IN VITRO PROPAGATION OF ARAK (*Salvadora persica* L.) USING SINGLE NODE EXPLANTS

By

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Dedication

To my family, mother and brothers

To my wife Safia and Children,

Kamal, Reem and Mohamed

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ABSTRACT

In vitro propagation using single node explants was carried out to develop a protocol for mass propagation of Arak trees.

Different types of media: Murashige and skoog's (MS) Gamborg (B5) and White were tested. MS and B5 media were found to give a better propagation results than White medium. When different concentrations of MS medium were tested MS gave the best result. Sucrose and glucose as a source of carbohydrate were also tested, and 2% w/v sucrose proved to be the best source at this concentration for good explant proliferation. A comparative study for the use of MS vitamins and Nitsch and Nitsch vitamins indicated the significance of MS vitamins formulation. Two types of growth regulators auxins (IBA and NAA) and cytokinins (BA and kinetin) were studied singly and in combination (IBA with BA). A combination of IBA and BA at 0.5 mg/l each was found to be better for single node explant while using auxins singly inhibited the growth. However the use of 10 mg/l $AgNO_3$ gave better explant proliferation

compared to other concentrations. To establish roots different types of auxins were tested and good rooting ability was achieved using 2 mg/l. NAA.

ملخص الأطروحة

استخدمت الزراعة المعملية للعقد السلامية للاكثار النسيجي لنبات الأراك.

تم اختيار عدة اوساط مغذية صناعية هيموراشيقي واسكوج (MS) وقـامبورج (B5) وهوايت ووجد أن بيئتى موراشيقي واسكوج وقامبورج همـا الأفضـل للإكثـار النسيجي. وعند استخدام تراكيز مختلفة من بيئة موراشـيقي واسـكوج وجـد أن بيئـة موارشيقي واسكوج مضاعفة التركيز هي الأفضل لتكاثر شجرة الأراك النسيجي. تمـت مقارة السكروز والجلوكوز كمصادر للطاقة واثبت تركيز ٢% سكروز كفائة عالية. عنـد مقارنة تأثير فيتمينات موراشيقي واسكوج وفيتيمينات نتش ونتش فـي بيئـات مراشـيقي واسكوج وبيئة قامبورج وجد أن فيتمينات موارشيقي واسكوج هي الأفضل. تـم اختيـار منظمات نمو مختلفة كالاوكسينات NAA، IBA والسايتوكاينينات BA وK منفردة او مخلوطة IBA مع BA في وسط موراشيق واسكوج الخالي مـن منظمـات النمـو زاد التفرع لنبيتات شجرة الأراك عند التركيز ٠,٥ ملجرام/لتر لكـل هـذا ومعاملـة العقـد السلامية بالاوكسينات لها أثرا سلبيا علي النمو.

تمت دراسة تأثير محلول نترات الفضة $AgNo_3$ ووجد أن ١٠ ملجمرام/لتر هو الأفضل لإكثار الأراك النسيجي.

لتحفيز نمو الجذور استخدمت أنواع مختلفة من الاوكسينات، ولقد كانت النتيجـة إيجابية فقط عند استخدام NAA بتركيز٢ ملمجرام/لتر..

CHAPTER ONE

INTRODUCTION

Trees are important component of the ecosystem which provides food, fuel, construction and industrial material, in addition, trees are recognized as the critical element in maintaining stability in world atmosphere (NCR, 1991). Forest resources are disappearing at an unprecedental rate. It has been estimated that about 500000 square kilometers of the semi arid land of the Sudan are directly affected by drought and desertification in the last four hundred years (Ibrahim, 1978). Though, the rural populations depend on forest for their existence and to maintain their life system, forest have been destroyed by cutting (fuel wood), over cultivation and over grazing. This widespread devastation resulted in colossal environmental degradation and desertification which in turn, lead to drought and famine in many parts of the country (Nasroun, 1989).

Salvadora persica L. (Arak) is a highly salt tolerant tree. It is grown in coastal regions and inland saline soils (Makwana *et al*., 1988). In Sudan Arak is distributed in the land area of the flood plain along valleys and khors in the North and East Sudan, White Nile,

Duiawm, Getaina, Khartoum, Red Sea hills, Sinkat and Kassala (El Amin, 1990).

Human for cleaning teeth (Muswak) uses the tender branches and roots of the Arak tree. The leaves extract is applied against oral infestation in animal (Suliaman *et al*., 1986). The wood is used for fire and charcoal, the trees also provide fodder for animal and the fruits are cooked and eaten by humans (FAO, 1968). It was also reported that, the salt contained in the leaves might affect the taste of cow milk, and can increase the lactation period (Vegt, 1995). Arak is also used as medicinal plant for treating fever, liver, ailment, rheumatism and for dental care (Mustafa and El Menshawi, 1994).

In Sudan Arak tree became rare due to extensive use as tooth brush, prosing purpose and due to drought in last decays. Difficulties face conventional methods for its propagation. The objective of this study is to develop a reliable *in vitro* propagation protocol for mass production of Arak plantlets. To achieve this goal various combinations of mineral salts, carbohydrates, vitamins and plant growth regulators were tested.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy

Salvadora persica L. belongs to the family *Salvadoraceae*. The generic name was given in 1749 in the honor of an apothecary of Barcelona Juan Salvador, Y. Bosca (1998-1681) by Dr. Laurent Garcin, Botanist, traveler and plant collector. The tree specimen of this species came as the specific name indicates, from *persica* (ICRAF, 2002).

2.2 Botany:

2.2.1. Tree

Salvadora persica L. appears as a shrub or small tree to 9.5 m high. Bark smooth, light grey, whitish of green slash (FAO, 1968; Von Maydell, 1990).

2.2.2. Leaves:

Leaves lanceolate to ellipitic, mucronate, 3-7 cm long, 1.5 - 3 cm broad, grey when dry, glabrous, with few lateral nerve equally prominent on both sides. Petiole nearly I cm long, thick to fleshy, with very small stipules (FAO, 1968; Von Maydell, 1990).

2.2.3. Flowers:

Flowers are greenish to yellowish, very loose, slender branched axillary or terminal panicales up to 10 cm long. Flowers with four petals –1.5 mm long, stamens 4, ovary-1 celled, calyx –4 lobed (Von Maydell, 1990).

2.2.4. Fruits:

Fruits are spherical, fleshy, 5 - 10 mm in diameter, pink to scarlet when mature. Single seeded, seeds turn from pink to purple-red and are semi transparent when mature (ICRAF, 2002).

2.3. Propagation:

2.3.1. Conventional methods:

2.3.1.1. Seed propagation:

Seedling is used in establishing most species, cultivars and botanical varieties than any other propagation methods. It is more economic and practical for mass production. In plant breeding it is the most important mean to produce new cultivars (Hartmann and Kister, 1975). With few exceptions such as poplars (*Puplus* spp.), Willows (*Salix* spp.) and some tropical species, trees are mostly propagated by seeds. The quality of seeds has great influence on the success of plantation raised (Willan, 1985). *Salvadora persica* readily germinates from seeds. Seeds exhibit no dormancy, but the fruit pulp contains germination inhibitors, that should be removed before sowing (ICRAF, 2002). The process of seed germination starts with the inbibition in water at 25-30°C for 24-72 hours. Soaked depulped seeds of S. *persica* will germinate in 24 hours. Seeds have been raised in the

nursery for up to three years prior to transplanting in the field. Mohamed El Kheir (1998) reported that, Arak seed does not need any pregermination treatment, as untreated seeds gave better results compared to treat seeds. Sider regenerates naturally by sprouting, natural seeding and coppice (Hussein, 1994). Artificially sider tree propagates by seeds, which are slow and difficult to germinate (Abo-Hassan and Mahros, 1979; Said, 1986).

2.3.1.2. Vegetative propagation:

It is a type of asexual propagation that involves mitotic cell division that duplicates genotypes of the parent plants. Such genetic duplication is known as cloning. The population of the offspring plant is known as clone. The character of the new plant will be the same as the mother plant (Hartmann and Kester, 1984). In nature higher plants reproduce sexually (by seed), but asexual (or vegetatively) reproduction occurs readily (Hartmann and Kester, 1984). Naturally, many plants reproduce vegetatively by different means, including coppicing, sucker, runners, stolen and rhizomes (Hartmann and Kester, 1975).

