation in shoot length (p=0.0001), number of nodes (p=0.0001), number of leaves (p=0.0057), number of shoot (p=0.0001), and growth vigor (p=0.0001) (Table 5, Figure 3, and Plate 3). The highest shoot length, number of nodes, number of leaves, number of shoot, and vigor were achieved on 1.0x. (Table 5, Figure 3). 1.5x and 0.5x were also good for leaf growth, and growth vigor.

4-4-2 Effect of modified Whites vitamins (1939)

The modified Whites vitamins (1939) on nodal explants tested at 0.0, 0.5x, 1.0x, 1.5x, and 2.0x strength (Table 6), showed no significant effect on shoot length (p=0.1708). Their effect on number of nodes, number of leaves, number of shoots, and growth vigor (p=0.0003, 0.0001, 0.0001, and 0.0094) respectively.
4-5 Effect of adenine sulfate

Nodal explants grown on basal MS medium supplemented with adenine sulfate at concentration of 0.0, 40.0, 80.0, 120.0, and 160.0mg/l as shown in Table (7), Figure (4), and Plate (4). 40.0mg/l and 80mg/l adenine sulfate gave significantly higher values of shoot length, number of nodes, number of leaves, number of shoot, and vigor. But higher concentration 120 and 160mg/l significant reduced the number of nodes and number of leaves and number of shoots and 160mg/l also reduced the vigor.

Table (5) Effect MS vitamins concentration on the mean of shoot growth parameters of *A.seyal* nodal explants during 8 weeks of incubation.
<table>
<thead>
<tr>
<th>MS vitamins strength (x)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoot</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.98b</td>
<td>2.35c</td>
<td>3.87b</td>
<td>1.0c</td>
<td>1.85c</td>
</tr>
<tr>
<td>0.5x</td>
<td>2.32b</td>
<td>2.87bc</td>
<td>4.67a</td>
<td>2.12b</td>
<td>2.18bc</td>
</tr>
<tr>
<td>1.0x</td>
<td>2.80a</td>
<td>3.82a</td>
<td>4.85a</td>
<td>2.6a</td>
<td>2.90a</td>
</tr>
<tr>
<td>1.5x</td>
<td>2.12b</td>
<td>3.34ab</td>
<td>4.75a</td>
<td>2.20b</td>
<td>2.75a</td>
</tr>
<tr>
<td>2.0x</td>
<td>2.12b</td>
<td>3.20ab</td>
<td>4.56ab</td>
<td>1.95b</td>
<td>2.45ab</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0057</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.
Figure 3. Effects of MS vitamins strength of some growth parameters of A. seyal segment plants after 8 weeks incubation.

A) Shoot length

B) Number of nodes

C) Number of leaves

D) Number of shoots

MS vitamin strength
Table (6) Effect of modified Whites vitamins (1939) concentration on the mean of shoot growth parameters of *A.seyal* nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>Whites vitamin strength (x)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoot</th>
<th>Vigor</th>
<th>Callus %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.0x</strong></td>
<td>1.90a</td>
<td>2.46b</td>
<td>3.65a</td>
<td>1.0b</td>
<td>2.01b</td>
<td>10</td>
</tr>
<tr>
<td><strong>0.5x</strong></td>
<td>2.00a</td>
<td>2.81ab</td>
<td>3.90a</td>
<td>1.50a</td>
<td>2.04b</td>
<td>60</td>
</tr>
<tr>
<td><strong>1.0x</strong></td>
<td>2.19a</td>
<td>3.21a</td>
<td>4.07a</td>
<td>1.59a</td>
<td>2.51a</td>
<td>50</td>
</tr>
<tr>
<td><strong>1.5x</strong></td>
<td>1.96a</td>
<td>3.42a</td>
<td>4.0a</td>
<td>1.5a</td>
<td>2.32ab</td>
<td>40</td>
</tr>
<tr>
<td><strong>2.0x</strong></td>
<td>1.98a</td>
<td>3.34a</td>
<td>2.78b</td>
<td>1.5a</td>
<td>2.32ab</td>
<td>30</td>
</tr>
<tr>
<td><strong>p</strong></td>
<td>0.1708</td>
<td>0.0003</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

