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Differentiation between male and female of jojoba plant *in vivo* and *in vitro* using PCR techniques

A thesis
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وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم

التمييز بين الذكور والاناث لنبات الهوهوبا خارج وداخل الجسم الحي باستخدام تقنية ال PCR

رسالة

مقدمة الى كلية العلوم في جامعة النهرين

و هي جزء من متطلبات نيل درجة الماجستير علوم

في علوم التقانة الاحيائية

من قبل

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Conclusions

-The proper plant growth regulators combinations of BA 0.5mg/l, 2,4-D 2.5mg/l used for callus induction from jojoba male and female leaves .

-CTAB method is suitable for DNA isolation from jojoba plant.

-Eleven primers were used to give variation between DNA in jojoba sexes. Six primers showed no amplification due to absence of suitable priming site for these primers in the genome of jojoba lines tested. Five primers of RAPD results produce amplified products, using these primers a represantable degree of DNA variation between jojoba sexes were detected .

- RAPD marker technology can be used to the genetic analysis of many plant species including jojoba.

Recommendations

-Using different plant growth regulator may give better response in callus production or even regenerate a new plant.

-In future RAPD experiments for jojoba plants can be distinguish using either these primers used in this experiment or other primers.

-Repeating the primers used in this experiment with control of differing experimental condition like DNA concentration $MgCl_2$ concentration ,*Taq* polymerase concentration ,and cycles number .

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بِسم اللهِ الرَحمنْ الرَحيم

إن الـذينَ امَنُـوا وَعَمِلُـوا الصَـالِحاتِ كَانَـتْ لَهُـمْ جَنَـاتُ الفِـ ـردوسِ نُزُلا ۝ خَالِدينَ فِيها لا يَبْغَونَ عَنها حِولا ۝ قُل لَو كانَ البَحرُ مِداداً لِكلماتِ رَبي لَنِفَدَ البَحْرُ قَبْلَ أنْ تَنْفدَ كَلِمَاتُ رَبْي ولو جِئْنا بِمِثْلِه مَدَدا ۝ قُل إنما اَنا بَشر مِثلَكم يُوحى إلي إنما الهَكُم الهٌ واحدٌ فمَنْ كَانَ يرْجوا لقاءَ رَبهِ فليَعمل عَملاً صالحاً ولا يُشرك بعِبادةَ ربه أحداً ۝

صَدقَ اللهُ العَظيمْ

سورة الكهف
الاية ١٠٧-١١٠

List of Abbreviations

Abbreviations	Meaning
RAPD	**Random amplified polymorphic DNA**
PCR	**Polymerase Chain Reaction**
MS	**Murashige and Skoog**
BA	**Benzyl Adenine**
2,4-D	**2,4-dichlorophenoxy acetic acid**
TE	**Tris –Ethylene Diamine Titra acetic acid**
G,C	**Guanine ,Cytosine**
ssDNA	**Single strand DNA**
2ME	**2-mercaptoethanol**
P.G.R.	**Plant Growth Regulator**
OD	**Optical Density**
EDTA	**Ethylene diamine tetra acetic acid**
TBE	**Tris ,EDTA, Boric acid**
MS	**Murashige and Skoog**
S.D.W	**Sterile distilled water**

الخلاصة

إن الهدف من هذه الدراسة هو للتمييز بين ذكر وانثى نبات الهوهوبا *Simmondsia chinensis* بواسطه الكشف عن التغاير بالدنا باستخدام مؤشرات تضاعف سلسله الدنا العشوائي متعدد الاشكال ال(RAPD) لغرض تشخيص الفروق.

-تم انتاج الكالس من اوراق الذكر والانثى لنبات الهوهوبا باستخدام تقنية زراعه الانسجه النباتية.

-كذلك تم عزل الدنا لنبات الهوهوبا باستخدام طريقة الCTAB حيث يتراوح تركيز الدنا من (١٠٠-١٥٠) مايكروغرام لكل من ذكر النبات وانثى النبات والكالس المنتج من ذكر والانثى المنتجة من ذكروبمعدل نقاوة يتراوح من (١,٠١-١,١٩) .

في هذه الدراسة استخدمت احد عشربادئاً(primers) لتقييم فائدتها في الكشف عن التغايرات الوراثية لذكر وانثى الهوهوبا ،وتضمنت التجارب في بداية الامر الى تحديد الظروف المثلى لتفاعلات ال(RAPD) كتركيز القالب الدنا وانزيم البلمرة والبادئات.

وكانت النتائج كالاتي:

تم انتاج الكالس من زراعة اوراق الهوهوبا على وسط زرعي MS ،باستخدام منظمات النمو (٠,٥ BA ملغم و٢,٥ ملغمD-2,4).

-ستة بوادئ(primers) لم تظهر أي تضخيم للدنا في كل من ذكر وانثى الهوهوبا .

-خمسة بوادئ (primers) والتي هي (E_7,A_1, A_{10}, D_{20}, C_5) اظهرت تضخيم ،وظهرت على انها وسيله للتمييز بين الذكر والانثى ،سجلت حزم الدنا المضخمة باستخدام هذه البادئات من خلال وجودها او غيابها او الاختلاف في الوزن الجزيئي .

-هذه النتائج بوضوح امكانية استخدام مؤشرات ال (RAPD) للتمييز بين ذكر وانثى الهوهوبا.

Summary

The objective of this study is to distinguish between male and female of *Simmondsia chinensis* by a mean of the detection of DNA variation using Random Amplified Polymorphic DNA (RAPD) markers in order to identify differences.

- Callus tissue was produced from both male and female jojoba leaves using plant tissue culture technique .

-Genomic DNA of jojoba plant was extracted using CTAB method.
The concentration of DNA ranging between (100-150) µg and0.7g weight of male fresh plant, female fresh plant, male callus, and female callus with purity ranging from (1.01-1.19).

-In this study, eleven different random primers were evaluated for their usefulness in detecting DNA variation between male and female of jojoba. This involved first optimization of RAPD reaction condition including, DNA template ,*Taq* polymerase ,and primers .

The results obtained were as follows:
-A callus produced from *Simmondsia chinensis* cultured on MS medium, using hormone combination of (0.5 mg/l **BA**, 2.5 mg/l **2,4-D**).

-Six primers showed no amplification products between jojoba male and female.

-Five primers (A_1, A_{10}, C_5,D_{20},E_7) showed amplification and represent a mean of distinguishing between male and female, using these primers wre

scored by the presence or absence of DNA bands and the differences in their molecular weight.

-The results clearly demonstrated the feasibility of using RAPD-PCR in distinguishing between jojoba male and female.

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الإهداء

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إلى من ارتقى مراتب الذرى ومرافئ الشمس.....

منارات أضاءا في ليل طويل ....أبي وأمي

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جميل العبيدي

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Great thanks to Allah, mercy and prays upon his prophet Mohamed the messenger of Allah.

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To my family members who supported me during my study.

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

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Scientific Degree:

Date:

(Chairman)

Signature:

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Date:

(Member)

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1.Introduction

Jojoba (*Simmondsia chinensis*) is considered as one of the mot important shrubs world wide. There are many products that have been isolated ,manufactured ,and used in many industrial applications .This plant was introduced by Francisco J.clavijero in 1789 (Hartwell,1971).

The plant has two major products jojoba oil and jojoba protein (simmondsin).

Jojoba oil considered as an important source of wax, which has a wide range of industrial uses in cosmetics. It is used mainly in the formulation and preparation of skin care products such as lotions, moisturizers, massage oil and soothing creams.

Simmondsin ,has been reported as epitate suppression natural drug. it form about 30% of the total seed, therefore it will be very good for investments (Benzioni,1995).

Generally, there are two sex types in Jojoba male and female. However, it can not distinguished at seedlings and vegetative stage of its growth.
Selection of the appropriate sex of the progeny for commercial planting would be useful, since only the female plants are grown for fruit.
The identification of sex type before cultivation would result in higher fruit production and increase profitability.
Jojoba flowering stage start after three years .The early diagnosis of the female will be of great importance for the economic side. While the females will form all of the product population, this will lead to more product percentage(Benzioni,1995).

The aim of this study

1.Establishing callus from male and female tissues, and see if the differences persist in callus or not?

3.Distinguishing between males and females by detection of DNA variation using RAPD-PCR technology.

3.Finding simple profile to distinguish between males and females of *Simmondsia chinensis* in vegetative state.

2. Literature Review

2.1 Jojoba (*Simmondsia chinensis*):-

Its pronounced (ho-HO-ba) which is an English name , its also called wild hazel, , quinine nut, deer nut, pignut, goat nut, coffee berry, gray box bush (a spanish name) (Bianchini and corbetta ,1976; Armstrong, 1998).

2.2History:-

Jojoba (*Simmodsia chinensis* (Link) Schneider) is a perennial woody shrub native to the semiarid regions of southern Arizona, southern California and northwestern Mexico. Jojoba is being cultivated to provide a renewable source of unique high-quality oil.

The shrub is first mentioned in the literature by the Mexican historian Francisco J. Clavijero in 1789, who noted that the Indians of Baja California highly prized the fruit for food and the oil as a medicine for cancer and kidney disorders. Indians in Mexico use the oil as a hair restorer (Hartwell, 1971).

Native Americans extracted the oil from jojoba seeds to treat sores and wounds centuries ago. Collection and processing of seed from naturally occurring stands in the early 1970s marked the beginning of jojoba domestication. In addition, the ban on the importation of sperm whale products in 1971 led to the discovery that jojoba oil is in many regards superior to sperm oil for applications in the cosmetics and other industries.

Today, 40,000 acres of jojoba are under cultivation in the southwestern U.S. Much of the interest in jojoba worldwide is the result of the plant's ability to survive in a harsh desert environment.

The utilization of marginal land that will not support more conventional agricultural crops could become a major asset to the global agricultural economy.

The oil of jojoba was reported to be emetic, jojoba is a folk remedy for colds, obesity, parturition, poison ivy, sores, sorethroat, warts,and wounds. Seri Indians applied jojoba to heal sores aching eyes, colds and to facilitate parturition (Barua *et al*, 1993) also for cooking, hair care, and for treatments of many medical problems such as kidney malfunction (Benzioni, 1995; Hartwell, 1971).

2.3 Botanical classification:-

(Table 1 jojoba classifications) cited from (Underschold, 1990**).**

Kingdom:	*Plantae*
Division:	*Magnoliophyta*
Class:	*Magnoliopsida*
Order:	*Caryophyllales*
Family:	*Simmondsiaceae*
Genus:	*Simmondsia*
Species:	*Chinensis*

2.4 physical properties :

Jojoba seed contains a light-gold colored liquid wax ester which is the primary storage lipid of the plant. This is unlike conventional oilseed crops, such as soybean, corn, olive, or peanut which produce oils as the primary storage lipid. Jojoba wax (called oil) makes up 50% of the seed's dry weight. The physical properties of jojoba oil are: high viscosity, high flash and fire point, high dielectric constant, high stability and low volatility. Its composition is little affected by

temperatures up to 570°F (300°C). Jojoba oil contains straight-chained C20 and C22 fatty acids and alcohols and two unsaturated bonds, which make the oil susceptible to many different types of chemical manipulations. The extracted oil is relatively pure, non-toxic, biodegradable, and resistant to rancidity (Brown,. and Dwyer, 1988).