Artificially, man tried different ways to propagate plant vegetatively. The common methods adopted are cutting, grafting, budding, layering, runner, sucker separation, crown division and *in vitro* culture system.

Foresters employed vegetative propagation for production of uniform plant of known genotypes (Fielding, 1963), and for the provision of genetic and physiological information (Hinds and

Crugman, 1974). Sometimes vegetative propagation was imperative e.g. when a plant produces no viable seeds or produces seeds of short longevity. Also some plants grow slowly in juvenile stage, some have complex seed dormancy. Vegetative propagation of some species may be easier, more rapid and more economic than propagation by seeds (Hartman and Kester, 1975).

Sunil *et al*. (1988) reported that *Bobinia pseudoacacia* gave good germination and survival percentage of 60% and 75% respectively when propagated sexually by seeds. Stem cutting can also propagate it. *Berberis ariststa* DC, as noted by Parmer and Khama (1989) can be propagated sexually and asexually. Eltahir (1997) reported that *Balanites aegyptiaca* can be propagated by stem cuttings in winter using growth regulators IBA and NAA. Mohamed ELKheir (1988) failed to propagate *Salvadora persica* by stem cutting.

2.3.2. Tissue culture:

The capacity for clonal propagation is closely linked to the genetic and physiological factors that control the transition from juvenile to mature growth in the tree (Bonga, 1980; 1982a). In trees (of few species) some tissue remain capable of organogenesis for many years after the tree reaches maturity, such as leaf tissue of 60

years old *Populus balsamifera* (Bychenkova and David, 1978) and terminal bud of 100 years old *Techtona grandis* (Gupta *et al*., 1980). In mature trees of other species, such as *Sequola sempervirens* some parts like basal orthotropic shoots are juvenile and have tissue capable of organogenesis (Ball *et al*., 1979).

Section of immature inflorescence of various herbaceous plants has produced adventitious embryos or organs *in vitro* (Dale *et al*., 1981). Similar results were obtained with several tree species. Section of stalk of immature inflorescence of palm produced adventitious plantlets *in vitro* (Reynold, 1982). Stalks of immature female cones of *Laria decidua* formed multiple shoots (Bonga, 1982b).

Explant of some difficult to propagate mature trees, produced adventitious structures that resembled shoot, roots and embryos, but had an abnormal morphology. Adventitious shoot and embryo like structure in culture of mature *Abies balsamoa* (Bonga, 1977), and shoots and roots that formed in callus culture of *Populus termata, Betula rerrucosa, Tilia parvifolia, Ulmus campestris* and *Quereus sessiflora* showed a lack or delayed development of vascular system (Jacquiot, 1964).

Sarita *et al*. (1988) investigated micropropagation of a leguminous timber tree *Pterocarpus santalinus*. Shoot multiplication was obtained from single node and terminal cuttings derived from aseptic seedling and shoot differentiated from cotyledon callus using 0.25 MS medium supplemented with Benzyladenine (3 μM) and adenine (0.4 μM).

Nirmala *et al*. (1989) cultured cotyledonary nodes of *Ficus auriculata* on MS medium supplemented with BA (1 mg/l) and NAA (0.01 mg/l) to induce shoot formation. Microshoots were dipped in IAA solution 100 ppm and rooted in a mixture of sand and powder of dry leaves.

Lal *et al*. (1988) cultured callus from *Ficus religiosa* leaf explants from approximately 20 years old tree on MS medium, containing 0.5 mg/l 2,4-D. The calli were transferred to medium supplemented with various combination and concentration of 0.05-1.0 mg/l of BA and NAA. In the presence of 0.1mg/l BA and 0.05 mg/l NAA, globular, heart and torpedo shaped somatic embryiods were formed on the surface of the callus 4-5 weeks after transfer.

Jones *et al*. (1990) cultured node, internode and phyllode explant from each of five *Acacia* species on solidified MS with

combination of auxin IAA or IBA and cytokinin BA. Callus developed on all explant types of each species with limited regeneration of roots from *A. holosericea* internodes. Axillary shoot growth occurred from nodal explants of all species and with the exception of *A. sclerosperma*. These shoots developed roots on medium containing 1.8 mg/l IBA

Fougal *et al*. (1997) achieved best shoot proliferation from nodal segments of *Z. mauritiana* on MS medium supplemented with 0.25 mg/l NAA and 1.0 mg/l BA, but they failed to reproduce roots on the microshoots, they only produced callus at the basal cutting of each shoot. High callus was obtained from the leaf explant on MS medium supplemented with 0.25 mg/l IBA and 2.5 mg/l BA. The formed callus from both explants on shoot regeneration medium failed to differentiate into shoots.

Rhathore *et al*. (1992) developed protocols for colonal propagation of *Z. mauritiana* and *Z. nummularia* by tissue culture. From a nodal explants of *Z. mauritiana* 4-5 shoots were developed from the nodal region of each explant on MS medium containing 7.5 mg/l BA and 0.1 mg/l IAA for *Z. nummularia* 5-7 shoots were proliferated on MS medium containing 5 mg/l BA and 0.5 mg/l IAA.

Shoots regenerated *in vitro* could be further multiplied on fresh medium. The isolated shoots were placed for rooting on filter paper bridge in White liquid medium containing 25.0 mg/l IBA. After 48 hours these shoots were transferred to solid White medium without growth regulators. Regenerated plants were hardened off and successfully transferred to pots.

Bonga and Pond (1991) cultured primordial shoot explants excised from buds of one *Laria decidua* tree of about 30 years old. The explants produced more adventitious buds, elongating into shoots, when grown on half strength Litvay medium than when grown on other basal media. Thidiazuron and 6-BA were equally effective in adventitious bud induction. In a comparative study of 30 years old *Laria decidua*, *L. leptolepis, L. eurolepis* and *L. decidua* produced numerous cultures with adventitious buds that elongated into shoots. Those from *L. leptolepis* were less productive, and those from *L. laricina* failed to form adventitious buds. The highest response was obtained with material collected in August and September and in March and April. The lowest response occurred in the explants from the October collection.

Zaheer *et al*. (1990) investigated culture from nodal bud segments excised from a mature (40 years old) *Melia azedrach* tree. The highest proliferation rate was observed on MS medium supplemented with BA 1.0 mg/l. Some shoot formation occurred in media supplemented with kinetin, but the shoots did not proliferate on subculture. GA3 promoted only callus formation, while to either did not promote shoot growth or in some cases caused formation of thin and delicate shoots with no photosynthetic capacity. Rooting of shoots *in vitro* was achieved on MS medium containing 1.0 - 2.0 mg/l of IBA while IAA was ineffective.

Mathew and Hariharan (1990) investigated the effect of Gamborg (1962) and 0.50 MS strength salt media, supplemented with BA and NAA on micropropagation of clove tree *Syzygium aromaticum.* Multiple shoots were induced from nodal segments of 1 month seedling. Six to 8 shoots were obtained with 3.0 mg/l BA and 0.5 mg/l NAA in the media. No significance difference between the 2 media was noted.

2.4. Fundamental requirements for establishment of *in vitro* trees culture:

2.4.1. Choice of explant:

When dealing with trees that are difficult to propagate *in vitro* it is very essential to determine first the type of explant to be used, because there could be juvenile cells in the tree that have hitherto not been utilized (Bonga, 1981). Considerable differences in organogenic capability often exist between different parts of the plant (Bonga, 1982a). Such differences occur between neighboring tissue or cell, even with such small organ as the shoot apical meristem (Ball, 1980). Furthermore, the morphogenic ability of tissue is either stimulated or inhibited by neighboring tissue (Tranthanhvan, 1980).

A major advantage of manipulating angiosperm trees *in vitro* is the wide range of materials which can be used as explants, such as seed, seedling parts, leaves, shoots and buds from both mature and juvenile trees, floral parts and in some cases roots. The shoot apex and nodal cutting have been the most frequently used explant materials of several genera of woody species. Only plantlets produced through axillary shoot proliferation are genetically stable (Murashige, 1974). Cutting shoot segment 1 cm long excised from 20 years old *Eucalyptus citriodera* trees gave a rate of 10 - 15 plantlets (Gupta *et al*., 1981). Apical meristem cultures, shoot tip culture and stem tip cultures were used for orchids (Murashige, 1974). Ruredzo and Hanson (1993) used seedling shoots for micropropagation of *Acacia*

albida. Bray *et al*. (1994) used axillary buds for propagation of *Acacia ehrenbergiana.* El-Tigani and Ali (2001) used seedlings of *Acacia senegal*. El-Hadeeb (1995) used axillary buds for micropropagation of *Salvadora persica*.