Means with the same letter in the same coloum are not significantly different according to Tukey- Kramer test.
Table (7) Effect of adenine sulfate concentration on the mean of shoot growth parameters of \textit{A.seyal} nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>Adenine sulfate concentration (mg/l)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoot Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.05c</td>
<td>2.78bc</td>
<td>3.56bc</td>
<td>1.25d</td>
</tr>
<tr>
<td>40.0</td>
<td>2.76a</td>
<td>3.87a</td>
<td>4.9a</td>
<td>2.56a</td>
</tr>
<tr>
<td>80.0</td>
<td>2.41b</td>
<td>3.29ab</td>
<td>4.58a</td>
<td>2.17b</td>
</tr>
<tr>
<td>120.0</td>
<td>2.10bc</td>
<td>2.71bc</td>
<td>2.71bc</td>
<td>2.21b</td>
</tr>
<tr>
<td>160.0</td>
<td>1.84c</td>
<td>2.32c</td>
<td>2.32c</td>
<td>1.62c</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.
4-6 Effect of activated charcoal
Activated charcoal concentration had significant effect on shoot length, number of
nodes, number of leaves and growth vigor (p=0.0001, p=0.0001, p=0.0001, p=0.0001)
respectively as showed in table (8), figure (5), and plate (5). The concentration of
2.0g/l charcoal gave the highest shoot length, number of nodes, leaves and growth
vigor follow by 3.0g/l and 1.0g/l as in Table (8). But high concentrations at 4.0 and 5.0g/l reduced the length of shoot.

4-7 Effect of silver nitrate (AgNO₃)

Table (9), and Plate (6) show the effect of AgNO₃ at concentrations (0.0, 0.5, 1.0, 5.0, and 15mg/l), on number of nodes showing no significant differences (p=0.216) and have significant effects on number of leaves, shoot lengths and growth vigor (p=0.081, p=0.001 and p=0.0001) respectively. The concentrations more than 0.5mg/l and 5.0mg/l gave highest shoot lengths and vigor. No significant effect of AgNO₃ on abscission of leaves (p=0.1), that increased when the medium devoided of AgNO₃ and with high concentration of AgNO₃. No significant differences obtained on number of shoot with all concentrations of AgNO₃.