Most jojoba oil produced in the U.S. today is sold at a high price for use in cosmetics and hair care products. As many as 300 products containing jojoba have appeared in the U.S. in recent years. As the supply of oil increases and price decreases, more uses will become economically feasible. For example, the viscosity index of jojoba oil is much higher than that of petrolium oil; therefore, it may be used as a high temperature, high pressure lubricant. The stability of jojoba oil makes it attractive to the electronic and computer industries. And since jojoba oil contains no cholesterol or triglycerides and is not broken down by normal metabolic pathways, it may become an important low-calorie oil for human consumption. The oil can be used as an antifoam agent in antibiotics production and as a treatment for skin disorders. Other proposed uses include candles, plasticizers, detergents, fire retardents, transformer oil, and for the leather industry. The meal contains up to 30% protein, but toxic compounds (simmondsins) make it currently hazardous as an animal feed (Brown, J. and Dwyer, K. 1988).

2.5 Environmental Requirements:-

Native jojoba populations can be found in areas receiving a rain fall about 500-600 mm and having temperatures ranging from 9°C to 50°C, When temperatures drop below 0 °C flowers and terminal portions of young branches of most jojoba plants are damaged (Underschold, 1990).

Jojoba is found in diverse climatic, geographic and edaphically conditions, at altitudes from sea level to about 1,200 m. During early seedling development, excessive cold may kill an entire plantation. Frost may not damage taller plants to the same degree, but it can reduce yield. Jojoba is very tolerant of high temperatures. Irrigation has produced more luxuriant vegetative growth, this increased growth results in higher seed yield. Jojoba requires the most water during late winter and early spring (Benzioni, 1995).

2.6 Plant morphological description:-

Jojoba is a woody green shrub ,wind-pollinated, reaching a height of 1-5 meters and having a long life span (100-200 years),jojoba leaves are leathery, thick, grayish green in color, and elliptical in shape (Kohorn, 1990).

The plant is drought resistant and to some extent also salt resistant **(**Mills *et al*., 2004). It is adapted to relatively dry climates with annual rain of 500-600 mm and temperatures ranging from (9-50̊) C.

Where annual rains are less than 350 mm, supplementary irrigation is required. In the arid areas of occupied Palestine, with less than 200 mm of annual rain, supplementary irrigation of about 300-500 mm is applied. Effluents and saline water may be used to irrigate Jojoba. (Yermanos, 1977; Newall, *et al* 1996).

These leaves are vertically oriented, an adaptation which lessens sun impact at summer high noon, while exposing each leaf to the less extreme temperatures of morning and evening, their occurrence reflecting the ability of the plant to conduct its leaf functions both in cold months and in dry ones ,the leaves are xerophytic which contain

high concentration of phenol. The length of the branch divided by the number of internodes and branching frequency, is 8 branches per plant in the main (Kohorn, 1990).

2.7 Morphological Differences between male and female:-

Female and male flowers occur on separate plants and the male pollens are pale-greenish in color, occurring in a dense cluster. Blossoming times for Jojoba are extremely variable (from December to July), female flowers are also tiny, and greenish yellow in color, eventually forming hard-shelled, acorn-like seeds up to 2.4 cm long, which initially are pale green The seeds eventually ripen to a strong brown color (Dimmit, 2000).

In some populations in xeric sites, males have smaller leaves and more compact canopies than females, female shrubs have larger leaves, more open canopies, and are, on average, smaller plants than males, But while sexes are dimorphic in terms of mean values of these morphological parameters, there is substantial overlap between sexes and considerable within population variation within each sex (Kohorn, 1990).

Sex ratios (52 males: 48 females) obtained from seed in horticultural propagation are consistent (Yermanos, 1977). There are no reports of plants changing sex, confirming that environment does not play a role in sex expression. Hermaphroditic plants are only rarely found (Buchmann , 1987). Sex chromosomes have not been identified, nor has their presence been ruled out (Lawson *et al*, 1994).

The female flowers are usually solitary, one per two nodes although flowers every node or in clusters are not rare. The male flowers are clustered. Flower buds form in the axiles of leaves on the new vegetative growth occurring during the warm season under favorable temperatures and water regime (Brawn, 1988).

Female plants also have a more open structure which allows more photosynthesis to take place. Together these two strategies permit the female plants to produce the high amounts of energy needed for fruit production. In overall energy expenditures male shrubs only reserve 10-15% of their tissue for reproductive use while female plants utilize 30-40%. Males and females of jojoba can differ in their vegetative structure as well as in floral morphology. Male flowers occur in axillary inflorescences containing from 3 to 20 flowers, while the larger female flowers generally occur singly, also in an axillary position. (Buchmann, 1987).

2.8 Seed Preparation and Germination:

Jojoba can be planted by direct seeding or by transplanting seedlings to the field. In the southwestern U.S., many growers prefer direct seeding because it is less expensive, faster and requires less hand labor. Seed can be germinated in vermiculite or sand at about 31 ° C. Emergence occurs in 15 to 20 days, and the seedlings are ready for transplanting when they are 15-30 cm tall (8 to 10 weeks).

Jojoba may be propagated by direct seeding, in which case about half of the seedlings are males which should be roughed as 10% of males are sufficient for pollination. The fact that only 50% of the plants bear seed and the large heterogeneity of the plants those originate from seed lead to low average yields. Hence, vegetative propagation that

enables the planting of desired proportion of female plants of superior clones is preferred. Rooted cuttings are used for this purpose (Benzioni, 1995).

2.9 Harvesting

All seeds on a jojoba shrub do not mature at the same time, and more than one harvest may be necessary. Most jojoba is currently harvested by hand. Over-the-row fruit and berry harvesting equipment is adaptable to jojoba harvesting.(undersander,1990).

cultivation, hedge-row, or orchard-like plantations, without undergrowth, seeds could be raked from under the bushes and then picked up by suction. Pruning the lower branches might be advantageous if this method be used. A device could be designed to pick the seeds from the bush prior to the time of falling. Cost of harvesting would depend on the method(Underschold, 1990).

2.10 Variety Selection and weed control:-

There are no improved varieties of jojoba. Some yield components that vary among wild jojoba stands include: seed size, oil content, number of flowers per node, early flowering, precocious seed production (starting before the fifth year), consistent high production from year to year, upright growth habit, and degree of frost tolerance. Work is underway to select for desired traits and plants suitable for mechanical harvest.

Weeds must be controlled early in the establishment of the plantation. Weed control prior to planting and/or cultivation between rows during growth is needed until the jojoba plant is large enough to shade competing plants (Underschold, 1990).

2.11 Diseases, insects and Their Control:-

On poorly drained soil, jojoba is susceptible to fungal wilts, including *Verticillium ssp, Fusarium ssp, Pithium ssp* and *Phytopthorsa ssp.*

More than 100 species of insects have been identified on jojoba, but few cause known economic damage. Infestations of spider mites, grasshoppers, and thrips may result in yield losses.

Fences may be necessary to eliminate browsing by wild animals who find the plant very palatable. This has been a major factor in the distribution of jojoba(Undersander ,1990).

2.12 Economics of productions:-

The value of *Simmondsia* oil as a hard wax was estimated at $ 55 per kg. Because of the present demand for the wax and oil. Jojoba is being considered as a non-competitive crop that could replace wheat and cotton in Texas and Southern California, with as much as the yield from 70,000 kg is being absorbed by industry. The Chemical Marketing Report (Dimmit, 2000). stated that jojoba prices doubled in 6 months to $200 / kg, the cost of establishing a plantation can vary from $3,000/ km^2 on land with irrigation available to $5,600/ km^2 on rough desert terrain, maintenance costs are low only $200/ km^2. One km^2 can yield 1,125–2,250 kg oil per km^2 .

(N.A.S., 1975; Yermanos, 1977) suggested that after 5 year the yield which is about 82.5 kg of nuts for km^2 will increase to 412.5 kg / km^2 in the 12th year. Such yields may be optimistic, even for well managed plantations. Estimates of the amount of wild nuts available each year range from one hundred million to one billion pounds, the

plants growing over 2 million km^2 in California, Mexico, and Arizona. Usually plants in cultivation yield oil in 6–7 years, the best specimens in Middle East yield 2 or more kg of seed in the fourth year, wild plants yield about 1 kg of nuts per year, and cultivars should yield twice that amount or more.

A number near 1650 kg was recorded for production of seed cultivated in 1 km^2 Individual plants may yield 5 kg (dry weight) seeds and more, of which 50% (43–56%) by weight is a colorless, odorless liquid wax commonly called "jojoba oil" (Buchanan and Duke, 1981).

2.13 Products and chemistry of jojoba:-

2.13.1. Jojoba products:-

2.13.1.1:- The oil:-

Jojoba oil is one of the finest cosmetic ingredients in the world. Its excellent inherent emolliency, moisturization and oxidative stability properties rank it as one of the top cosmetic lipid materials, natural or synthetic, in use today (N.A.S, 1975). Jojoba wax (called oil) makes up 50% of the seed's dry weight, Jojoba oil esters are designed to resist hydrolysis and oxidation for their intended use in non-occlusive moisture control and photo protection on external surfaces of skin, hair, eyes and plant leaves. Extensive use of jojoba as an ingredient in a wide diversity of products by millions of consumers over the past 15 years, as well as widespread use of 100% pure jojoba on skin and hair without adverse effects also verifies its safety

Jojoba oil is a complex mixtures of organic compounds that include carboxylic acids, long chain alcohols and to a much lesser extent, alkanes, its also a mixture of longed chained unbranched liquid wax

esters that result from the esterification of an Omega-9 (double bond located between the ninth and tenth carbon atom).

Jojoba oil is quickly absorbed into the skin. Absorption is apparently via the transappendegeal mechanism and occurs through the pores and hair follicles. Additionally, because jojoba is rapidly absorbed, the pores and hair follicles can remain open and thus maintain their proper functioning ability. From the pores and hair follicles, jojoba diffuses into the corneal layer of the skin probably via a pilosebaceous mechanism (Kadish, 1984).

Many of the most effective ingredients for skin care formulations are those with chemical composition and physical properties similar to the skin's own surface layers. Since jojoba is completely miscible with sebum, when it is applied to the skin, a very thin, non-greasy lipoid layer of jojoba and sebum forms. This partially porous layer provides exceptional transepidermal respiration and moisture control. Unlike greasy occlusive materials such as petrolatum, mineral oils and some lanolin products, jojoba provides an absolutely non-tacky and non-greasy, dry emolliency. At the same time jojoba significantly reduces transepidermal water loss without totally blocking transpiration of gases and water vapor. This function is enhanced by the kinking at jojoba's cis configuration that helps avoid tight packing of hydrocarbon chains. Jojobas oil serves as an excellent moisturizing agent with exceptional spread and lubricity, and leaves a rich velvety non-oily feel on the skin while retarding water loss and enhancing the flexibility and suppleness of the skin.