Hypocotyl was used as explant for direct and indirect organogenesis. Good results were obtained from hypocotyl culture of *Eucalyptus alba* (Kitahara and Cladas, 1975), *Eucalyptus grandis* (Goncalves, 1975), *Morus alba* (Ohyama and Oka, 1982). *Acacia senegal* (Ibrahim, 2000).

Leaves of hard wood species can be used as source of explant for producing adventitious buds. Park and Son (1988) punctured leaves from *in vitro* stock culture of *Populus negra* x *P. maximowiczii* to regenerate shootbuds. Leaves were also used in embryogenic culture of *Cocos nucifera* (Branton and Blake, 1983).

Cotyledons were also used in embryogenesis as in *Morus multicalis* (Minamizawa and Hirano, 1974). *Tamarindus indica* (Mascarenhas *et al*., 1987).

2.4.2. Media composition:

2.4.2.1. Inorganic salts:

A variety of media and salt concentrations have been used, but Murashige and Skoog's (MS)(1962) still remains the most widely used formulations for angiosperm (Harry and Thorpe, 1994). Several researchers used MS or its modifications. Ibrahim (2000) used 0.50 MS for *Acacia senegal*. Mascarenhas *et al*. (1987) used MS for *Tamarindus indica*, Oka and Ohyama (1978) used it for morus, Hussein (2002) used it for *Zizphus spina-christi*. El-Hadeeb (1995) used MS for *Salvadora persica*.

Woody Plant Medium (WPM) which derived by Lloyd and Mc Cown (1980) is becoming a popular of tissue culture of some woody species (Garton *et al*., 1983). The total ionic concentration of WPM is low compared to MS. Many researchers such as, Mc Granhan and Driver (1987) for Junlans nigra, Sommer and Wetzstion (1987) have used it for Liquidamber.

Both Whites medium (1943) and Gamborg (B_5) developed by Gamborg *et al*. (1968) have been used to a lesser extent in tissue culture of woody species.

Guta *et al*. (1980) used whites medium for culturing explant from teak tree, Mc Granhan and Driver (1987) for *Juglans regia*, Nagmani and Venketwaran (1987) used B5 medium for hypocotyls

culture of *Leucaena leucocophala*. Mc Granhan and Driver (1987) for embryo culture of *Juglans regia*.

The salt combinations are used either at full strength or with modification based on experimental results. Several researchers have combined the macro salt or organic component of one formulation with micro of another. The medium for larch induction using whole excised embryo, was half strength Quoirin and Leporvers (QP) (1977) salts with Schenk and Hildel brandt (SH) (1972) organic (Harry *et al*., 1991).

Bonga and Von Aderkas (1992) stated that, the nutrient and tissue interaction are influenced by environmental factors such as light intensity and quality, photoperiod, temperature, agar and medium pH. Further more, tissue response varies with changes in the physiological conditions of the explant or subculture tissue.

2.4.2.2. Growth regulators:

Five principal classes of plant growth regulators have been recognized, namely the auxins, gibberellins, cytoknins, abscisic acid and ethylene. None of these is associated alone with a single physiological or developmental process but each growth process is controlled by interaction of two or more of these growth regulators (Leopold, 1987).

2.4.2.2.1. Auxins:

Auxins are involved in cell division, elongation and cell wall synthesis. The principal natural auxin is indole acetic acid (IAA). It is not usually used in tree tissue culture media, as it is unstable (Nissen and Suffer, 1988). Iron in the medium stimulates light-catalysed destruction of IAA (Dunlap and Robacker, 1988). Many researchers used IAA. Chang (1976) for *Tsuga heterophylla*, Rao and Bapat, (1978) for *Santatum album*, Gupta *et al*. (1980) for rooting of *Tectona grandis.*

Indole butyric acid (IBA) is less destroyed by heat and light than IAA, (Nissen and Suffer, 1988). IBA is metabolized to IAA, an investigation of rooting of *Castanea salivra* shoots indicated that, IBA can change endogenous levels of IAA (Mato and Vieitez, 1986).

Naphthalene acetic acid (NAA) has been widely used to induce callus in gymnosperms (Straus and Epp, 1960). It is also used to induce root on calli, embryos and explants (Zaerr and Mapes, 1982), but it inhibits shoot formation in some cases (Chalupa, 1974). Many forest trees formed callus as a result of 2,4-D treatment such as *Acacia koa* (Skolman and Mapes, 1976). *Santalum album* (Rao and Bapat, 1978).

In some explants the production of endogenous auxin is sufficient for shoot induction, and addition of even low concentration to the medium is inhibitory, such as the case in culture of *Larix decidua* (Bonga and Von Aderkas (1988) and *Picea abies* (Borman, 1983).

2.4.2.2.2. Cytokinins:

Cytokinins are involved in induction of adventitious shoots, callus formation and cell division (Minocha, 1987). Cytokinins sometimes mimic the effect of light and stimulate the uptake of potassium (Green and Mair, 1979). A cytokinin is sometimes required for the induction of somatic embryogensis, in culture of *Vitis vinifera* embryogensis was induced by 2,4-D, only if used in combination with BA (Matsuta and Hirabaynshi, 1989). Light has a certain effect on the action of cytokinin on shoot formation, *Prunus institia* shoot formation was produced by BA in culture exposed to light, however shoot elongation was inhibited by BA in cultures kept in dark or light (Baralde *et al*., 1988). Culture over exposed to BA sometimes fail to elongate or roots, this happens in culture of *Pinus radiana* (Biondi and Thorpe, 1982) and *Vitis rotundifolia* (Lee and Wetzstus, 1990).

Explants of some species are very sensitive to BA, a very low concentration of BA and other cytokinins inhibited adventitious and axillary bud development in *Arucaruia sp.* Explants (Burrows *et al*., 1988). Over exposure to BA lead to excessive callus formation in culture of *Pinus strobus* (Flinn *et al*., 1986).

Adventitious shoot formation generally requires treatment with auxin and cytokinin. However, auxin should be used sparingly because excessive auxin application favours callus growth and causes shoot abnormalities (Bonga and Von Aderkas, 1992).

2.4.2.2.3. Gibberellins:

Most reports dealing with gibberellins (GA_3) have stated that, it was ineffective or inhibitory (Zaerr and Mapes, 1982), low concentrations of GA_3 were inhibitory to bud development in explant of *Araucaria spp*. (Burrows *et al*., 1988), and registered no effect on cotyledon culture of Pinus radiana (Biondi and Thorpe, 1982).

2.4.2.2.4. Abscisic acid:

Abscisic acid (ABA) is the most prominent among natural inhibitors. When trees are exposed to stress, they respond by producing ABA (Johnson, 1988). ABA was required for normal development of somatic embryos of several conifers. *Picea abies* (Arnold and Hakman, 1988), Picea glauca (Boulary *et al*., 1988). ABA inhibited axillary shoot multiplication, but not shoot elongation in culture of *Robinia pseudoacacia* (Barghchi, 1987).

2.4.2.2.5. Ethylene:

Ethylene is a natural plant growth regulator that was produced in many tissues, most notably in ripening fruits and senescing tissue. An important aspect of ethylene in tree physiology is its effect on phenyl alanine aminomolyase PAL, an acidic peroxidase that regulate lignin formation (Gasper *et al*., 1985) Ethylene has profound effect on regulation of growth and morphogenesis (Yang, 1980). Ethylene production is often stress response e.g. response of wounding, toxin, heat, chilling and desiccation (Yang and Hoffman, 1984). It has been reported to inhibit root elongation but not initiation in cutting of *Hedera helia* (Hacket *et al*., 1988).

2.4.2.3. Vitamins:

Plant tissue produced most of the vitamins required in their metabolism. Under *in vitro* condition endogenous production of some of the vitamins in particular thiamine is often insufficient for optimal growth, therefore a few vitamins, such as niacin (nicotinic acid) pyridoxine and thiamine are routinely added to the nutrient medium. Calcium panthothenale and riboflavin are added occasionally (Bhojwani and Razdan, 1983). Niacin is involved in hydrogen and electron transfer dehydrogenase, liquid catabolism and photosynthesis, and it is heat and light stable (Kutsky, 1973; Hagen *et al*., 1991).