<table>
<thead>
<tr>
<th>Charcoal concentration (g/l)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoot</th>
<th>Vigor</th>
<th>Abscission</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3.08c</td>
<td>2.79bc</td>
<td>3.14c</td>
<td>1.0</td>
<td>2.32c</td>
<td>0.50a</td>
</tr>
<tr>
<td>1.0</td>
<td>3.83b</td>
<td>3.59b</td>
<td>3.75b</td>
<td>1.0</td>
<td>2.81b</td>
<td>0.39a</td>
</tr>
<tr>
<td>2.0</td>
<td>4.61a</td>
<td>4.26a</td>
<td>4.48a</td>
<td>1.0</td>
<td>3.31a</td>
<td>0.35a</td>
</tr>
<tr>
<td>3.0</td>
<td>3.98b</td>
<td>3.64b</td>
<td>3.98ab</td>
<td>1.0</td>
<td>2.64bc</td>
<td>0.42a</td>
</tr>
<tr>
<td>4.0</td>
<td>3.14c</td>
<td>3.15bc</td>
<td>3.53bc</td>
<td>1.0</td>
<td>2.56bc</td>
<td>0.35a</td>
</tr>
<tr>
<td>5.0</td>
<td>3.18c</td>
<td>3.10bc</td>
<td>3.54bc</td>
<td>1.0</td>
<td>2.45bc</td>
<td>0.39a</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>___</td>
<td>0.0001</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Means with the same letter in the same coloum are not significantly different according to Tukey- Kramer test
Table (9) Effect of AgNO₃ concentration on the mean of shoot growth parameters of *A. seyal* nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>AgNO₃ concentration (mg/l)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoot</th>
<th>Vigor</th>
<th>Abscission</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3.20b</td>
<td>3.41a</td>
<td>3.66ab</td>
<td>1.0</td>
<td>2.51bc</td>
<td>0.82a</td>
</tr>
<tr>
<td>0.5</td>
<td>3.54ab</td>
<td>3.64a</td>
<td>3.85ab</td>
<td>1.0</td>
<td>2.76ab</td>
<td>0.69a</td>
</tr>
<tr>
<td>1.0</td>
<td>3.85a</td>
<td>3.94a</td>
<td>4.08a</td>
<td>1.0</td>
<td>3.16a</td>
<td>0.48a</td>
</tr>
<tr>
<td>5.0</td>
<td>3.60ab</td>
<td>4.12a</td>
<td>3.73ab</td>
<td>1.0</td>
<td>2.75ab</td>
<td>0.62a</td>
</tr>
<tr>
<td>15.0</td>
<td>3.19b</td>
<td>3.01a</td>
<td>3.46b</td>
<td>1.0</td>
<td>2.08c</td>
<td>0.78a</td>
</tr>
<tr>
<td><strong>p</strong></td>
<td>0.001</td>
<td>0.21</td>
<td>0.081</td>
<td>0.0001</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.
4-8 Effect of growth regulators
4-8-1 Cytokinins
4-8-1-1 Effect of benzyl adenine (BAP): -

Table (10) and Figure (6) show the effect of BAP concentration (0.0, 0.5, 1.0, 1.5, 2.0 and 3.0mg/l) using MS basal medium on some growth parameters was significant. The highest length of shoots, number of nodes, number of leaves and growth vigor were obtained on concentration 1.0mg/l and 1.5mg/l. Medium devoided of BA or with high concentrations (0.0 and 3.0mg/l) gave the shortest shoots, the lowest number of leaves, the lowest number of nodes and the lowest growth vigor (Table 10, figure 6).

4-8-1-2 Effects of kinetin

The effect of kinetin at concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 6.0mg/l) was highly significant on length of shoot, number of nodes, number of leaves, number of shoots and growth vigor (p=0.0001, 0.0001, 0.0001 and 0.0001 respectively) (Table 11). The
highest shoot length, number of nodes, number of leaves and vigor were obtained on concentrations 1.5mg/l and 2.0mg/l. Similarly 1.0mg/l also gave the high number of leaves, number of shoots and growth vigor.

4-8-2 Auxins: -

4-8-2-1 Effect of indole butyric acid (IBA)

Indole butyric acid (IBA) concentrations (0.0, 0.5, 1.0, 1.5, 2.0, and 3.0mg/l) had no significant effect on shoot length, but have significant effect on number of nodes, number of leaves, number of shoot and growth vigor (Table 12). The concentration 0.5, 1.0, 1.5, and 2mg/l gave longer shoots, higher number of nodes and number of leaves, number of shoots compare to 0.0. 2mg/l gave better number of leaves (Table 12).

4-8-2-2 Effect of naphthalene acetic acid (NAA)

Table (13) show that, different concentrations (0.0, 0.1, 0.5, 1.0, 1.5, and 2.0mg/l) of NAA have significant effect on shoot length, number of leaves, nodes, shoot, and growth vigor p=0.0001. The concentrations (1.5 and 2.0mg/l) gave higher shoot length; number of leaves and growth vigor compare to others. The concentration (1.0 and 2.0mg/l) gave higher number of nodes.