Jojoba seed contains a light-gold colored liquid wax ester which is the primary storage lipid of the plant (its own common name, "Jojoba

Oil") and it's unlike conventional oil seed crops, such as soybean, corn, olive, or peanut which produce oils as the primary storage lipid.

Only the jojoba tree produces commercial quantities of this functional liquid material in its seeds (while for example solid carnauba wax is from the leaves of palm trees),

Acid value is the most frequently determined property of jojoba oil. Total acid value of jojoba is typically in the range of 0.2-0.5 (mg of KOH to neutralize acid in 1 gram of sample) and it can be reduced to less than 0.2 with refining methods (Johnson, 1992). Jojoba's oil oxidative stability, thermal stability and lack of support for microbial growth can also increase a products safety and decrease its dependence on antioxidants, It has a comparatively low saponification value and contains little to no lecithin. The iodine value is a measure of unsaturation specific gravity that indicates the heavy feel of an oil (Jelinski, G. and H ,Cheliak,. 1992).

2.13.1.2 Protein of Jojoba (Simmondsin):-

The other important product found in jojoba is simmondisin, meal contains up to 30% protein, but toxic compounds (simmondisin) make it currently hazardous as an animal feed (Undersander., 1990).The jojoba meal contain a series of molecules considered to be toxic, with simmondsin [2 - (cyanomethylene) -3-hydroxy- '4,5-dimethoxycyclo-hexylb-D-glucoside] as the most important. Indeed, the extracted and purified simmondsin from jojoba meal caused a food intake reduction in adult rats. Taste is apparently not involved because the same response was seen with intragastric intubation as with oral administration. The action of simmondsin is observed within the first hour after oral administration and lasts for several hours.

Simmondsin treated with beta-glucosidase and taken into the gastrointestinal tract seems to be more active than simmondsin itself with respect to inhibition of food intake. (Miwa, 1984).

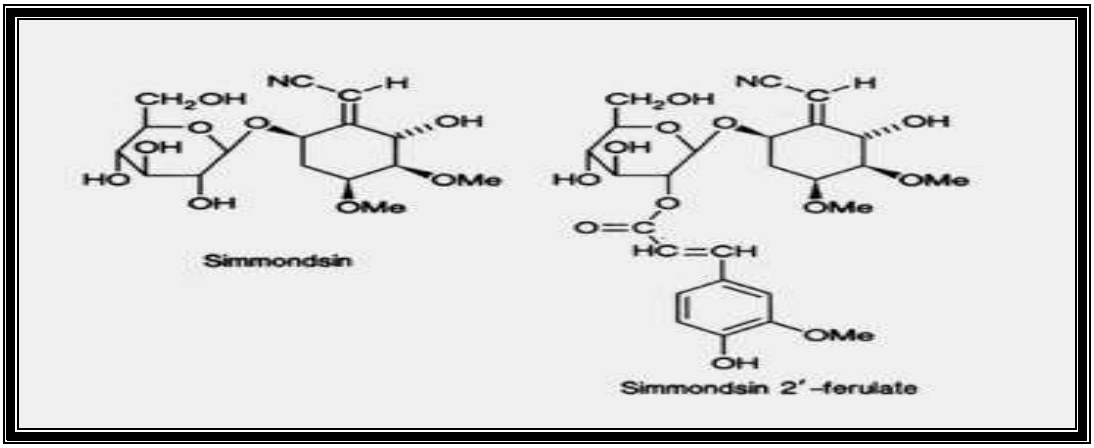


Figure (1) Simmondisin structures (Cokelaere, 1985).

2.13.2 Chemical composition of jojoba oil:-

The analysis showed that 100 g of jojoba seeds contain:

(1) 4.3–4.6 % H_2O.

(2) 14.9–15.1 %fat.

(3) 50.2–53.8 % protein.

(4) 24.6–29.1 % total carbohydrate.

(5) 3.5–4.2 % fiber.

(6) 1.4–1.6 % ash.

Three related cyanomethylenecyclohexyl glucosides have also been isolated from the seed meal. The acute oral LD_{50} for crude jojoba oil to male albino rats is higher than 21.5 ml/kg body weight. The amino

acid composition of deoiled jojoba seed meal is 1.05–1.11% lysine, 0.49% histidine, 1.6–1.8% arginine, 2.2–3.1% aspartic acid, 1.1–1.2% threonine, 1.0–1.1% serine, 2.4–2.8% glutamic acid, 1.0–1.1% proline, 1.4–1.5% glycine, 0.8–1.0% alanine, 1.1–1.2% valine, 0.2% methionine, 0.8–0.9% isoleucine, 1.5–1.6% leucine, 1.0% tyrosine, 0.9–1.1% phenyalanine, 0.5–0.8% cystine and cysteine, and 0.5–0.6% tryptophane . Per 100 g jojoba meal, there is 1.4 % lysine, 0.6 % histidine, 1.9 % arginine, 2.6 % aspartic acid, 1.3 % threonine, 1.3 % serine, 3.2 % glutamic acid, 1.5 % proline, 2.4 % glycine, 1.1 % alanine, 0.6 % cystine, 1.5 % valine, 0.1 % methionine, 0.9 % isoleucine, 1.8 % leucine, 1.1 % tyrosine, and 1.2 % phenylalanine (Pierik, 1987).

2.14 Plant tissue culture:-

The concept that the individual cells of an organism are totipotent is implicit in the statement of the cell theory.Schwann in 1839expressed the view that each living cell of multicellular organism should be capable of independent development if provided with the proper external conditions.A totipotent cell is one that is capable of development by regeneration into whole organism ,and this term was probably coined by Morgan in 1901(Pezzuto, 1996).the basic problem of cell culture was clearly stated by(Pierik, 1987). If all of the cells of a given organism are essentially identical and totipotent ,then the cellular differences observed within an organism must arise from responses of those cells to their microenvironment and to other cells within the organism .it should be possible to restore suppressed functions by isolating the cells from those influences responsible for their suppression .

The first plant tissue cultures, in the scenes of long term culture of callus ,involved explants of cambial tissue isolated from carrotand tobacco tumor tissue from the hybrid *Nicotiana glauca* X *Nicotiana Langsdorffii*.The latter tumor tissue requires no exogenous cell-division factor. Results from these laboratories published independently, appeared almost simultaneously. Fortunately, plant physiologists working in other areas had discovered some of the hormonal characteristics of indole-3-acetic acid, IAA, and the addition of this auxin to the culture medium was essential to the carrot cultures maintained by. According to(Pierik, 1987), the carrot cultures required knop's solution (Pierik, 1987)supplemented with salt mixture, glucose, gelatine, cysteine-HCL, and IAA.The goal at that time was to demonstrate the potentially un limited growth of a given culture, by repeated subcultures, with the formation of undifferentiated callus. The workers were fascinated by the apparent immorality of their cultures and devoted much effort to determining the nutritional requirements for sustained growth (Pezzuto, 1996).

2.15 Initiation and maintenance of callus:-

A callus consists of an amorphous mass of loosely arranged thin – walled parenchyma cells arising from the proliferating cells of the parent tissue. Frequently, as a result of wounding, a callus is formed at the cut end of a stem or root. The term callus may be confused with "callose,"an other botanical term. The latter refers to polysaccharide associated with sieve elements, and formed rapidly following injury to sieve elements and parenchyma cells (Barua, 1993). Although the major emphasis has been on an angiosperm tissues, callus formation

has been observed in gymnosperms,ferns,mosses,and liverwort (Yeoman and Macleod,1977).

Callus has no predictable organizational pattern, although localized centers of meristematic activity are present, and often rudimentary cambial region appears with zones of vascular differentiation.

(Pierik, 1987), had described some of the early observations on wound callus formation. The stimuli involved in the initiation of wound callus are the endogenous hormones auxin and cytokinin. Using tissue culture techniques, callus formation can be induced in numerous plant tissues and organs that do not usually developed callus in response to an injury.

The first successful prolonged cultures of experimentally induced callus were achieved almost simultaneously at the research laboratories of Gautheret in Paris, and White in Princeton in 1939. The term" tissue culture," as applied to such cultures, is a misnomer. A culture tissue does not maintain its unique characteristics as a plant tissue, but reverts to an organized callus. The most important characteristics of callus, from a functional view point, is that this abnormal growth has the potential to develop normal roots, shoots, and embryoids that can form plants (Pierik, 1987).

The nutritional requirements for the initiation of callus vary considerably for primary explants of different origin .Juice vesicles from lemon fruits and explants containing cambial cells exhibit callus growth without the addition of any exogenous growth regulator. The majority of excised tissues, however, require the addition of one or more growth factors to the medium in order to stimulate callus development (Yeoman and Macloed, 1977). After the callus has been

grown for a period of time in association with original tissue, it become necessary to subculture the callus to fresh medium. Growth on the same medium for extended period will lead to a depletion of essential nutrients and to a gradual desiccation of the agar because of water loss (Braunm, 2003).

2.16 Media selection:-

In 1934 White discovered that isolated tomato roots had the potential for unlimited growth, if they were provided with a liquid medium containing a mixture of inorganic salts, sucrose, thiamine, pyridoxine, nicotinic acid, and glycin.In addition to the somewhat selective biosynthetic activity of certain isolated tissues and organs, culture system may exhibit changes in their metabolic pathways over a period of time. Theses changes in metabolism require corresponding changes in nutritional requirments.The choice of particular medium depends on the species of plant, the tissue or organ to be cultured, and the purpose of experiment Several media formulation are commonly used for the majority of all cell and tissue culture work these media include (Murashige and Skoog ,Gamborg B-5,Nitsch and Nitsch,and Gautheret medium) (Anderson, W.C.1975).

A suitable starting point for the initiation of callus of a dicot tissue explants would be the preparation of the MS basal medium. One characteristic of this medium is its relatively high concentration of nitrate, potassium, and ammonium ions in comparison to other nutrient media, and also MS medium high in. macronutrient (Anderson, W.C.1975).

2.17 Molecular variation between dioeciously male and female plants:-

In contrast to most animals, many plant species generate both male and female organs on the same individual. Only a few plants are unisexual, that is “dioecious”. Male and female plants of dioecious species may differ in many leaves and whole plant traits, including life history, reproductive, allocation, phenology, and growth .

Some of these plants have heteromorphic sex chromosomes by which sex is genetically determined, In the well studied dioecious plant, *Silene latifolia*, the Y chromosome dominantly and positively induces male development by its presence in an XX/XY system similar to the mammalian pair of sex chromosomes. However, the primary structure and molecular function of the sex chromosomes in plants are largely unknown (Ohima, 2001).

Other plants have the same chromosomes number like the jojoba (the project plant) which had 13 chromosomes (Robin, 1954).

Gene expression variation mechanisms in plant could include many reasons, sex related expression is one of them in which the identification of certain ribosomal protein that is related with male and female was reported ,certain gene from *Mercurialis annua* by mRNA differential display was investigated in which sequence analysis of a cDNA clone of this gene showed that it encodes a protein that belongs to the eukaryotic ribosomal protein family, Northern blot analysis showed a stronger expression of this gene in female flowers as compared with male flowers. Analysis of the genomic DNA sequence showed the presence of one untranslated and three translated exons, the association of habitat (substrate type and light availability), plant size, and inflorescence production included also as gene expression

efficient ,the expression of sexually antagonistic alleles generates intersexual genetic conflict during ontogeny because they produce a phenotype that moves one sex toward its optimum, but when expressed in the other sex, moves the phenotype away from that sex's optimum (Rice and Chippindale, 2001).