Pyridoxine regulates protein, carbohydrates and lipid metabolism. It is heat stable, but light labile in alkaline solution. Thiamine is a co-enzyme in pyruvate metabolism, it is heat labile and decomposes in UV light. All these vitamins act synergistically with each other (Kutsky, 1973). Myo-inositol is one of the active ingredient in coconut milk (Street, 1979). It is involved in the synthesis of phospholipids and cell wall pectins (Amderson and Wolter, 1966; Street, 1979; Grey *et al*., 1987). Most media contain the vitamins of the MS medium. However, only a few studies have been carried out to determine if the MS vitamin combination is indeed optimal in tree tissue culture (Bonga and Von Aderkas, 1992). Risser and White (1964) tested 10 vitamins in culture of *Picea glauca* tumor tissue. Of these only thiamine, Myo-inositol, niacin and asorbic acid were needed for continuous growth. The development of cell colonies from *Pseudotuga menziesii* and *Picea glauca* protoplast was stimulated by high Myo-inositol in the medium, (Attree *et al*., 1989). Riboflavin stimulated rooting in culture of *Eucalyptus facifolia*, this stimulation occurs in both light and dark conditions (Gorst *et al*., 1981). Hussein (2002) used Nitsch and Nitsch vitamins for *Ziziphus spina-christi*. Ibrahim (2000) used white vitamins for *Acacia senegal*.

2.4.2.4. Gelling agent:

Agar is the most commonly used gelling agent. It is complex polysaccharide from some species of algae. During fabrication it is subjected to varying degree of purification, however, minerals and organic impurities remain (Romberger and Tabor, 1971). Some cultures grow better on media with low concentration of agar, for example shoot apices of *Picea abies* accumulate dry matter faster when cultured on medium with agar at 0.125% than at 1%. This inhibition at high concentration was properly due to accumulation of an excretion product immediately below the explant (Romberger and Tobor, 1971). The most popular alternative to agar is gelrite, it is a complex extra cellular polysaccharide produced by *Pseudomonas oledea* (Bonga and Von Aderkas, 1992). Gelrite contain less free minerals and organic impurities than agar. However, it contains potassium and magnesium at high concentration (Pasqualetto *et al*., 1988). One problem with gelrite is that, some cultures become vitrified more easily on gelrite than on agar. In a study comparing the effect of agar and gelrite on somatic embryogensis of *Picea abies*, it was found that, the response was similar on medium containing 0.7-1.3%, agar and medium with 0.2 – 0.5 % gelrite (Von Arnold, 1987). However cotyledons of *Pinus canariensis*, formed more buds on medium solidified with 0.8% Difco Bacto agar than on medium

solidified with various concentrations of gelrite (Martinez *et al*., 1990). Various starches have been used to solidify nutrient media, these include barley, corn, potato, rice and wheat starch (Henderson and Kimersly, 1988).

2.4.2.5. EDTA:

The sodium salt of ethylene diamine tetra acetic acid (EDTA) is normally used to chalet iron, thus making it more soluble (Bonga 1982; Teasdale, 1987). As was pointed out by Singh and Krikorian (1980) $FeSO_4$ and NaEDTA are often not used in equimolar concentration in the media. In most Fe EDTA formulation there is excess of EDTA that may lead to EDTA toxicity (Dalton *et al*., 1983). In *Pinus radiata* suspension culture 20μM NaFe EDTA was optimal for cell growth and excess of ETDA over iron resulted in inhibition. In few cases iron chalet stimulated embryogenesis and root growth, where iron dissolved in non-chaleted form did not (Said and Murashige, 1979). Pretreatment of shoots of mature conifers with ETDA without iron, stimulated morphogensis in these shoots after their transfer to culture medium (Bonga, 1981).

2.4.2.6. Silver nitrate ($AgNO_3$):

A chemical often used to inhibit ethylene formation is silver nitrate. Some cultures fail to form shoots on media supplied with various auxin cytokinin combination unless silver nitrate is added to the medium (Prunhauser *et al*., 1987).

Mekey, (1999) used $AgNO_3$ on culture of strawberry and found that, it did not significantly enhance the proliferation of strawberry culture, but usually induced runners *in vitro* at 10 mg/l. Ibrahim (2000) investigated the effect of $AgNO_3$ on *in vitro* multiplication of *Acacia senegal*. He found that the addition of $AgNO_3$ on MS basal medium 5 mg/l or above decreased the number of shoots but at the same time increased the number of leaves. Hussein (2002) used $AgNO_3$ at low, moderate and high concentrations on culture of *Ziziphus-spina-christi* and found that moderate concentration promoted shoot regeneration on nodal explants, while low or high concentration did not.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Plant material

Salvadora persica L. Arak tree is grown in Shambat area, at the Botanical Garden, Faculty of Agriculture, University of Khartoum. It is a mature tree, about 40 years old well irrigated and pruned for the production of new twigs. From these new twigs single nodes each with an axillary bud of about 0.5 cm were used as explant for all experiments.

3.2. Preparation of explant

New twigs were removed from the mother tree, and were washed with tap water to remove dust and to reduce contamination. By means of a sharp blade the leaves were removed and the plant material was subjected to thorough washing with running tap water for more than 30 minutes.

3.3. Sterilization

3.3.1. Explant

The explant were transferred to laminar air flow cabinet, then rinsed with 70% ethyl alcohol for 30 seconds followed by soaking in 5% sodium hypochloride solution (V/V), tween 20 added two drops for 100 ml, with continuous hand shaking. The explant were then carefully washed 3 times with sterile distilled water to remove any traces of sodium hypochloride.

3.3.2. Medium

The medium was sterilized by autoclaving for 15 minutes at 121^{o}C with pessure of 1.06 Kg/cm^{2}.

3.3.3. Equipments

All glass- wares were sterilized by dry heat in an oven at 160^{o}C for 3 hours. Forceps, scalpels and blades were sterilized by dipping in

90% ethyl alcohol and then were subjected to flaming before use each time.

3.3.4. Laminar air flow cabinet and transfer room:

Laminar airflow was sterilized by smearing with ethyl alcohol 70% and then switched on 20 minutes before use. The UV lamps were switched on for over night in the transfer room.

3.4. Culture media:

Three types of culture media were used Murashige and Skoog's (MS) (1962), Gamborg (B5) (1968) and White (1943) inorganic macro were used, then MS micro were added to each, the source of carbohydrates was sucrose 3% w/v, MS vitamins were added, the gelling agent was Defco Bacto agar 0.8% w/v, pH was adjusted to 5.7 ± 0.1 with 0.1 N HCl and 0.1 N NaOH before agar was added. 25 ml of the medium were distributed in a 100 ml sterilized test tube covered with screw cap.

3.5. Incubation condition:

Incubation room temperature was adjusted at 25 ^{o}C ± 2 under light intensity of 1000 lux and 16 hours photoperiod using flourscent lamp.

3.6. Experimentation:

3.6.1. Exp. 1: The effect of type of medium on morphogenesis. Three media were tested. Murashige and Skoog's (MS), Gamborg B5 and White.

MS medium: MS macro + MS micro + MS vitamins + 3% w/v sucrose + 0.8 w/v agar, pH 5.7 ± 0.1.

Gamborg B5: Gamborg B5, macro + MS micro + MS vitamins + 3% w/v sucrose + 0.8 w/v agar, pH 5.7± 0.1.

White: White macro + MS micro + MS vitamins +3% w/v sucrose + 0.8 w/v agar, pH 5.7± 0.1.

3.6.2. Exp. 2: **The effect of different MS macro strength on Arak morphogenesis:**

MS at 2x, 1x, 0.5x and 0.25x strength were used plus MS micro + MS vitamins + 3% w/v sucrose and 0.8% agar and pH adjusted to 5.7 ± 0.1

3.6.3. Exp. 3: Carbohydrates source:

Two different sugars (sucrose and glucose) were tested as carbohydrate source incorporated in MS and Gamborg B5 media. Different levels of concentrations were tested namely 2, 3, 4 and 5%.