4-9 Rooting Results

Two experiments were carried out for root induction of A.seyal shootlets. Using MS medium with different salt mix strengths and different concentration of IBA, no roots were observed on 0.5x MS salt mix medium supplemented with 20g/l sucrose, and
IBA at the concentration 0.0, 1.0, 2.0, 3.0, and 4.0 mg/l. When used 0.25x MS salt mix medium supplemented with 15 g/l sucrose, 100 mg/l ascorbic acid +150 citric acid, and IBA at the concentration 0.0, 0.5, 2.0, 4.0, and 8.0 mg/l, rooting was achieved on 0.5 mg/l IBA and only rate of 30% after 8 weeks incubation, Plate (7) show that.

Table (10) Effect of BAP concentration on the mean of shoot growth parameters of *A. seyal* nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>BA concentration (mg/l)</th>
<th>Length of shoots (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoots</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.98c</td>
<td>1.75c</td>
<td>2.79d</td>
<td>1.0c</td>
<td>1.42d</td>
</tr>
<tr>
<td>0.5</td>
<td>2.39b</td>
<td>2.48b</td>
<td>3.39bc</td>
<td>1.82ab</td>
<td>2.23c</td>
</tr>
<tr>
<td>1.0</td>
<td>2.93a</td>
<td>3.10a</td>
<td>3.81ab</td>
<td>2.07a</td>
<td>2.70ab</td>
</tr>
<tr>
<td>1.5</td>
<td>3.09a</td>
<td>2.89ab</td>
<td>4.01a</td>
<td>2.15a</td>
<td>2.82a</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.57b</td>
<td>2.45b</td>
<td>3.28bcd</td>
<td>2.09a</td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>3.0</td>
<td>2.29bc</td>
<td>2.43b</td>
<td>3.50abc</td>
<td>1.57b</td>
<td>2.21c</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.
Figure (6). Effects of BA concentration of some growth parameters of \textit{A. seyal} nodal explants after 8 weeks incubation.

Table (11) Effect of Kinetin concentration on the mean of shoot growth parameters of \textit{A. seyal} nodal explants during 8 weeks of incubation.
<table>
<thead>
<tr>
<th>Kin concentration (mg/l)</th>
<th>Length of shoot (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoots</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.22d</td>
<td>2.40c</td>
<td>3.25b</td>
<td>1.0c</td>
<td>1.84b</td>
</tr>
<tr>
<td>0.5</td>
<td>2.47cd</td>
<td>2.68bc</td>
<td>3.35b</td>
<td>1.45b</td>
<td>2.09b</td>
</tr>
<tr>
<td>1.0</td>
<td>2.89bc</td>
<td>3.09ab</td>
<td>3.96a</td>
<td>1.92a</td>
<td>2.85a</td>
</tr>
<tr>
<td>1.5</td>
<td>3.24ab</td>
<td>3.34a</td>
<td>4.42a</td>
<td>2.09a</td>
<td>2.90a</td>
</tr>
<tr>
<td>2.0</td>
<td>3.35a</td>
<td>3.48a</td>
<td>4.43a</td>
<td>2.09a</td>
<td>2.84a</td>
</tr>
<tr>
<td>6.0</td>
<td>2.32d</td>
<td>2.54bc</td>
<td>3.38b</td>
<td>1.35b</td>
<td>1.92b</td>
</tr>
<tr>
<td>p</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.

Table (12) Effect IBA concentration on the mean of shoot growth parameters of *A.seyal* nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>IBA concentration (mg/l)</th>
<th>Length of shoots (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoots</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.39a</td>
<td>2.62b</td>
<td>3.46c</td>
<td>1.0b</td>
<td>2.07d</td>
</tr>
<tr>
<td>0.5</td>
<td>2.59a</td>
<td>3.20ab</td>
<td>4.39ab</td>
<td>1.70a</td>
<td>2.53a</td>
</tr>
<tr>
<td>1.0</td>
<td>2.37a</td>
<td>3.57a</td>
<td>4.12bc</td>
<td>1.84a</td>
<td>2.39ab</td>
</tr>
<tr>
<td>1.5</td>
<td>2.55a</td>
<td>3.76a</td>
<td>4.62ab</td>
<td>1.84a</td>
<td>2.45ab</td>
</tr>
<tr>
<td>2.0</td>
<td>2.35a</td>
<td>3.34a</td>
<td>4.87a</td>
<td>1.83a</td>
<td>2.32ab</td>
</tr>
<tr>
<td>p</td>
<td>0.1818</td>
<td>0.0002</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different according to Tukey-Kramer test.
Table (13) Effect of NAA concentration on the mean of shoot growth parameters of *A. seyal* nodal explants during 8 weeks of incubation.