The alleles expression dominance is a good example of this effect (London, *et al* 1993).

The different genes that may either be completely absence in one sex and presence in another or either suppressed totally or temporary will result in the differences in these gene products (London,*et al* 1993).

2.18 Polymerase Chain Reaction (PCR)

The (PCR) principles which were first described in 1985 by Kary Mullis, has revolutionized the way molecular biology is being carried out (Mohan ,1991).This process takes its name from DNA polymerase, the enzyme that carries out DNA replication in the cell. This process is a chain reaction because DNA polymerase is allowed to carry replication over and over again until there are million or more copies of target DNA.

PCR can be defined as in vitro method that uses DNA polymerase and primers to amplify specific DNA segment from complex mixture (Mader, 1993).The typical PCR reaction component are DNA template, primers, *Taq* DNA polymerase, deoxy nucleoside tri phosphate (dNTPs), and buffer. These components could all be assembled and therefore simple cycling of temperature within the reaction tube carries out the amplification reaction(Mader, 1993)\.

A few drops of mineral oil are often added to seal the reaction and prevent evaporation (Saiki, 1990).

The PCR amplification process generally consists of 30 to 40 repeated cycles of (Mohan, 1991) consisting these stages:-

(1) DNA denaturation: where the template DNA is first denatured by heating usually at 94 ° C. It is very important that the reaction reaches a temperature at which complete strand separation occurs. In sufficient heating during the denaturation step is a common cause of failure in a PCR reaction.

(2) Annealing of primers to ssDNA: In this stage the reaction mixture is cooled to a temperature usually at 36°C that allows the primers to anneal to their target sequences. The temperature at which annealing is done depends on the length and the guanine, cytosine content of the primers.

(3) Primer extension: The annealed primers are extended with *Taq* polymerase usually at 72°C .The addition of dNTPs will be at the *5'* end of the template DNA. The time of incubation at 72°C varies according to the length of target sequences being amplified. The cycles of denaturation, annealing, and DNA synthesis is repeated many times which result in the exponential accumulation of specific fragments whose termini are defined by the *5* 'ends of the primers and whose length are defined by the distance between the primers. Thus,

for example (20) cycle of PCR yield about a million folds amplification (Saiki, 1990)

The original protocol for PCR used the klenwo fragments of *E.coli* DNA *polymerase I* to extend the annealed primers. This enzyme was inactivated by high temperature required to denature DNA. Consequently, fresh enzyme had to be added during every cycle (Saiki *et al.*, l988b). This problem was solved with the introduction of thermo stable DNA polymerase *(Taq* DNA polymerase) that was first isolated from the thermophilic bacterium *Thermus aquaticus,* (Chien *et at.*, 1976). Recently the recombinant *Taq* DNA polymerase is produced from a genetically engineered *E. coil* where the desired gene is introduced in to this bacterium after recovering it from *Thermus aquaticus.* The *Taq* DNA polymerase unit can be defined as One unit of enzyme catalyses the incorporation of 10 n mol of total nucleotides in to acid — insoluble product in 30 minutes at 70°C using M 13 m p 18 (+) DNA as a template (Saiki *et al.*, 1988).

The recombinant *Taq* DNA polymerase has a number of advantages over other DNA polymerases mainly its stability at high temperature required for DNA denaturation step, i.e., it can withstand temperature up to 94°C for about 2 hours(Saiki,1990). However, the optimum temperature for this enzyme ranges between 70-75°C. Therefore, there is no need to add fresh enzyme to the reaction during each cycle. Further more, this enzyme is very processive, so that relatively long molecules may be produced (Saiki *et al.*, 1988).

There are number of factors that may affect enzyme activity in the PCR reaction such as: the *Taq* DNA polymerase is sensitive to the concentration of magnesium ions. 2_mM magnesium chloride maximally stimulates *Taq* polymerase activity at 0.7-0.8 mM total dNTPs. However, higher concentrations of Mg^{2} may have inhibitory effect. Another factor that may affect enzyme activity is the dNTPs concentration. Since dNTPs can bind Mg^{2}, therefore, low, balanced concentrations of dNTPs have been observed to give satisfactory yield of PCR product. It's likely that insufficient dNTPs concentration may adversely affect the processivity of *Taq* DNA polymerase. The *Taq* DNA polymerase is also influenced by the concentration of KC1. The modest concentration of KC1 stimulates the synthesis rate of the *Taq* DNA polymerase with an apparent optimum at 50mM. However, higher KC1 concentration begins to inhibit enzyme activity. (Saiki, 1990).

The primers used for PCR determine the success or failure of an amplification reaction more than any other components (Saiki, 1990). PCR has already found wide applications in the characterization of the genetic structure of many organisms. (Mohan, 1991).In human, this technique extensively Used to reveal the genetic disorders such as cystic fibrosis (Ballabio *et al*.,1990) and sickle cell anemia (saiki *et al*, 1988). It's also used to serve forensic medicine to exclude a person suspected of committing a crime using DNA extracted from a few white blood cells (Roewer *et al* 1991), hair (Higuchi *et al.*, 1988) or a small amount of sperms (Li *et al*., l988).Routine clinical microbiological diagnosis is also performed using PCR, where it is used to identify many pathogenic microorganisms of medical importance for example, HIV (Ou *et al.*, 1988*)* and *Entamoeba histoliytica* (Mirelman *et al.*, 1991*).* Recently the data from PCR

amplification of mitochondrial DNA sequences from different human populations were used to construct a human phylogenetic tree (Mader, 1993). PCR techniques have been used in the genetic analysis of many fungi species, e.g. 19 isolates of *Aspergillus fumigatus* were fingerprinted by (London *et* al., 1993) using these techniques. In animals, PCR used to the estimation of genetic diversity of Swiss goats (Saitbekova *et al.*, 1999) and the genome mapping of bovine (Vaiman *et al*, 1997).

In plants, PCR techniques have a wide range of applications. They have been used for variety identifications of plant species including wheat (Dovidio *et al.*, 1992), arid also cultivated potato.(Provan *et* aL,1996). PCR molecular techniques have been used in breeding programs such as the plant height and growth habitat in barely (Barua *et al,* 1993). They are also used to select disease- resistant plants e.g. Root cyst nematode resistant of potato (Niewohner *et al***,** 1995) and leaf mold resistant of tomato (Thomas *et al.*, 1995). Genetic maps for many plant species were established using PCR techniques, for example, barely (Tragoon Rung *et al***.,** 1992) and rice (Kurata *et al***.,** 1994). PCR facilitate the estimation of genetic diversity for many plant species, where, it's used to the estimation of genetic diversity of banana *(Musa)* species(Niewohner *et al***,** 1995).

PCR techniques have found many applications to improve both cultivation and productivity of chickpea germplasm. They have been used for DNA fingerprinting of chickpea *(Cicer arietinum*.) accession by (Sharma *et al*, 1995).Chickpea is affected by numerous disease. *Ascochyta* blight and fusarium wilt is the most devastated disease to chickpea. Therefore, the use of resistant cultivars is considered the most economical mean of

controlling the disease. Mayer *et al., (1997)* have been used PCR to genome analysis of *fusarium* wilt resistant germplasm lines. Genome mapping of chickpea *(Cicer arietinumL.)* was performed using PCR techniques. (Winter *et al.,* 1995).

The development of Molecular Biology has led to an increase in the range of molecular marker techniques available that are based on PCR such as RAPD, Sequence -Tagged Site (STS), Arbitrary Primed -PCR (AP-PCR), and DNA Amplification Fingerprinting (DAF) (Winter *et al.,* 1995).

2.19 Random Amplified Polymorphic DNA (RAPD):-

RAPD is a PCR-based molecular marker technique that was developed by (Williams *et al.,* 1990). This technique is based upon the fact that a short oligonucleotide or randomly chosen sequence (primer) of usually 10-bases, when mixed with genomic DNA (template) and a thremostable DNA polymerase *(Taq* polymerase) in the presence of all required materials and subjected to the temperature cycling conditions of PCR will allow the amplification of several DNA fragments (Innis *et al,* 1990).

During a RAPD-PCR reaction primers will bind to DNA in two regions that are separated by a short length. The sequence between these priming sites will be amplified.

The use of low annealing temperature give rise to products that are usually separated on agarose gels and detected by ethidium bromide staining. The number of generated fragments depends on the size of the genome and the length of the primer (Rafalski, 1997).Sequence differences between

different individuals give rise to bands that are present or absent and also may give bands of different molecular weight. These differences may be attributed to several reasons. They may be resulted from insertions or deletions in amplified regions or from base changes altering primer binding sites.(Weigand *et al.*,1993).
In addition to the PCR advantages, RAPD analysis offers other advantages (Rafalski, 1997):

1-Non-radioactive detection.

2. No prior DNA sequence information for genome is required.

3. It works with universal primer at any genome.

4. Muliplix detection of polymorphism.

5. Expermental simplicity.

6. No need for expensive equipment beyond a thermocycler and transillumminater.

7-The RAPD product can be digested with four-base cutter endonuclease enzyme to reveal more polymorphism among species that have little genetic variation (Mohan, 1991).

Although only several years ago there is a rapidly growing interest in RAPD technology .The technique became a very powerful tool and has found wide applications in different organisms including agricultural species.

RAPD has been used to differentiate between two *Lactobacillus* species (Van-Reenen and Disks, 1996) and to detect genetic variations

among several *Fusarium avenaceurm* strains.The genetic analysis for parasites, insects; and mammals were achieved using this technique. It has been used to identify four *Meloidogynes* species (Cenis, 1993), the genetic analysis of silk worm (Hwang *et al*, 1996) and the estimation of the genetic variations in Holstein dairy cattle.(Chung *et al*, 1995). Furthermore, it has been used for human genome analysis (Benter *et al.*, 1995).

Simplicity of this technique compared with other DNA marker techniques facilitated its use to reveal the genetic structure of different plant species. This technique was being used to create high-density genetic maps for tomato. (Klein-Lankhorst *et al.*, 1991), lettuce (Michiemore *et* at, 1991), pine (Chaparro *et al.*,1992), sugarcane (Al-Janabi *et al.*,1993) and lentil (Eujayl *et al.*,1998) .

RAPD-PCR was demonstrated to be an efficient technique to detect the inter-specific and intra-specific variations among plant species and varieties, where it was used for estimation of genetic variations between *Phaseolus vulagris* varieties (Graham *et al.*,1994),sunflower varieties(Lawson *et al*.,1994),*Medicago* species(Brummer *et al,1995),* and *Triticum* accessions (Cao *et al*.,1999).The genetic diversity of various plant species was also estimated by the data obtained from RAPD analysis where it's used to estimate the genetic diversity in willows *Salix* species and the genetic diversity in Cuban rice (Funtes *et al.*,1999).