3.6.4. Exp. 4: Effect of vitamins:

Two vitamins mixture were used (MS and Nitsch and Nitsch), added to two basic macro salts of MS and Gamborg B5. Two strength

of each vitamin mixture was tested 1x and 2x. For all treatments MS micro + 3% w/v sucrose + 0.8% w/v agar were added, pH was adjusted to 5.7 ± 0.1.

3.6.5. Exp. 5: Effect of growth regulators:

Two groups of growth regulator were tested.

3.6.5.1. Auxins:

Indole butyric acid (IBA) and naphthalene acetic acid (NAA), each was added to MS basal medium. MS macro + MS micro + 3% w/v sucrose + 0.8 w/v agar, pH 5.7± 0.1, at concentrations of 0.0, 0.1, 0.5, 1.0 and 2 mg/l.

3.6.5.2. Cytokinins:

6-furfurylamine (kinetin) and 6-benzyl amino purine (BA) were tested. Concentrations tested were 0.0, 0.1, 0.5, 1.0 and 2 mg/l using Ms basal medium. MS macro + MS micro + MS vitamins + 3% w/v sucrose + 0.8 w/v agar, pH adjusted to5.7± 0.1.

3.6.6. Exp. 6: Combined effect of indole –3 butyric acid (IBA) and 6- benzyl amino purine (BA):

Four levels of each growth regulator were tested using MS basal medium in factorial experiment in the following combinations (concentration mg/l).

IBA 0.0, 0.5, 1.0 and 2.0
BA 0.0, 0.5, 1.0 and 2.0.

3.6.7. Exp. 7: Effect of silver nitrate concentrations ($AgNO_3$).

Silver nitrate in different concentrations 0.0, 5.0, 10.0, 15.0 and 20.0 mg/l was tested. These concentrations were added to MS basal medium.

3.6.8. Exp. 8: Rooting

For rooting nodes were allowed to grow on basal medium till about 20-30 mm long and then transferred to rooting media.

The following experiments were carried out

1) MS basal medium + IBA at 0.0, 0.1, 0.5, 1.0, 2.0, 4.0 and 6.0 mg/l.

2) 0.25 MS macro strength + MS micro + 3% w/v sucrose + 0.8% agar + MS vitamins, pH adjusted to5.7 ± 0.1. IBA concentration levels were 0.1, 0.5 and 1.0 mg/l.

3) 0.25 MS macro strength + MS micro + 3% w/v sucrose + 0.8% agar + MS vitamins, pH adjusted to7± 0.1. pH was adjusted to 5.7± 0.1 then 2-4-D at 0.1, 0.5 and 1.0 mg/l. for 2 weeks then transferred to MS basal medium.

4) MS macro + MS micro + MS vitamins + 3% w/v sucrose + 0.8% w/v agar, pH was adjusted to 5.7± 0.1 with IBA at 0.0, 0.5, 1.0 and 2.0 mg/l. Shoot tips from *in vitro* shoot lets were used.

5) 0.5 MS macro + MS micro + MS vitamins + 3% w/v sucrose + 0.8% w/v agar, pH was adjusted to5.7± 0.1. NAA auxin at 0.0, 0.5, 1.0 and 2.0 mg/l.

3.6.9. Data records and experimental design:

Data were weekly recorded for number of leaves and shoot length. However, for callus formation, number of shoots, number of nodes and number and length of roots. The data were recorded at the end of the incubation period of 10 weeks.

Complete randomized design with 10 replicates for each treatment. Duncan multiple range test was used for separation of means.

CHAPTER FOUR

RESULTS AND DISCUSION

4.1. Effect of different types of media:

Table (1) illustrate that, number of leaves and shoots length were significantly higher when MS and B5 media were used compared to White medium, but they were not significantly different from each other. The leaf size was observed to be very much reduced when White macro elements were used (Plate 1). The number of leaves increase with time (Fig. 1). The shoot length increase with incubation in all media (Fig. 2).

Number of shoots was not significantly different in all treatments, but was slightly higher on MS medium (Table 2 and Plate 1). Number of nodes was significantly higher on MS and B5 media (5.6 and 4.3 respectively) when compared to White. The callus was formed on both MS and B5 media amounting to 16% of the cultured tubes, and no callus was observed when white medium was used.

These results showed that, Arak can grow successfully on MS and B5 media, compared to poor growth on White medium. These results agree with Gleba and Gordzievaskaya (1978) cultured *Platycerium bifurcatum* and obtained good proliferation on full MS.

Table 1: Effect of different inorganic salt Murashige and Skoog's (MS) Gamborg (B5) and White macro on morphogenesis of Arak after 10 weeks.

Parameters / Treatments	No. of leaves	Shoot length	No. of shoots	No. of nodes	Callus %
MS	8.5 a	22.6 a	2.1 a	2.6 a	16
Gamborg B5	7.6 a	18.8 a	1.3 a	4.3 a	16
White	2.1 b	8.3 b	1.1 a	1.3 b	0

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

Zaheer *et al*. (1990) cultured *Melia azadrach* successfully on MS. Mittal, *et al*. (1989) cultured *Acacia auriformis* successfully on B5 medium. Gupta and Agrawal (1992) cultured *Acacia nilotica* on B5 and obtained shoot and root. These results do not agree with Gupta, *et al*. (1980) who successfully cultured Teak tree on White medium. This may be due to genotypic differences, which were reflected, in variable morphogenetic responses.

4.2. Effect of strengths of MS medium:

MS macro with different strengths 0.25, 0.5, 1.0 and 2.0 were tested. Results illustrated on Table (2) show that, significantly highest number of leaves was obtained on 2.0 MS (11.6), which was significantly different from all other treatments. 0.5 MS gave a significantly low number of leaves, but the leaf size was much smaller and only normal leaves were obtained on 1.0 MS (Plate 2). There was a sharp increase in number of leaves induced on 2.0 MS during week 6 to 10 (Fig. 3).

Shoot length was significantly high on 0.25 MS than that obtained on 0.5, 1.0 and 2.0 MS, which were not significantly different from each other (Table 2). Shoot length increase with time

Table 2: Effect of MS strength of macro nutrient on *in vitro* morphogenesis of nodal explants of Arak after 10 weeks.

Parameter / Treatment	No. of leaves	Shoot length	No. of shoots	No. of nodes	Callus %
0.25 MS	8.0 b	32.8 a	1.4 a	4.2 b	0.0
0.50 MS	5.6 b	18.0 b	2.2 a	3.2 b	0.0
1.0 MS	8.6 b	22.4 b	1.8 a	5.2 b	20
2.00 MS	11.6 a	20.2 b	3.2 a	7.6 a	40

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

on all treatments (Fig. 4). Sarita, *et al*. (1988) obtained good growth of *plerocarpus santalinus* on 0.50 MS macro.

The results given on Table (2) show that, the number of shoots obtained on all media strengths did not show any significant difference, although 2.0 MS gave slightly high number (3.2), this result is in agreement with that of Cauvin and Salesses, (1988) who used 2.0 MS to improve the multiplication rate of chestnut tree

However results given on Table (2) show that, medium strength of 2.0 gave significantly higher number of nodes (7.6) than all other concentrations which were not significantly different from each other. Callus formation increased by increasing salt concentrations from 1.0 (20%) to 2.0 (40%), but no callus was observed in lower concentrations of 0.25 and 0.50. This agrees with Bonga and Von Aderkas, (1992) who found that generally callus formation increased with increasing salts concentration. Many investigators used 0.50 MS macro salts such as Mathew and Hariharan, (1990) in *Syzygium aromatiam* culture and Badji *et al*. (1993) in *Acacia senegal* micropropagation.

Abnormal leaf growth was noticed on weak strengths of MS and this may be due to sub-optimal quantities of essential element.

Best propagation parameters were on 2.0 MS due to high number of nodes, leaves and number of shoots while on other media elongation of internodes was formed.

4.3. Carbohydrate source:

4.3.1. Sucrose:

Tables (3) and (4) show the influence of sucrose in different concentrations 2, 3, 4 and 5% w/v using MS and B5 media. The number of leaves was significantly higher when sucrose concentrations of 2 and 3% were used on MS medium compared to 4 and 5% concentrations (Table 3, Fig. 5 and Plate 3). On B5 medium the results showed somewhat the same trend (Table 4, Fig. 6 and Plate 4). Many researchers used low sucrose concentrations. Miller *et al*. (1982) used 20 g/l sucrose for proliferation of peach. Mehrapta *et al*. (1977) used 20 g/l sucrose for *pinus taeda* and Gupta, *et al*. (1983) for *Eucalyptus spp*.