<table>
<thead>
<tr>
<th>NAA concentration (mg/l)</th>
<th>Length of shoots (cm)</th>
<th>Number of nodes</th>
<th>Number of leaves</th>
<th>Number of shoots</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.88c</td>
<td>2.59c</td>
<td>3.51b</td>
<td>1.0b</td>
<td>2.20d</td>
</tr>
<tr>
<td>0.1</td>
<td>2.25bc</td>
<td>3.59b</td>
<td>4.09ab</td>
<td>2.0a</td>
<td>2.56cd</td>
</tr>
<tr>
<td>0.5</td>
<td>2.34b</td>
<td>3.20bc</td>
<td>4.15ab</td>
<td>2.01a</td>
<td>2.81b</td>
</tr>
<tr>
<td>1.0</td>
<td>2.35b</td>
<td>4.29a</td>
<td>4.82a</td>
<td>1.89a</td>
<td>2.70c</td>
</tr>
<tr>
<td>1.5</td>
<td>2.87a</td>
<td>3.48b</td>
<td>4.67a</td>
<td>2.06a</td>
<td>3.18ab</td>
</tr>
<tr>
<td>2.0</td>
<td>2.91a</td>
<td>4.39a</td>
<td>4.34a</td>
<td>1.98a</td>
<td>3.34a</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Chapter Five  
Discussion  

5-1 Basal Media  
5-1-1 Effect of MS Mix salt strength  

In-vitro culture of *Acacia seyal* nodal explants on 1.0x and 0.5x (full and half) strength MS media gave best performance of shoot length, number of nodes, number of leaves and vigor compared to 0.25x MS, 2.0x MS and 3.0x MS strength medium. This agrees with Zhang et al., (1995) work on *Acacia auriculiformis*, buds from 4-year-old trees on full MS medium that gave 66.7% multiple shoots. Also it agrees with Beck et al., (1998, and 2000) using nodes from mature trees, and meristem from *in vitro* grown plantlets of *Acacia mearansii*, on half and full MS strength medium that gave better result. In all strengths of MS medium, *A. seyal* gave one shoot per explant that was found similar with Aziz et al., (2002) work on *Acacia tortilis* which failed to produce more than one shoot per explant. Flinn et al., (1986) reported that shoot formation occurred in embryo explants of *Pinus strobus* on half strength MS medium but failed on full strength MS medium due to its high concentration of ammonium.
5-1-2 Effect of sucrose


5-1-3 Effect of glucose

The addition of glucose to *in-vitro* culture of *Acacia seyal* nodal explants at concentrations of 20 or 30.0g/l gave better shoot length, number of nodes, number of leaves and vigor. This agrees with findings of Mohamed, (2003) who found that 20g/l glucose on MS medium gave higher shoot length while 30.0g/l and 40.0g/l gave better number of leaves, number of nodes, number of shoot and shoot length, when adding glucose to B3 medium. High concentrations of sugar therefore may have affected explants growth as a result of high osmotic pressure whereas low concentrations may result in low carbon and energy essential for growth and development.
5-1-4 Effect of myo-inositol

The induction of myo-inositol at concentration of 100mg/l gave better shoot length, number of nodes, number of leaves, and vigor. Similar results were reported by Mohamed (1994) worked on guava and Ibrahim (2000) on *A. senegal*.