RAPD technique is applied to many breeding programs for many important plant species, e.g. establishing of somatic hybrids identity (Xu *et al.*, 1993). Diseases are major constraints to crop product, therefore, breeding of resistant plant varieties allows this limit on crop production to be overcome.RAPD marker were found to be liked to gene responsible for this resistance and, therefore, theyare used to get disease-resistance plant.

In Iraq, this DNA marker technique was used to detect DNA polymorphism in the fungus *Aschochyta rabiei* and for genetic relationships estimation among a number of barely cultivars/lines (Jubrael, 1995). It was also used for genetic characterization of a number of date-palm varieties (Jubrael, 2000). Recently, RAPD marker technology was employed to serve many diverse applications where it is used for sex determination of date-palm varieties, to estimate the genetic relationships among several potato varieties. Furthermore, it has been used as epidemiological marker to differentiate between a number of *Pseudomonas aeroginosa* strains and also to differentiate between human papilloma viruses in cervical carcinoma (Jubrael, 2000).

2.20 DNA Isolation:-

Isolation of DNA is an essential step in many molecular biology experiments. The structure and composition of different organisms varied greatly. Thus, the DNA isolation procedure must be tailored to the organism from which the DNA is to be obtained (Frifilder, 1987) .The nucleic acids have two important characters, they don't dissolve in organic solvents and the second is they precipitated in the presence of alcohol and high concentration of salts. These characters permit the DNA isolation from other cell components. (Parish,1972). All procedures for DNA isolation have common steps, a cell is first broken down chemically or mechanically and then the DNA is separated from other cell components by centrifugation (Parish, 1972).

The plant cells have rigid walls that need a great force to be broken down. Therefore, the isolation of DNA from these cells will be a difficult procedure. Many procedures have been adopted to perform DNA isolation from different plant cells such as the use of Homogenizer to break down the plant cell wall. Chemical methods are also used for this purpose. This is usually done using enzymes to digest the plant cell walls (Calza & Scoeder, l983).Other physical methods used to break down the cell walls are either by means of liquid nitrogen or using lyophilization material .Lyophilization makes the cell to lose their flexibility and they will be more fragile. In plant cells, the presence of polysaccharide and Tannins make the extraction procedure more complex. Therefore, using the density gradient solutions using cesium chloride salt with different extraction buffer contents may counteract the negative effect of these materials. More recently, new methods of plant DNA isolation have been developed are based on the use of combination of earlier

e.g. using CTAB in the extraction buffers. (Weigand *et al* 1993). CTAB method has an advantage in forming a Nucleic acid /CTAB complex which prevents nucleic acids from negative effect of the other cell component. This method was being successfully used for DNA isolation from many different plant cells such as barely (Jubrael,1995)and date palm (Jubrael,2000).

3. Materials And Methods

3.1 Apparatus and chemicals

Table (2) Instruments used in experimental work.

Apparatus	Source
Autoclave	TAKIAWA (Japan)
Oven	Galenkamp (England)
Balance	Sartorius (Germany)
Sensitive Balance	Sartorius (Germany)
pH meter	Metler Toledo (England)
Centrifuge	Heraeus (England)
Water Bath	Atom (England)
Microfuge	Eppendorf (Germany)
Electrophoresis	Fisher (Germany)
Spectrophotometer	CECIL Instrument LTD/UK
Refrigerator	Concord
Laminar flow air cabinate	ESCO
Incubator	Sanyo / Japan

Table (3) Chemicals Used in experimental work

Material	Origin
Tris-HCL	Sigma
NaCl	BDH
EDTA	BDH
CTAB	BDH
2-Mercaptoethanol	BDH
Chloroform	BDH
Ethanol	BDH
Bromo- phenol blue	BDH
Ethidium bromide	BDH
Alumina ($Al\ O_3$)	BDH
Octanol	BDH
Glycerol	Merk-Darm(Germany)
Boric acid	BDH

3.2 Preparation of solutions:

3.2.1 Solutions for MS medium:

3.2.1.1 Stock 1 (micronutrient stock):

Approximately 400 ml of DDH_2O was added to 1 liter beaker. Each salt in table 5 was weighed and dissolved in the first column. The solution was transferred to a 1liter volumetric flask, and DDH_2O was added to the final volume. The solution stored under refrigeration, 10 ml of stock 1 was pipetted for 1 liter MS nutrient medium (Bonga, J.M.1982).

3.2.1.2 Stock 2 (iron stock):

Approximately 80 ml of DDH_2O was added to 100 ml beaker .Salts in the order indicated in table 5 were weighed and dissolved. The solution was transferred to 100 ml volumetric flask, and DDH2O was added to the final volume. Then 5 ml of iron stock pipetted for 1 liter of MS nutrient medium (Bonga, J.M.1982).

3.2.1.3 Stock 3(vitamin stock):

Approximately 50 ml of DDH2O was added to 100 ml beaker each of the vitamins indicated in table 5 were weighed and dissolved. Vitamin mixture was transferred to 100 ml volumetric flask, and then DDH_2O was added to the final volume. Because of the possibility of heat degradation of the vitamin, the vitamin supplement should be added before sterilizing by autoclave. The desired amount of vitamin was sterilized with ultrafiltration with syringe equipped with sterile Swinney filter unit containing a Millipore filter disc (0.22) mm pore diameter (Bonga, J.M.1982).

3.2.2.1 2M Tris - HC1 (pH8.0)

It was prepared by dissolving 60.55 g of Tris Base in 200m1 of distilled water. The pH was adjusted to (8.0) by adding concentrated HC1. The volume was completed to250ml with distilled water and sterilized by autoclaving (Maniatis *et al.*, 1982).

3.2.2.2 O.5 M EDTA (pH8.0)

36.55 g of EDTA was dissolved in 200ml of distilled water and stirred vigorously on a magnetic stirrer.The pH was adjusted to (8.0) using (1 N) NaOH.. 250 using distilled water and sterilized by autoclaving (Maniatis *et al.*, 1982).

3.2.2.3 5 M NaC1

It was prepared by dissolving 73.05g of NaC1 in 200m1 of distilled water. The volume was completed to 250ml using distilled water and sterilize by autoclaving. (Maniatis, *et al.*,1982).

3.2.2.4 1 N NaOH

4g of NaOH was dissolved in 80ml of distilled water and the volume completed to100ml using distilled water (Maniatis, *et al.*, 1982).

3.2.3 Solutions for RAPD-PCR:

3.2.3.1 TBE (pH 7.8) 10 x

It was prepared by dissolving 108g of Tris Base, 55g of Boric acid and 40ml of 0.5M EDTA in 800m1 of distilled water The pH was adjusted to (7.8) .The volume was completed to (l000ml) using distilled water and sterilized by autoclaving. (Maniatis, *et al.*, 1982).

3.2.3.2 Loading buffer (6x)

0.25 g of Bromo-phenol blue dye and 30ml of glycerol was dissolved in 70m1 distilled water and stored at 4°C. (Maniatis *et al.*, 1982).

3.2.3.3 TE (pH 8.0)

It was prepared by mixing l0ml of Tris-HC1 (pH8.0) with 2ml of 0.5M EDTA (pH8.0). The volume was completed with distilled water and sterilized by autoclaving (Maniatis *et al.*, 1982).

3.2.3.4 dNTPs mixture

It was also provided by Boehringer Mannheim Company with concentration of (100 m M-pH 7.0).

3.2.3.5 Primers:

This primers were provided by Operon Incorporation lyophilized form which can be redissolved in 500µl, in a of sterile distilled water to give a final concentration of 5pmol /µl. numbers of primers were used in this study to test the differentiation of Jojoba. These primers and their sequences are listed in Table (4)

Table (4) Primers and their sequences

Primers	Sequences
A1	**5′ CAGGCCCTTC 3′**
A10	**5′ GTGATCGCAG 3′**
C5	**5′ GATGACCGCC 3′**
N7	**5′ CAGCCCAGAG 3′**
N16	**5′ AAGCGACCTG 3′**
O2	**5′ ACACACGCTG 3′**
E13	**5′ CCCGATTCGG 3′**
A18	**5′ AGGTGACCGT 3′**
A10	**5′ GTGATCGCAG 3′**
D20	**5′ ACCCGGTCAC 3′**
E7	**5′ AGATGCAGCC 3′**

3.2.3.6 *Taq* DNA Polymerase

Provided from Boehringer Mannheim Company as a tube that contains100µL, each µl contains 5U of the enzyme.

3.2.3.7 PCR Buffer 10 X :

This buffer was provided by Boehringer Mannheim company and it is contain 10m M Tris-HCL (pH 8.3),50mM KCl and 2mM $MgCl_2$,0.0001% gelatin.

3.2.4 Solutions for DNA isolation:

3.2.4.1 CTAB extraction buffer 1x : for 100 ml

2.0 M Tris-HCL PH 8.0	2.5 ml
5.0 M NaCL	14.0 ml
0.5 M EDTA	2.0 ml
CTAB	1.0 g
2-mercaptoethanol	140.0 µl

This solution was made up without 2-mercaptoethanol, avoiding foaming and then autoclaved and stored above 15 C. then 2-ME added immediately before use.

3.2.4.2 CTAB precipitation buffer 1X : for 100 ml

2.0 M Tris-HCl	2.5 m
0.5 M EDTA	2.0 ml
CTAB	1.0 g

Made up as CTAB extraction buffer.

3.2.4.3 10% CTAB: for 100 ml

CTAB	10.0 g
5.0 NaCl	14.0 ml

Chloroform –Ooctanol (24:1) 3.2.4.4

1 ml of octanol was taken and added 24 ml of chloroform in glass Universal bottle.

3.3 Steps in the preparation of the MS medium:

1.400 ml of DDH_2O to a 1-liter beaker. Each salt of the macronutrients in table (5) were weighed and dissolved.

2. From each of the stock solutions previously prepared, 5ml of iron stock, 10ml of micronutrient were added.

3.100 mg of myo-inositol weighed and dissolved in the medium mixture.

4.DDH_2O was added until the total volume of liquid is approximately 950 ml.While stirring the solution ,pH of the medium was adjusted to 5.7 by delivering 1 N NaOH or 1 N HCL with separate pasture pipettes.

5. The medium was transferred to 1-liter volumetric flask , DDH_2O was added to the final volume.

Table(5) MS medium composition (Murashige and Skoog , 1962).

Ingredient	Concentration
(A) Macronutrient	(g/liter)
$(NH_4)NO_3$	16.5
$CaCl_2.2H_2O$	4.4

KNO_3	19.0
$MgSO_4.6H_2O$	3.7
KH_2PO_4	1.7
(B) Iron	(g/liter)
Na_2EDTA	3.36
$FeSO_4.7H_2O$	0.278
(C)Micronutrient	(g/liter)
$MnSO_4.4H_2O$	1.7
$ZnSO_4.4H_2O$	0.86
H_3BO_3	0.62
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCL_2.6H_2O$	0.025
(D)Vitamins	(mg/liter)
Glycine	200
Nicotinic acid	50
Pyridoxine.HCl	50
Myo-inositol	1000
Sucrose	30.000

2-4-D	(1.5,2.0.2.5)
Benzyl Adenine(BA)	0.5

3.4 DNA isolation using CTAB method (Weigand *et al* 1993):

1. About 0.7 g from both freeze-dried callus and freeze dried leaf were grinded with 0.3 g of alumina in small mortar and pestle.