The shoot length that induced on 2% was significantly different from 5 and 4% on both media however, it was comparable with 3% (Table 3 and 4, Figs. 7 and 8 and Plates 3 and 4). These results indicate that, shoot elongation of Arak explants requires low levels of sucrose in the medium. This agrees with the finding of Aitken *et al*. 1980) who showed that *Binus radiata* required 2% sucrose for shoot

Table 3: Effect of sucrose concentrations on MS morphogenesis of nodal explants of Arak *in vitro* on MS medium after 10 weeks.

Parameter / Sucrose %	No. of leaves	Shoot length	No. of shoots	No. of nodes	Callus %
2	7.5 a	25.7 a	1.8 a	4.0 a	0
3	7.0 a	19.7 ab	1.5 a	4.5 a	14
4	5.4 b	12.5 b	1.7 a	3.2 b	14
5	4.5 b	10.2 b	1.2 a	3.2 b	14

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

Table 4: Effect of sucrose concentrations on morphogenesis of nodal explants of arak *in vitro* on B5 medium after 10 weeks.

Parameter / Sucrose %	No. of leaves	Shoot length	No. of shoots	No. of nodes	Callus %
2	7.1 a	22.3 a	1.5 ab	4.6 a	16
3	7.0 a	19.0 ab	1.8 a	4.3 ab	0
4	7.0 a	16.8 bc	2.1 a	5.3 a	0
5	4.8 b	13.5 c	1.0 b	3.0 b	16

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

elongation and 3% for bud induction. Table (3) shows that, there was no significant difference between all treatments in number of shoots

on MS medium on all concentrations of sucrose. Number of shoots on B5 was comparable on all concentrations of sucrose expect 5% which was significantly lower than those induced on 3 and 4% sucrose (Table 4).

The number of nodes were significantly higher on 2 and 3% (4.0 and 4.5 respectively) compared to 4 and 5% (3.2 and 2.8 respectively) on MS medium. Inconsistent results were obtained with different sucrose concentrations on B5 medium on which, 2 and 4% (4.6 and 5.3 respectively) gave significantly higher number of nodes compared to 5% (3.0) but not significantly different from 3% (Table 4).

The optimal concentration of sucrose may vary depending on the plant type. The normal level 3% w/v is of universal application. Amin and Jaiswal (1987) used this level for both initiation and proliferation phase of guava. Von Arnold and Eriksson (1981) found that sucrose was the only sugar necessary for bud induction in *Pinus contora*. The inhibitory effect of high sucrose concentrations may be due to increase of medium osmotic pressure. This may interfere with the uptake of substances that activate plant growth.

4.3.2. Glucose:

Tables (5 and 6) show the effect of glucose on Arak using MS and B5 media. However, the results given on Table (5) show no significant differences in all parameters measured using different glucose concentrations and more shoot length with 2% concentration (Figs 9 and 10 and Plate 5). Table (6) on the other hand shows the effect of different glucose concentrations using B5 medium. Positive effect of glucose on number of leaves occurred. Concentration of 3 and 4 (5.6 and 5.8 respectively) were a significantly higher than 2% but was not significantly different from 5% (3.1) (Fig. 11 and Plate 6).

Plant height number of shoots and nodes per explants were comparable on all concentrations of glucose on B5.

Despite the wide spread use of sucrose, it is not always the most effective carbohydrate source for shoot induction. Oka and Ohyama (1982) reported that fructose and glucose were the best source for mulberry bud induction. Fadoul (1996) found that, concentrations of glucose of 60 g/l and higher resulted in decrease of number of leaves, plant height and number of suckers in banana. Mekey (1999) found that, different concentrations of glucose had no effect on plant height in strawberry. However, high concentrations of glucose may affect

Table 5: Effect of glucose concentrations on morphogenesis of Arak *in vitro* on MS medium after 10 weeks.

Parameters / Treatment Glucose %	No. of leaves	Shoot length in mm	No. of shoot	No. of nodes
2	5.5 a	16.0 a	1.0 a	3.4a
3	6.2 a	15.0 a	1.2 a	3.1 a
4	5.8 a	14.1 a	1.2 a	3.1 a
5	6.0 a	15.5 a	1.2 a	3.0 a

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

Table 6: Effect of glucose concentrations on morphogenesis of Arak on B5 medium after 10 weeks.

Parameters / Treatment Glucose %	No. of leaves	Shoot length in mm	No. of shoot	No. of nodes
2	3.1 b	12.8 a	1.1 a	2.3 a
3	5.6 a	14.8 a	1.6 a	4.1 a
4	5.8 a	14.0 a	1.8 a	3.3 a
5	4.0 ab	14.8 a	1.3 a	3.0 a

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

explant growth as a result of high osmotic pressure, while low concentrations may result in low carbon as energy source in the medium.

4.4. Vitamins:

4.4.1. Effect of MS vitamins:

MS vitamins were tested in two concentrations 1 x and 2 x on MS and B5 media. Table (7) illustrates that, highest number of leaves was achieved by using 1 x MS vitamins on MS medium, which was significantly different from2 x on MS and 1 x on B5, but comparable with 2 x on B5 (Fig. 13, Plate 7). The highest shoot length was achieved by 1 x MS vitamins on MS medium (19.7 mm) which was not significantly different from 1 x and 2 x MS vitamins on B5 medium, but was significantly different from 2 x MS vitamins on MS medium (8.4) (Table 7, Fig.14 and Plate 7). 1 x MS vitamins on MS medium (2.2) also achieved the highest number of shoots.

This was significantly higher than that obtained on 2 x MS on MS and 1 x MS on B5 media but not 2 x MS on B5 medium. These results agree with Quoirin *et al*., (2002) who used MS vitamins to improve the multiplication rate, to reduce leaf necrosis and to raise the elongation rate of black wattle tree. The highest number of nodes was achieved when 1 x MS vitamins were used on MS medium (4.5),

Table 7: Morphogenesis of nodal explants of Arak on MS and B5 media supplemented with different concentrations of MS vitamins after 10 weeks.

Parameters	No. of leaves	Shoot length in mm	No. of shoots	No. of nodes	Callus %

Treatment					
MS medium 1 x MS vit.	9.1 a	19.7 a	2.2 a	4.5 a	14
MS medium 2 x MS vit.	4.8 b	8.4 b	1.2 b	2.1 b	14
B5 medium 1 x MS vit.	4.1 b	13.1 ab	1.1 b	2.4 b	0.0
B5 medium 2 x MS vit.	6.0 ab	17.0 a	1.8 ab	3.2 ab	0.0

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

which was not significantly different from 2 x MS vitamins on B5 (3.2) but was significantly differences from 2 x MS vitamins on MS and 1 x MS vitamins on B5. Callus formation was observed only when MS medium was used (14%).

Many researchers used MS vitamins for micropropagation of many species. Baolin *et al*. (1987) investigated the importance of MS vitamins on rooting of *Euphorbia fulgens* and found that, it was important for rooting. Sandra and Morehart, (1988) used MS vitamins with the modification of 6.7 ***μ***M thiamine HCl for successful propagation of Osage-orange tree. Raymond and Pool, (1988) used 3 μM thiamine, HCl, 55.5 μM Myo-inositol, 8 μM nicotinic acid and 5 μM pyridoxine. Hcl for rooting of *vitis*.

4.4.2. Effect of Nitsch and Nitsch (NN) vitamins:

Nitsch and Nitsch vitamins in different concentrations (1 X and 2 X) were tested on MS and B5 media. The results on Table (8) show that, the maximum number of leaves was achieved by 1 x NN using B5 medium (1.3) which was not significantly different from 1 x NN on MS medium (1.1) but was significantly different from 2 x NN (0.0) on MS medium and 2 x NN on B5 medium (0.0) (Fig. 15). The shoot length responded differently to the addition of different NN vitamins. The maximum length was achieved by 1 x NN using MS medium

Table 8: Morphogenesis of nodal explants of Arak on MS and B5 media supplemented with different concentrations of NN vitamins after 10 weeks.