5-1-5 Effect of MS vitamins

MS vitamins mixture at strength of 10ml/l (1.0x) gave better shoot length, number of nodes, number of leaves, number of shoot, and vigor. Similar observation were reported by Mohamed, (2003) work on *Silvadora persica*, he obtained the highest number of leaves and length of shoots by using 1.0x MS vitamin.

5-1-6 Effect of modified White vitamins (1939)

On the other hand, addition of modified Whites vitamins (1939) had no significant effect on shoot length but had significant effect on number of leaves, number of nodes, number of shoot, and vigor. One dose (1.0X) of Whites vitamins gave better number of nodes, leaves, shoots, and growth vigor. Similarly Ibrahim (2000) found that Whites vitamins had significant effect on shoot length and leaves necrosis on *A.senegal* shoot tips, but had no significant effect on nodal segments. Also Mohamed (1994) found that half and full-dose (0.5X and 1.0X) of Whites vitamins resulted in minimum leaf necrosis and vitamins at all tested concentrations improve vigor.

5-1-7 Effect of adenine sulfate

Addition of adenine sulfate had significant effect at all tested concentrations whereas 40.0 and 80.0 mg/l gave better shoot length, number of nodes, number of leaves, number of shoots, and growth vigor. This agrees with the result of Pack and Han (1988) working with *gloxinia*. Also, Cheong et al., (1987) found that addition of
adenine sulfate at 40.0mg/l to shoot tips, and 80.0mg/l to nodal explants, enhanced the height of shoot and the number of the nodes, of *jojobe* species. Ziv *et al.*, (1970) found that adenine sulfate promote direct adventitious shoot formation on the explants in presence of recognized cytokinins. However, it has been suggested by Thom *et al.*, (1981) that this effect can be attributed to an available source of nitrogen to the cells that rapidly can be taken compared to inorganic ones.

### 5-1-8 Effect of activated charcoal

Activated charcoal assists in absorption of toxic substances that are present in media as a result of autoclaving or produced by cultured tissues when first transferred to media or during further growth (Wheatherhead *et al.*, 1979). For *Acacia seyal* addition of 2.0g/l of activated charcoal gave better shoot length, number of nodes, number of leaves, and vigor compared to 0.0, 1.0, 3.0, and 4.0g/l. This agrees with Ibrahim (2000) work on *A. senegal*, who found that 2.0g/l of activated charcoal gave better response in enhancing shoots number, number of leaves, and growth vigor. This positive response can be attributed to its positive effect on culture medium as shown by Rumary and Thorpe (1984) work on *Picea glauca* shoots, they found that activated charcoal promoted elongation and rooting of in vitro produced adventitious of shoots. Moreover, Tisserat, (1979) suggested that addition of charcoal improved growth by reducing browning of the tissues during micro propagation of *Phoenix dactylifera*. It could be suggested that addition of charcoal is essential to enhance *in-vitro* culture of *A. seyal* shoots.
5-1-9 Effect of silver nitrate

The results obtained by the addition of the AgNO₃ on in-vitro culture of A. seyal shoots was not significant on number of nodes but was significant on shoot length, number of leaves and vigor. The better response was observed between 0.5mg/l and 5.0mg/l. This agrees with Mohamed (1994) in cultures of guava where highest shoot number and vigor were obtained by the 1.0mg/l. Moreover, this concentration resulted in the least number of necrotic leaves. The same result was confirmed by Ibrahim (2000) work on Acacia senegal, he found the addition of AgNO₃ at 5.0mg/l or above decreased the number of shoots but at the same time increased the number of leaves. Addition of AgNO₃ to the culture medium of A.seyal, decreased the necrosis of leaves at 1.0mg/l of AgNO₃ (0.482) compared to medium devoided of AgNO₃ and high concentration of AgNO₃ (0.821 and 0.785 necrosis of leaves respectively.