2. The powdered placed on Eppendorf tube.600 µl of 1X CTAB extraction buffer was added, and then the mixture gently dispersed with plastic stirring rod, the mixture incubated 20 min in 56 C.

3.600 µl of chloform- octanol (24:1) were added, emulsified by shaking. The samples centrifuged for 2 min in microcentrifuge then the aqueous phase was removed.

4. The denatured protein interface washed in the original tube with 100 µl of extraction buffer, then the tube recentrifuged and the aqueous phase removed.

5. 70 µl of 10%CTAB were added to the pooled aqueous phase then the sample was mixed.

6. Avoiding the debris the aqueous phase removed, and then an equal volume of precipitation (600µl) was added, mixed and leaved 20 min at room temperature.

7. The samples were centrifuged (2000 ***g***, 15 min) to collect the precipitate. The supernatant poured off and the pellet drained.

8. The nucleic acid/CTAB pellet was redissolved in 400µl of 1 N NaCl, heated to 56 C then the solution was removed to t a sterile eppendorf tube.

9. 800 µl of ethanol was added when the pellet is fully dissolved, then the samples placed in -20 over night.

10. The precipitated nuclic acid pelleted in microcentrfuge for 2 min then washed with 65%ethanol for 1 min.

11. The nucleic acid redissolved in 50µl of sterile distilled water .the samples then stored in -20°C.

12. DNA was measured by spectrophotometer

3.5 Spectrophotometer Determination of Nucleic Acids*:*

10µl of DNA sample was added to l.99 mlof TE buffer, mixed thoroughly, and the optical density (OD) was measured in a spectrophotometer at wavelengths of 260nm and 280nm. The DNA concentration in the solution was calculated according to the following formula:

DNA concentration.(µg/µl*)* = (OD260x100x50µl/ml)/1000

Theoretically, OD of one corresponds to approximately (50µg/ml) for double-stranded DNA. The ratio between the readings at 260nm and 280nm (OD 260, OD280) provides an estimate of the purity of nucleic acid. (Sambrook *et al*., 1989).

3.6 Agarose Gel Elecrophoresis

Agarose gels (1.0 and 1.2 %) were run horizontally in 1X TBE. Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells on the gel. Generally, the gel buffer added up to the level of horizontal gel surface and gels were run for 2-3 hours at 3-5 v/cm. Agarose gels were stained with ethidium bromide by immersing them in distilled water containing the dye of a final concentration of 0.5µg/ml for 30-45 minutes. DNA bands were visualized by U.V. illumination at 366nm wavelength on U.V. Transillumminater visualized wavelengths. Gels were destained in distilled water for 30-60 minutes to

get rid of the back ground before photographs were taken using Polarietocamera. (Maniatis *et al.*, 1982).

3.7 RAPD-PCR procedure:

The following reagents were mixed in a sterile 1 .5m1 eppendorff tubes.

Table (6): represents the component required for RAPD-PCR reaction.

Addition Order	Component	Volume	Concentration
1.	10 x PCR buffer	2.5µl	1X
2.	dNTPs	2.5µl	0.2mM
3.	Primer	2µ1	10 Pmol
4.	*Taq* Polymerase	0.2µl	1U
5.	S.D.W.	15.8 µl	
6.	DNA	2µl	25—50ng
Total		25µ1	

Since four Jojoba samples were analyzed, so, it was easier to prepare a master mixture of reagents and the enzyme enough for all DNA samples. It was mixed gently and spun own in a 1.5ml -eppendorff tube. 2µ1 of the

master mixture was aliquoted into other tubes.2µlof DNA template solution was added to each tube and mixed gently .The reaction, mixture overlaid with 20µl of mineral oil .The tubes were placed in the thermal cycler to carry out amplification.

The amplification program was run as follow:

1 cycle of 94° C for 2 minutes.

40 cycles of each 92° C for 1 minute,

36°Cfor l minute,

72° C for 1 minute,

1 cycle of 72° C for 10 minutes.

From the reaction mixture, the amplified DNA was withdrawn into another tube and analyzed by gel electrophoresis (1.2%). (Weigand *et al*., 1993).

4. Results And Discussion

4.1-Sterilization of Explants:

Explants of *simmondsia chinensis* used in this experiment was sterilized by rinsing under running tap water for about 15 min then these explants (leaves,roots,and stems)submerged in alcohol (ethanol 70%)for about 30 second, then NaOCl was used in next step of sterilization of the explants (leaves,roots,and stems) of *Simmondsia chinensis*. Fig (2) shows that the most effective concentration of NaOCl was 3% for 10 min. which gave the highest percent (100%) of survival, increasing time to 15 min. caused damage to plant tissues, whereas lowering the concentration of NaOCl led to high rate of contamination. Most concentrations of NaOCl used for15 min reduced survival rate(Brown,1984).

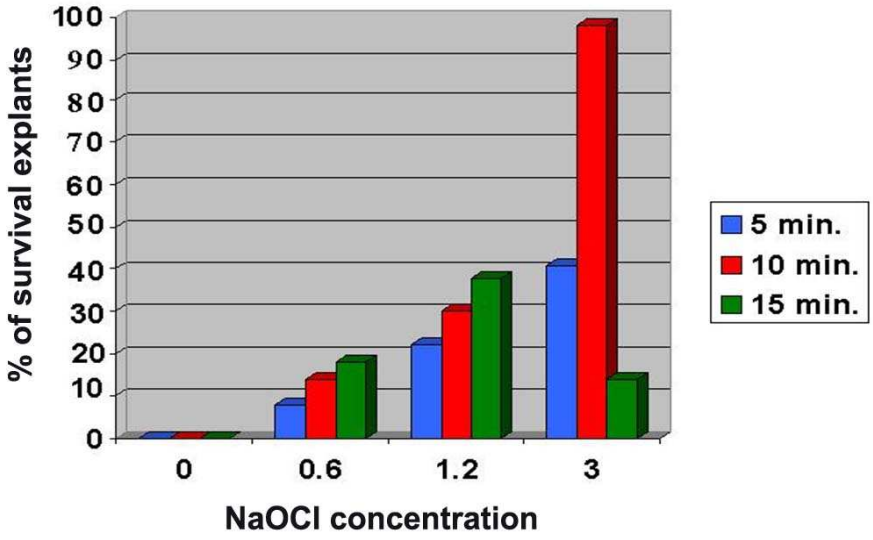


Fig.(2) Effect of different concentrations of NaOCl(percentage) on explants survival at sterilization periods of 5, 10, and 15.

Using NaOCl was important to decrease the contamination. It is used widely for tissue sterilization. The selection of sterilizing material depends on the source of explants, roughness of its surface. The sterilization material should be easy to remove from explants when washed with sterilized DDH_2O (Yeoman and Macleod, 1977; Sateesh, 2003).

The result is in agreement with (Pierik, 1987) who referred to the importance of sodium hypochlorite in explants sterilization. Increasing the sterilization period and concentration often lead to serious reduction in survival rate. Optimization experiment is therefore necessary to achieve maximum survival rate with minimum contamination. All surfaces must be in contact with the sterilant, after the using time for sterilization, the sterilant should be decanted and the explants washed at least three times with sterilized DDH2O.

4.2- Callus Induction:

In callus induction experiment three parts of *Simmondsia chinensis* (leaf, root and stem) used as explants for each male and female using differing combinations between the growth regulators (2, 4-D and BA).

The only explants that give response on MS medium to these combinations were leaves in both male and female explants.

The effect of combination between different concentrations of 2,4-D and BA on the percentage response to callus induction on Jojoba male leaf explants is shown in table (7) and picture (1).

Table (7): Effect of different concentrations of 2, 4-D and BA on the (%) response of callus induction on *Simmondsia chinensis* male leaf explants (n= 12).

2,4-D (mg/l)	BA(mg/l)		Mean
	0.0	0.5	
0.0	0.0	8.33	8.33
1.0	16.33	25.0	15.66
1.5	8.33	25.0	16.65
2.0	16.33	41.6	28.96
2.5	33.3	83.3	58.30
Mean	11.59	36.64	

The highest response to callus induction using BA only was achieved on explants treated with 0.5 mg/l (8.33%). Lowest response was recorded on explants treated with 0.0 (control ones) 0.0%.

Maximum response percentage in 2,4-D treated explants (58.3) appeared on explants treated with 2.5 mg/l of 2,4-D, followed by 2.0, 1.5, 1.0 mg/l, giving 28.96, 16.65, and 15.66 % respectively.

All response were different except between those treated with 1.0 and 1.5 mg/l of 2,4-D.

The interaction between the two growth regulators achieved 83.3% response in a combination of 0.5 mg/l BA and 2.5mg/l 2,4-D .While no callus induction was reported on control treatments.

The effect of different concentrations of BA and 2,4-D on the percentage of female leaf explants responded to callus induction is shown in table (8) and picture (2).

Table (8): Effect of different concentrations of 2,4-D and BA on the (%) response of callus induction on *Simmondsia chinensis* female leaf explants (n=12).

2,4-D (mg/l)	BA(mg/l)		Mean
	0.0	0.5	
0.0	0.0	16.66	16.66
1.0	16.66	25.0	20.83
1.5	16.66	33.3	24.98
2.0	25.0	50.0	37.5
2.5	41.6	91.6	66.6
Mean	19.98	43.31	

There was a significant increase in the percentage response with increasing 2,4-D concentrations up to 2.5 mg/l. Maximum response 66.6% with explants treated with 2.5 mg/l, which different from the level 1.0 mg/l. Minimum response percentage induction was recorded in a medium deficient of 2,4-D (16.66 %).

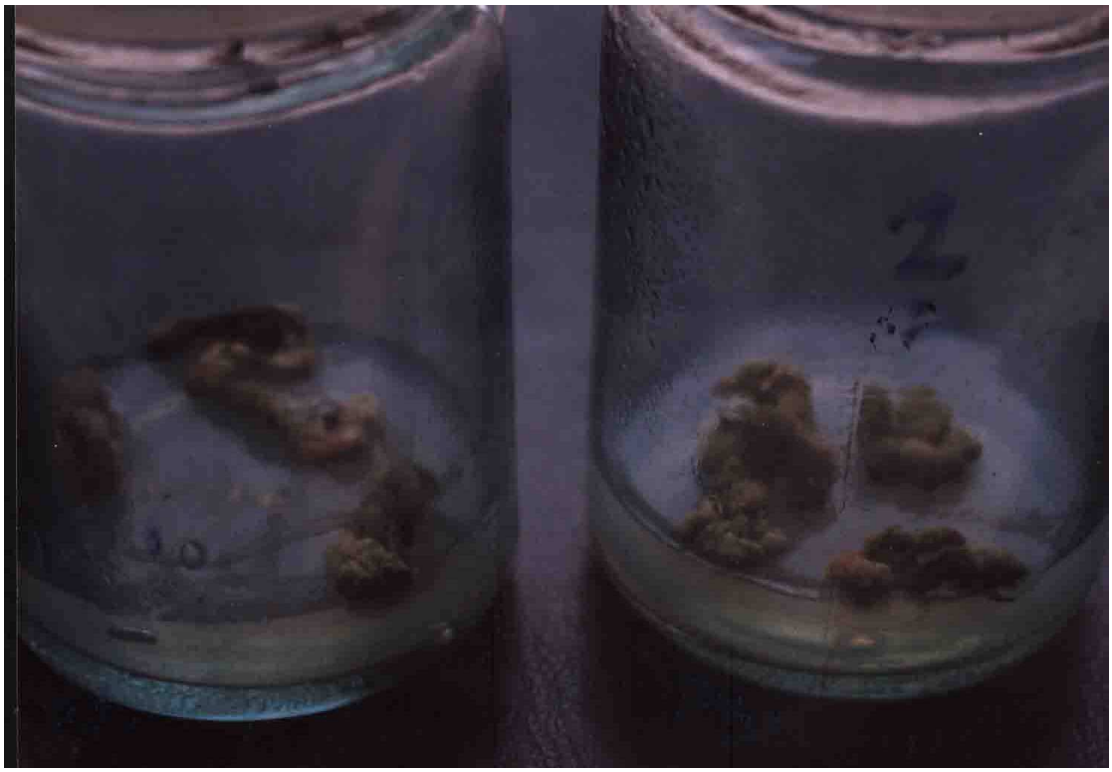
Response to callus induction fluctuated with the inclusion of BA in the nutrient medium. The level 0.5 mg/l exhibited the highest mean value (43.31%) which differed from the medium deficient of BA (19.98).