Parameters	No. of leaves	Shoot length	No. of shoot	No. of nodes	Callus %

Treatment		in mm			
MS medium NN. 1 x	1.1 a	4.5 a	0.6 a	0.6 a	-
MS medium NN. 2 x	0.0 b	0.06 b	0.0 b	0.0 b	-
B5 medium NN. 1 x	1.3 a	3.5 a	0.5 a	0.8 a	-
B5 medium NN. 2 x	0.0 b	0.0 b	0.0 b	0.0 b	-

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

(4.5 mm). This was not significantly different from that obtained when explants were cultured on B5 containing 1 x NN, but was significantly different from the other two treatments. No growth was observed on 2 x NN on MS and B5 media (Table 8 and Fig.16). There was no

significant difference in number of shoots between 1 x NN on MS medium (0.6) and 1 x NN on B5 medium (0.5) but there was a significant difference from 2 x NN using MS and B5 media (0.0). The maximum number of nodes was obtained by 1 x NN on B5 medium (0.8) which was not significantly different from 1 x NN on MS medium (0.6) but was significantly different from 2 x NN on MS and B5 media (Table 8). The results obtained show that, the addition of NN vitamins in the higher concentrations tended to inhibit the growth of the explants on MS and B5 media, and better growth occurred in the lower concentrations using MS and B5 media. Roberto and Gribando (1987) added NN vitamins for rooting of *Ficus lyrata* at concentration of 5 μM. Hussein (2002) used NN vitamins for *Ziziphus spina christi* and found that 0.5 x NN vitamin was optimal for shoot induction from nodal explant using 2 x MS medium.

4.5. Effect of growth regulators:

4.5.1. Auxins:

4.5.1.1. Effect of indole butyric acid (IBA):

The effect of different concentrations of indole butyric acid (IBA) on some growth parameters was studied using MS medium. Table (9) show that, the maximum number of leaves was achieved on auxin free medium or when the lowest concentration of 0.1 mg/l was

used, though no significant differences were recorded between all treatments.

Shoot length responded differently to the different IBA concentrations. The highest shoot length was obtained when the medium was devoid of IBA (16.5 mm). This was not significantly different from 0.1 mg/l (14.8 mm) but these two values were significantly different compared to the higher concentration of 0.5, 1.0 and 2 mg/l (Table 9). These results illustrate that, the levels of endogenous auxins of the explants might have been sufficient for shoot induction and elongation and that addition of exogenous auxin to the medium could have been inhibitory. Such results were obtained by Bonga and Von Aderkas, (1988) in culture of *Larix decidua* and Bornman, (1983) in culture of *Picea abies* who found that addition of auxin to such explants inhibited the growth.

Table 9: Effect of different concentrations of IBA on morphogenesis of nodal explants on MS medium after 10 weeks.

Parameters / IBA Mg/l	No. of leaves	Shoot length in mm	No. of shoots

0.0	4.3 a	16.5 a	1.1 a
0.1	4.3 a	14.8 a	1.3 a
0.5	3.5 a	9.5 b	1.1 a
1.0	2.8 a a	6.3 c	0.8 a
2.0	2.3 a	7.8 c	0.8 a

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

Number of shoots per explant showed no significant differences between all treatments, though highest number was achieved when 0.1 mg/l was used. Addition of IBA had no effect on number of shoots per explants and this result is in agreement with Rumary and Thorpe (1984) in epicotyl explant of *Picea glauca* who found that addition of

auxin to such explant inhibited the growth. Flinn *et al*. (1986) in culture of *Pinus strobus* reported the same result.

4.5.1.2. Effect of Naphthaleneactic acid (NAA):

Table (10) and Fig. (19) illustrate that, different concentrations of NAA had different effects on explant growth. The highest NAA concentrations (1.0 and 2.0 mg/l) resulted in low number of leaves (2.5 and 3.5 respectively). The highest value was (6.8) obtained on auxin free medium and was significantly different from 1.0 and 2.0 mg/l.

Shoot length responded differently. Significantly highest shoot length was achieved at the medium free of NAA. On the other hand, treatments with concentration of 0.5, 1.0 and 2.0 mg/l resulted in explants producing significantly shorter shoots compared to control and 0.1 mg/l (Table 10 and Fig. 20). Similarly NAA tended to reduce the number of shoots though not significantly. These results are in

Table 10: Effect of different concentrations of NAA on morphogenesis of nodal explants on MS medium after 10 weeks.

Parameters / NAA Mg/l	No. of leaves	Shoot length in mm	No. of shoots

0.0	6.8 a	18.1 a	2.0 a
0.1	5.3 ab	14.6 b	1.8 a
0.5	4.0 ab	9.8 c	1.8 a
1.0	2.5 b	7.5 c	1.8 a
2.0	3.5 b	11.0 c	1.6 a

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

agreement with Skoog (1944) who showed that auxin could inhibit shoot formation. Fadoul (1996) in *Musa spp*. failed to induce shoots by using NAA auxin. Raymond and Pool, (1988) showed the influence of NAA on *vitis* where increased rooting occurred by increasing NAA concentration. Hendrik and Stadenal (1988) in *Ixia flexuosa* also found that NAA increased rooting %.

4.5.2. Cytokinins:

4.5.2.1. Effect of Benzyladenine (BA):

Table (11) shows the effect of different concentrations, (0.0, 0.1, 0.5, 1.0 and 2.0 mg/l) of BA using MS medium on some growth parameters. The highest number of leaves was obtained when the highest concentration of BA 2.0 mg/l was used. This was significantly higher than the number of leaves produced when lower concentrations were used (Fig. 21). However, the highest shoot length was obtained on 2.0 and 0.5 mg/l (19.2 and 18.8 mm respectively) with no significant difference between them. These were significantly higher than that obtained by 1.0, 0.1, and 0.0 mg/l. This generally indicates that lower concentrations of BA induced a significantly shorter shootlets (14 mm and 12.5 mm for concentration of 0.0 and 0.1 mg/l respectively) (Table 11; Fig. 22). Idris (1994) reported the optimum

Table 11: Effect of BA on morphogenesis of nodal explants of Arak on MS medium after 10 weeks.

Parameters / BA Mg/l	No. of leaves	Shoot length in mm	No. of shoots
0.0	5.0 c	14.0 c	1.14 c

0.1	5.28 c	12.5 c	1.5 c
0.5	7.8 b	18.8 a	3.0 a
1.0	7.2 b	16.1 b	2.0 b
2.0	9.1 a	19.2 a	2.2 b

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

level for bud induction and elongation of guava to be 0.5 and 1.0 mg/l. The highest number of shoots was obtained at 0.5 mg/l. This result was significantly higher than that obtained from all other concentrations tested. The numbers of shoots were 2.2, 2.0, 1.5 and 1.14 for treatments of 2.0, 1.0, 0.1 and 0.0 mg/l respectively (Table 11).

These results indicate that, both number of leaves and plant height increased by increasing growth regulator concentration. This is in agreement with Youn and Ohaba, (1990) in culture of *Tilia amurensis* who successfully used BA. Stapfer and Heuser, (1986) used BA for rapid and maximum multiplication of *Heuchera sanquinea*. Lisa and Fred, (1986) also used BA to produce high number of shoots from *Querus shumardii*. Hamza and Belo, (1999)

used BA for multiplication of *Origgnum valgare* and obtained high rate at 2.0 mg/l.

4.5.2.2. Effect of kinetin (k):

Table (12) illustrates the effect of k in different concentrations of 0.0, 0.1, 0.5, 1.0 and 2.0 mg/l on MS medium. From this table it is rather clear that the number of leaves were not affected significantly with the use of k on MS medium (Fig.23). However, the shoot length were significantly reduced with addition of K at all concentrations

Table 12: Morphogenesis of nodal explants of Arak supplemented with kinetin on MS medium after 10 weeks.

Parameter / K. Mg/l	No. of leaves	Shoot length in mm	No. of shoots
0.0	6.14 a	17.0 a	1.2 a
0.1	4.2 a	10.7 b	1.0 a
0.5	5.0 a	13.4 b	1.4 a
1.0	4.1 a	11.7 b	1.4 a

2.0	4.5 a	12.4 b	1.4 a

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

tested (Fig. 24). Similar to the response of number of leaves, shoots were unaffected significantly with addition of k to the medium. This result agrees with Hamza and Bilo (1999) who showed that kinetin had low effect on bud induction of *Lavandula angustifolia*. Zaheer, *et al*., (1990) found that only some shoot formation occurred when they propagated *Melia azadarach* with medium supplemented with K.