5-2 Growth regulators

5-2-1 Effect of BAP

Growth regulators play a very important role for successful in vitro culture in terms of establishment and morphogenesis. In all cytokinins tests of this study (BAP and kinetin). The concentration of BAP at 1.0 and 1.5mg/l gave better shoot length, number of nodes, number of leaves, number of shoot, and vigor. This agrees with Beck et al., (1998) findings with A.mearnsii, where the concentrations 2.0 and 1.5mg/l resulted in optimal shoot production with 85% of the explants producing shoots with an average of 2 shoots per node. Also Toda et al., (1995) obtained 71.4% shoot production from axillary buds of 2-years old seedling of A. mangium on MS medium supplemented with 1.0 mg / 1 BA. Also, Amin and Jaiswal (1987) working with guava, also obtained only 2 shoots after 6-8 weeks in media enriched with 1.0mg/l, and the same result was
confirmed with Mohamed (1994), where 1.0mg/l gave maximized shoot number, length, and vigor of explants.

5-2-2 Effect of kinetin

Kinetin at 1.5 and 2.0mg/l gave better shoot length, number of nodes, number of leaves, number of shoots, and vigor. This agree with Ibrahim (2000) who found that the concentration 2mg/l gave better response on node proliferation. In Acacia species, the range (0.5-3.0mg/l) has been found the optimum for culture of explants in vitro, (Quoirin et al., 1998, and Rout et al., 1995).

5-2-3 Effect of IBA

The auxin, IBA, had significant effect on single node explants of A.seyal. The concentration of 1, 1.5, and 2mg/l of IBA gave better number of nodes and number of shoot compare to other one. On the other hand no root formation was observed.

5-2-4 Effect of NAA

The concentrations (1.5 and2.0mg/l) of NAA gave higher shoot length; number of leaves and vigor compare to others concentrations. On the other hand no root formation was observed.

5-3 Rooting

Rooting was achieved on 0.25x (¼) MS medium with 0.5mg/l IBA and the rate of rooting was 30% after 8weeks incubation. This agree with Zhang et al., (1995) work on buds from 4-yr-old tree from A. auriculiformis, obtained 55 shoots produced 29.8% roots; Toda et al., (1995) work on A. auriculiformis, resulted 38.5% roots, and Mohamed (2003) obtained 40% roots of single node explants of mature tree of *Silvadora persica.*
This study show that plantlets produced by culturing mature explants material of *A. seyal* shootlets, were somewhat difficult to root *in vitro*. This agrees with other research workers findings and conclusions that generally rooting is more difficult when explants from mature trees were used e.g. Bonga and Aderkas, (1992); Vietiez *et al.*, (1987) reported failure of rooting of Castanea tree to form roots *in vitro*, and Aziz *et al.*, (2002) reported *A. tortilis* did not develop roots in any media tested. The difficulty of mature tree explants to form roots may be due to rooting inhibitors that is not present in juvenile explants, or due to change in carbohydrate metabolism in old trees.
Chapter Six

Conclusion

The observations made in this study indicate that mature material of *A.seyal* can be regenerated through the use of single node explants. From the previous discussed and results, It is concluded that the following basal media and growth regulators were better for *in vitro* micropropagation of *A.seyal* trees.

1-MS medium at full and half strength.

2-The concentration 30and 20g/l of sucrose.

3-Myo-insitol at 100mg/l.

4- MS vitamins at single dose.

5-Adenine sulfate at 40mg/l.

6-Charcoal at 2.0g/l was better for shoot elongation and to prevent browning of *A.seyal*.

7- AgNO₃ at 1.0mg/l and 5.0mg/l promoted elongation of explants and decreasing the necrosis of leaves.

8- BA at 1.0 and 1.5mg/l was better for shoot multiplication of *A.seyal*.

9-Kinetin at 1.5 and 2.0mg/l were better for shoot multiplication than other concentrations.
10-Rooting of *A.seyal* explants was only possible on ¼MS supplemented with 0.5mg/l IBA (30% rooting).

More research and investigation are needed especially for rooting, increasing the number of shoots, and to transfer the plantlets to *in vivo*. 
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