The lowest response appeared at 1.0 mg/l 2,4-D giving a mean value of (20.83%). The interaction between BA (0.5 mg/l) and 2,4-D (2.5 mg/l) showed a maximum response reached 91.6%. Other interactions values ranged between this value and no response at all.

Results in tables 2 and 3 showed that the best callus induction response for both explants occurred at a combination of 0.5 mg/l BA and 2.5 mg/l 2,4-D.Also these results showed that the response of female explants were higher than those explants from male plant when using the same concentration. Callus induction requires a balanced ratio from auxin(s) and cytokinin(s) as stated by Skoog and Miller (1957). In a number of plant species callus induction favors higher auxins than cytokinins (Ramawat, 2004).

Simmondsia chinensis male and female explants may contain some levels of endogenous regulators that made a balanced ratio with the exogenous regulators.

The process of callus establishment from the explants was divided into three stages, induction, cell division and differentiation. The length of these phases depends mainly on the cultural condition including the optimum combination of plant growth regulator as well as physolgical state of explants (Dodds and Roberts 1995).



Picture 1: Callus induction of male leaf explants grown on MS medium containing a combination of 0.5 mg/l BA and 2.5 mg/l 2,4-D,30 days after culture(16hr light/daily).

Picture 2: Callus induction of female leaf explants grown on MS medium containing a combination of 0.5 mg/l BA and 2.5 mg/l 2,4-D, 30 days after culture(16hr light/daily).

4.3-Maintenance of Callus Cultures:

Callus cultures induced on leaf explants from the best combination of BA and 2,4-D (0.5 and 2.5 mg/l) respectively, were subculture into the same combinations of plant growth regulators used for callus induction to determine the appropriate concentration for leaf male callus maintenance (Table 9).

Adding of BA at the concentration of 0.5 mg/l gave significantly higher callus fresh weight (388.79g) than other concentrations, while the lowest

was in the treatment where no BA was added to the culture medium. The highest callus fresh weight obtained in 2,4-D treated callus cultures (677.62 mg) was at the concentration 2.5 mg/l (Picture 4). This fresh weight was higher than other treatments.

Table (9): Effect of different concentrations of 2,4-D and BA on callus fresh weight (mg) initiated on leaf male explants of *Simmondsia chinensis* grown on a maintenance medium.

2,4-D (mg/l)	BA(mg/l)		Mean
	0.0	0.5	
0.0	0.0	113.45	113.45
1.0	100.1	165.37	132.91
1.5	163.28	208.26	185.77
2.0	342.18	582.64	462.41
2.5	481.01	874.23	677.62
Mean	217.31	388.79	

Picture 3: Callus cultures originated from male leaf explants grown on maintenance medium containing 0.5 mg/l BA and 2.5 mg/l 2,4-D. Cultures were continuously cultured on fresh medium at 21 days intervals (16hr light/daily).

Picture 4: Callus cultures originated from female leaf explants grown on maintenance medium containing 0.5 mg/l BA and 2.5 mg/l 2,4-D. Cultures were continuously cultured on fresh medium at 21 days intervals(16hr light/daily).

Combination between the two growth regulators resulted in maximum callus production reached (874.23 mg) at the levels of 0.5 and 2.5 mg/l BA and 2,4-D respectively.

This combination was higher than all other interactions. Callus tissues showed a reduced growth when inoculated into media lacking BA. and 1.0 or 1.5 mg/l 2,4-D.

Table (10) indicates that the trend was similar in callus cultures initiated on female leaf explants since the highest fresh weight (913.14 mg) was obtained from the combination of 0.5 mg/l BA and 2.5 mg/l 2,4-D (Picture 5). This fresh weight was higher than other combinations.

Table (10): Effect of different concentrations of 2,4-D and BA on callus fresh weight (mg) initiated on female leaf explants of *Simmondsia chinensis* grown on maintenance medium.

2,4-D (mg/l)	BA(mg/l)		Mean
	0.0	0.5	
0.0	0.0	164.8	164.8
1.0	123.08	171.73	147.40
1.5	130.72	218.28	174.5
2.0	398.33	684.05	541.19
2.5	416.57	913.14	644.85
Mean	208.17	392.45	

According to the results stated above, callus was induced on male and female leaf explants then maintained for subculture on MS medium containing 0.5 mg/l BA and 2.5 mg/l 2,4-D for subsequent experiments. Induction and maintenance of callus cultures in *Simmondsia chinensis* seem to favor low levels of BA and rather higher levels of (2,4-D) .Increasing the levels of the two plant growth regulators suppressed callus growth. It would be convenient from the practical point of view to induce and maintain callus on the same growth nutrients and plant growth regulators requirements. Induction of callus on both types of explants using the same medium components is an additional advantage for plant biotechnologists.

4.4 DNA isolation from plants:-

Genomic DNA from *Simmondsia chinensis* plant were isolated according to the procedure described by (Weigand ,1993).Although PCR technique do not required large quantities and high purity DNA (Saiki *et al*.,1990),suitable quantities of DNA approximately (100-150 µg) from 0.7 g of both fresh plant tissue (male and female leaves) and callus culture (from them).DNA molecular weight were estimated on (1%and 1.2%) agarose gel electrophoresis .It was shown to be (35kbp) and also may reach around (45kbp) as seen in figure (3).The purity of isolated DNA from male fresh plant were 1.19, female fresh plant 1.15, male callus1.15, and for female callus 1.11. According to our results it was found that CTAB method represent a suitable method for DNA isolation from plants and fungi (Weising *et al*,1998).This method involves a number of advantages steps such as the lyophlyzation process that inhibit nucleases activity by a mean of low temperature during the grinding of plant tissues.

The extraction buffer containing CTAB helps better cell lyses and the CTAB complex will form the so called – Nucleic acid /CTAB complex which dose not dissolved in solvents and also nucleic acid can be separated from other cell component by chloroform –octanol precipitation. The presence of EDTA in the extraction buffer will act to withdraw the Mg+2 ions required for nucleases activity (Weigand *et al*.,1993).This method also uses the chloroform that considered being one of the efficient organic solvents that capable of protein denaturation and other cell materials(Maniatis *et al*.,1992).

The presence of high NaCl concentration with chloroform –octanol complex will act to precipitates the DNA and also to get rid of polysaccharides (Weising *et al* , 1998).

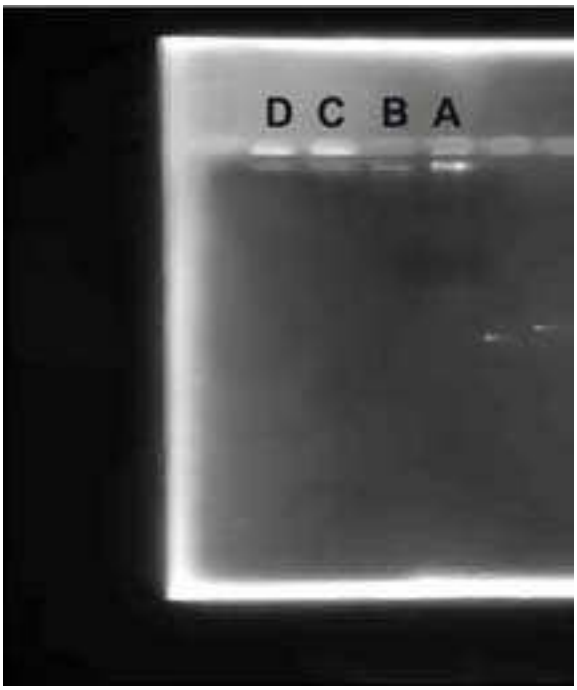


Figure (3): DNA isolation from four *Simmondsia chinensis* .Electrophoresis was performed on(1%) agarose gel and run with 5 volts /cm.The lanes (A,B,C,D)represent the female fresh plant, male fresh plant ,female callus , male callus ,respectively.

4.5 RAPD Analysis:-

In this study, eleven primers of decamer oligonucletides were screened for RAPD analysis using genomic DNA isolated from four sample of jojoba plant. The results were obtained from PCR reaction using these primers can be classified into two groups:

In the first group ,no amplification were detected .In spite of repeating a number of experiments ,similar results were obtained using six primers ,these primers include (A18,A10,N7,N16,O2,E13) .To be sure that all PCR component working properly, and no inhibitors within the reaction that could prevent the amplification the experiments repeated for these primers in different conditions like DNA concentration ,$MgCl_2$ concentration ,*Taq* polymerase concentration ,annealing temperature, and cycles number . The failure of these primers to amplify the genomic DNA may attributed to the absence of suitable priming site for these primers in the genome of jojoba lines tested (Devos and Gale 1992).

There were no complementary sequences for these primers in jojoba genomes .Similar result was obtained by (Ahmed.,1999),scored a number of RAPD primers that did not give amplification products in a study aiming to reveal genetic relationship among the annual *Cicer* species ,in other plant genomes such result also obtained by (Devos and Gale 1992).

The second group of RAPD results produce amplified products using five primers that were (A1,A10,D20,E7,C5).Using these primers ,a representable degree of DNA variation was detected among *Simmodsia chinemsis* sexes ,DNA variation can be detected and revealed generally in three forms (Mayer *et al* .,1997):

1. The presence or absence of DNA bands.
2. Differences of the molecular weight of bands.
3. Differences of the intensity of amplified bands.

The naturally occurring DNA polymorphism may be related to several causes such as the loss or alteration of one of the opposed pair of primer binding sites needed to produce the PCR product .Alternatively an insertion between the binding sites may place them at distance too great to allow amplification (Daves *et al*., 1999).

In this study, DNA variation was scored between jojoba male and female according to the differing in the number of bands and/or their molecular weights.