4.6. Combined effect of IBA and BA on *in vitro* morphogenesis of nodal explants of Arak:

Table (13a) shows that the effect of IBA in combination with BA on number of leaves was clear, and that 0.5 mg/l IBA with 0.5 mg/l BA gave the highest value (8.2) which was not significantly different from combinations of IBA/BA at 0.0 – 0.0, 2.0 – 2.0 and 1.0 – 2.0 mg/l (6.8, 6.4 and 6.2 respectively) but significantly different from all other combinations.

The highest shoot length was obtained when no growth regulators was used (17.4 mm) and this was not significantly different from IBA/BA combination of 0.5 – 0.5, 2.0 – 2.0 and 1.0 – 2.0 mg/l (16.8, 16.0 and 15.0 mm respectively). The shortest shootlets were obtained when IBA was used separately at 1.0 mg/l (Table 14b).

Many researchers used combinations of auxins and cytokinins for propagation of trees. Jones *et al*. (1990) used node and internode

Table 13a: Effect of factorial combinations of IBA and BA on number of leaves regenerated from Arak on MS medium after 10 weeks.

BA mg/l	IBA mg/l			
	0.0	0.5	1.0	2.0
0.0	6.8	3.7	3.1	3.00

0.5	4.4	8.2	5.0	3.7
1.0	3.8	4.4	4.1	4.1
2.0	4.7	4.5	6.2	6.4

Table 13b: Effect of factorial combinations of IBA and BA on shoot length regenerated from Arak on MS medium after 10 weeks.

BA mg/l	IBA mg/l			
	0.0	0.5	1.0	2.0
0.0	17.4	10.	8.8	10.2

0.5	11.8	16.8	14.2	10.2
1.0	11.5	11.8	12.8	11.7
2.0	12.1	11.4	15.0	16.0

Table 13c: Effect of factorial combinations of IBA and BA on number of nodes regenerated from Arak on MS medium after 10 weeks.

BA mg/l	IBA mg/l			
	0.0	0.5	1.0	2.0
0.0	4.1	2.1	1.8	2.2
0.5	3.1	4.5	3.4	2.4

1.0	3.0	3.1	3.0	3.1
2.0	3.0	3.5	3.8	4.1

explants of *Acacia solicina* and *Acacia saligna* on MS medium with IBA and BA and obtained callus and shoot growth. David (1987) indicated that the most effective range of BA is 2 – 4 UM and NAA 0.5 – 1.0 μM to promote axillary shoot proliferation from coppice and adult *Eucalyptus sideroxylon*.

No significant effect on number of shoots occurred when different factorial combinations of IBA/BA were tested. Number of shoots induced on all BA concentrations in factorial combination with IBA. The number of nodes varied significantly on the different concentrations of IBA in factorial combinations with BA. Similar

trend was found in number of nodes per explants (Table 13c). The best number of nodes was induced on MS without growth regulators.

Yadav *et al*. (1990) obtained a multiple shoots from *Syzygium cuminii* L. when they combined BA/IBA at 0.23 and 8.9 μM respectively. Paek *et al*. (1987) obtained high number of shoots from Chinese cabbage using BA 44.4 μM and IBA 1.5 μM. Omura *et al*. (1987) indicated that optimum combinations of BA and NAA for a pomegranats ornamental variety for adventitious shoot formation, was BA 5 μM and NAA 0.5 μM and for fruit caltivar was BA 1.0 μM and NAA 0.5 μM.

4.7. Effect of silver nitrate ($AgNo_3$) on morphogenesis of nodal explants:

Table (14) illustrates the effect of different $AgNo_3$ concentrations (0.0, 5.0, 10.0, 15.0 and 20.0 mg/l) on number of leaves showing no significant differences between 0.0, 5.0 and 10 mg/l, but each of these was significantly higher than 15.0 and 20.0 mg/l (Fig. 27). Fadoul (1996) obtained similar results in culture of banana indicating that, addition of 5.0 mg/l $AgNo_3$ increased the number of leaves and plant height of banana. The highest shoot length was obtained by using 10 mg/l (24.0 μM) which was significantly

different from all other concentration. However, 15.0 and 20.0 mg/l produced significantly shortest shoots (Table 14 and Fig. 25). There were no significant differences on number of shoots recorded when $AgNo_3$ was used (Table 14).

The significantly highest number of nodes was obtained when 10 mg/l were used. Significantly low number of nodes was obtained on sliver nitrate at 15.0 and 20.0 mg/l. It is clear that the best response of explants to $AgNo_3$ was achieved by using 10.0 mg/l in terms of shoot length and number of nodes. This result agrees with Mekey (1999) in culture of strawberry reporting the highest plant when 10.0 mg/l $AgNo_3$ was applied. Ibrahim (2000) in culture of *Acacia senegal*

Table 14: Effect of $AgNo_3$ on morphogenesis of nodal explants on MS medium after 10 weeks.

$AgNo_3$ Mg/l	Morphogenesis			
	No. of leaves	Shoot length in mm	No. of shoots	No. of nodes
0.0	10.2 a	20.5 b	3.1 a	5.2 bc
5.0	10.0 a	20.1 b	2.8 a	5.8 b

10.0	11.2 a	24.0 a	2.8 a	7.4 a
15.0	5.2 b	15.2 c	2.4 a	4.2 c
20.0	4.2 b	14.4 c	2.0 a	3.8 c

Means with the same letter(s) are not significantly different at P= 0.05 according to DMRT

found that addition of $AgNo_3$ 5.0 mg or more reduced plant high bud increased number of leaves. $AgNo_3$ is often added to inhibit ethylene formation and some cultures fail to form shoots with media supplied with auxin/cylokinin combination unless $AgNo_3$ is added to the medium (Roy and Mangat, 1989). Purnhauser *et al*. (1987) reported the same effect of $AgNo_3$ on growth of *Triticum aestiuum* and *Necotina*.

4.8. Rooting:

Five experiments were carried out for root induction and elongation of Arak shootlets, using MS medium with different strength and different auxins IBA, 2,4-D and NAA in different concentration. No roots were observed in 4 experiments using MS medium with different concentration of IBA and 2,4-D. also no roots were observed when shoot tip explants were used using IBA on MS in full strength.

When NAA was used rooting was achieved on 2.0 mg/l using 0.50 MS strength and only at a rate of 40% after 10 weeks of incubations with average root length of 15 mm (Plate 8).

This study shows that Arak shootlet, are somewhat difficult to root *in vitro*. This agrees with Elhadeeb (1995) who found that Arak shootlet were very difficult to root. Mohamed El Kheir (1995) fail to induce roots from *Salvadora persica* (Arak) macro cutting.

Generally, rooting is more difficult when explants from mature trees were used (Bonga and Von Aderka, 1992). Vietiez *et al*. (1987) reported failure of old castanea tree to form roots *in vitro*. Haissing, (1989) reported lack of rooting in stem cuttings of old conifers. The failure of mature tree explants to form roots may be due to rooting inhibitors that are not present in juvenile explants, or due to change in carbohydrate metabolism in old trees. Plant growth regulators

especially auxins have an important role in the *in vitro* rooting of explants. IBA and NAA are more effective than IAA (Gasper and Coumans 1987). This study is not in agreement with Vieilez and Vieilez (1983) in culture of *Castanea sativa* who found that IBA was more effective than NAA.

CHAPTER FIVE

CONCLUSION

From the previous discussed results we conclude:

1- MS and B5 media were better than White medium using single node explants for Arak propagation.

2- MS at double strength was better than other MS strengths for Arak propagation.

3- Low concentration (2%) of sucrose was better than high concentrations, and recommended as good source for carbohydrate than glucose.

4- MS vitamins at single dose were the best vitamin formation mixtures for nodal explants.

5- Addition of BA is recommended for the proliferation of shoots from nodal explants where K has no or little effect.

6- 0.5 mg/l IBA and 0.5 mg/l BA combination using MS medium have a significant increase in explant proliferation.

7- $AgNo_3$ at 10 mg/l increased number of nodes compared to other concentrations.

8- Rooting of Arak explants was only possible (40%) on 0.5 MS supplemented with 2 mg/l NAA.