Since each primer had produced differing DNA banding patterns. Thus, a detailed of RAPD results using these primers will be described according to the individual primer used.

4.6 The primer A_{10}:-

Using this primer which has the sequence of (5´GTGATCGCAG3´), a total number of 11 bands were detected in the four lanes as seen in the figure (4).The molecular weight of these bands ranged between (0.85 kbp-1.16 kbp). In this experiment by comparing the presence or absence of the first band (which has molecular weight1.5 kbp) in the lane C which represent female callus DNA, also DNA variation can be detected by the presence or absence of the first band (which has molecular weight 1.16 kbp) in the lane D which represent male callus DNA.

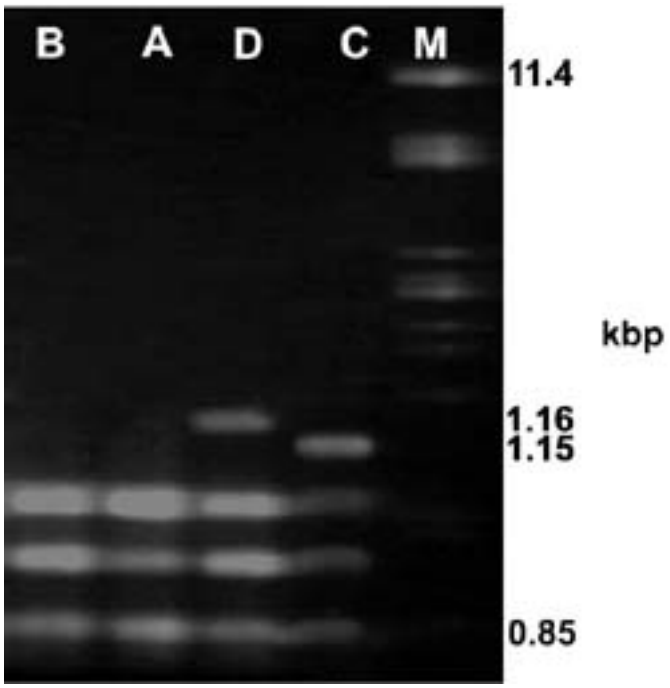


Figure (4): RAPD pattern of *Simmondsia chinensis* obtained with A10 primer. Electrophoresis was performed on (1.2%) agarose gel and run with 5 volt /cm. The lanes (A,B,C,D) represent female fresh plant, male fresh plant, female callus and male callus respectively ,lane M indicates the λ DNA as a marker digested with *Pst* I.

4.7 The Primer A1:-

Using this primer that has the sequence of (5′CAGGCCCTTC 3′), only one band was detected in the lane A as seen in figure (5).The molecular weight of this band (1.08 kbp) .The amplified products were not detected in the in three lanes (B, C, D) which represent male fresh plant, female callus and male callus.DNA variation can be detected easily be comparing the presence of the band in lane A that represent female fresh plant DNA with other lanes that showed no amplification product.

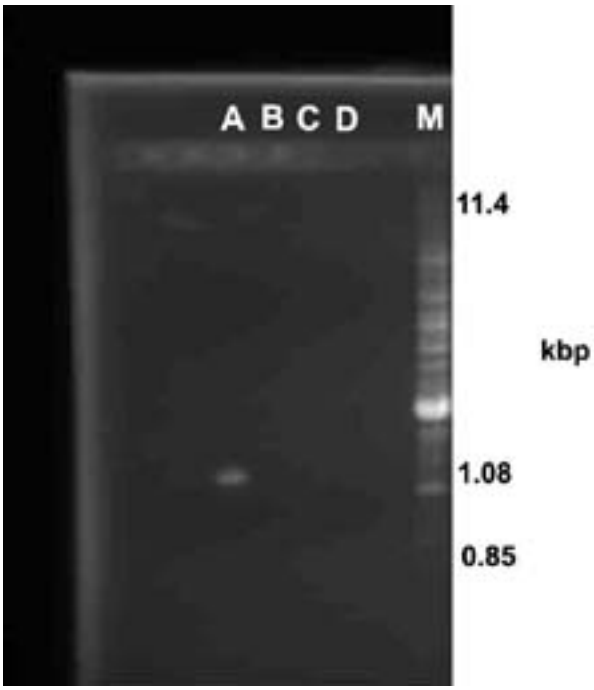


Figure (5): RAPD pattern of *Simmondsia chinensis* obtained with A1 primer. Electrophoresis was performed on (1.2%) agarose gel and run with 5 volt /cm. The lanes (A,B,C,D) represent female fresh plant, male fresh plant, female callus and male callus respectively .lane M indicates 1 kbp leader DNA as marker.

4.8 The primer C5:-

Using this primer that has the sequence (5´GATGACCGCC3´), a total number of 17 bands were detected as seen in figure (6) .The molecular weight of these band ranges between (0.85-1.0) Kbp. In this experiment a DNA polymorphic bands can be identified by comparing the presence or absence of band number 1 that present only in lane B (which has molecular weight 1 kbp) , using this primer lane B can be distinguished that represent DNA isolated from male fresh plant.

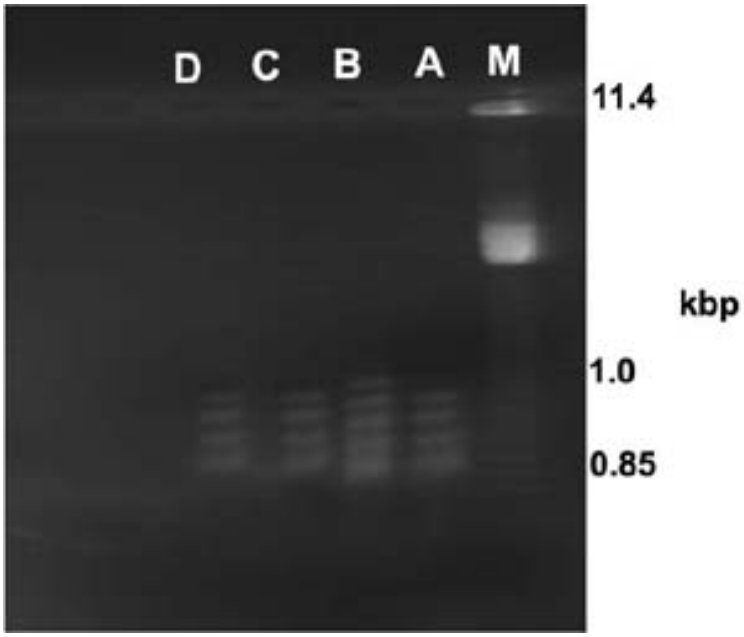


Figure (6): RAPD pattern of *Simmondsia chinensis* obtained with C5 primer. Electrophoresis was performed on (1.2%) agarose gel and run with 5 volt /cm. The lanes (A,B,C,D) represent female fresh plant, male fresh plant, female callus and male callus respectively ,lane M indicates the marker DNA digested with *Pst*I.

4.9 The primer D20:-

In this experiment, the use of this primer that has the sequence (5´ACCCGGTCAC3´) resulted a total number of 14 bands as seen in figure (7). Each lane contains four bands ranged between (0.85-1.4skbp), except the lane, A which represents female fresh plant DNA, have two bands.

The absence of the first and the forth band in lane A (which have molecular weight 0.85 and 1.4 kbp) respectively, lane A can be identified and DNA variation can be easily detected.

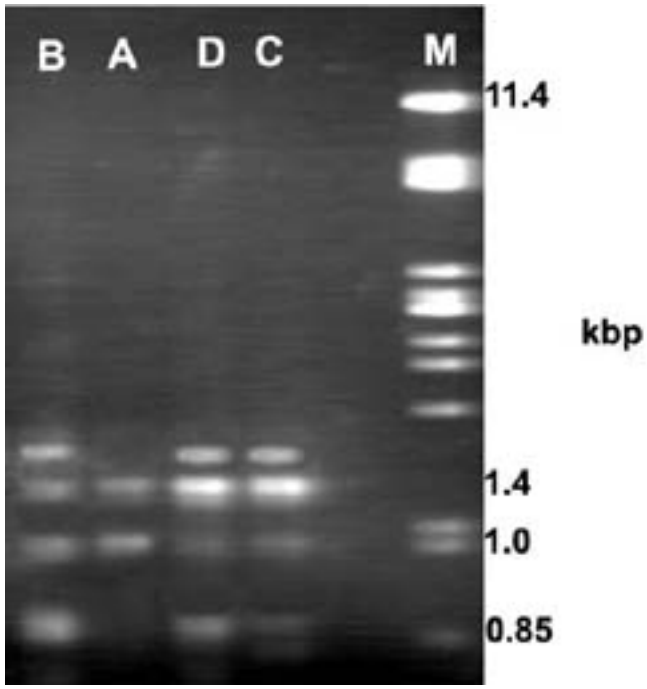


Figure (7): RAPD pattern of *Simmondsia chinensis* obtained with D20 primer. Electrophoresis was performed on (1.2%) agarose gel and run with 5 volt /cm. The lanes (A,B,C,D) represent female fresh plant, male fresh plant, female callus and male callus respectively ,lane M indicates the marker DNA digested with *Pst*I.

4.10 The primer E7:-

Using this primer that has the sequence (5´AGATGCAGCC3´), a total number of 22 bands obtained .The molecular weight of these bands ranged between (0.9-1.4s kbp) as seen in figure (8). Analysis of RAPD results using this primer are shown that DNA polymorphic bands can be identified by comparing the presence or absence of bands numbered (3,4)with molecular weight 0.95 and 1 kbp respectively ,these two bands present in the lanes(B,C, D) and absence in lane A whiche represent female fresh plant.

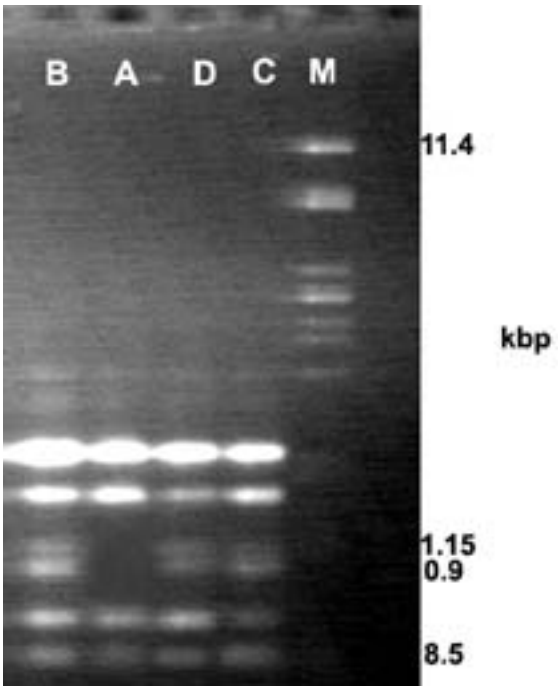


Figure (8): RAPD pattern of *Simmondsia chinensis* obtained with E7 primer. Electrophoresis was performed on (1.2%) agarose gel and run with 5 volt /cm. The lanes (A,B,C,D) represent female fresh plant, male fresh plant, female callus and male callus respectively ,lane M indicates the marker DNA digested with *Pst*I.

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