Ministry of Higher Education and
Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

Induction of Somatic Embryogenesis in *Solanum melongena* L.

A thesis

Submitted to the College of Science, Al-Nahrain University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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January **2009**

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D edication

I dedicate my work and achievements to my dearest mother who was always my guide.

To whom guided me towards success and never lost faith in me my father.

To my soul mate and my best friend my brother who will always stay side by side with me and never let me down.

To all my family members who supported me and kept believing in me.

To my best and for good friends Nenos and Noel who was more than friends to me.

Husam Ahmad

Acknowledgments

At the beginning, all praise to **Allah** who gave me health, faith, strength, patience and facilitated the ways for me to accomplish this work.

I would like to express my deep gratitude, thanks and appreciation to my supervisors **Prof. Dr. Kadhim M. Ibrahim** and **Ass. Prof. Dr. Abed Al Jasim M. Al Jibouri** for their advice and guidance through out the time course of this project.

My thanks to all Biotechnology department staff in Al-Nahrain University. Thanks are due to my colleges especially **Ahmad Khamees** and **Azad Sa'ad**.

My thanks to my colleuges **Rand Talal**, **Meriem Gadanfer**, **Noor Adel**, **Rajwan Khalel**, **Arwa Hamoode** and **Mostafa Khaeer**.

My sincere gratitude goes to my **family** who gave me the support during my study.

Abstract

This project was designed to induce somatic embryogenesis on different explants dissected from *Solanum melongena* L. plant.

In this study, calli was induced on stem segments grown on MS (Murashige and Skoog, 1962)medium supplemented with NAA at 8mg/l and Kin at 0.1mg/l, Decreasing the supplemented agar to 5g/l increased calli induction significantly in the rate and time of induction. Calli initiated on stem segments was photographed under light microscope after one week on MS free of PGRs which shown embryos at globular stage. Decreasing the agar concentration accelerated embryogenesis induction.

Shoots were developed from embryogenic calli after 30 days on MS free PGRs medium, then transferred to MS medium supplemented with 0.2mg/l NAA and 0.1mg/l BA where roots developed after 30 days. Anther culture was maintained on liquid R medium (Dumas de Vaulx *et al.*, 1981) in a cell suspension supplemented with 0.02mg/l 2,4-D and 0.01mg/l Kin at 100rpm in a shaker incubator and photographed under light microscope after two weeks. Embryos at the globular stage were seen and counted in embryogenic calli and the result was 2977000embryo/ml. Embryos cell suspension count was 3550000embryo/ml. Viability test was carried out for embryos cell suspension and the viable embryos percentage was 72%.

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List of abbreviations

Abbreviation	Full name
2, 4-D	2, 4-Dichlorophenoxy acetic acid
2,4,5-T	2,4,5-Tricholrophenoxy acetic acid
ABA	Abscisic acid
BA	Benzyl adenine purine
°C	Degree Celsius
DH	Double haploid
DDH_2O	Double distilled water
FDA	Flourescein diactate
g	Gram
hrs	Hours
IAA	Indol acetic acid
IBA	Indole butyric acid
IEDC's	Induced embryogenic determined cells
Kin	kinetin
L	Liter
mg	Milligram
MS	Murashige and Skoog medium, 1962
NAA	Naphthalene acetic acid
NaOCl	Sodium hypochlorite
n	Number of replicates
PEDC's	Pro-embryo-genic determined cells
PGRs	Plant growth regulators
SD	Standard deviation
UV	Ultraviolet (light)
wt	Weight

Chapter One

Introduction

and

Literature Review

1.1- Introduction

Plant cell culture technique occupies a key role in the second green revolution in which gene modifications and biotechnology are being used to improve crop yield and quality. This state of art technology has become possible by continuous efforts of many scientists, who carried out the basic work. (Kuang and Cheng, 1981; Misawa, 1985).

Plant cells are totipotent and can produce whole new plants under favorable conditions of nutrients and plant growth regulators. Reneirt in Germany reported for the first time, somatic embryo formation in carrot cell suspension cultures (Ramawat, 2008).

Eggplant was selected because it offer's a potentially good system to investigate plant growth and development *in vitro* (Gleddie *et al.*, 1986; Sharma and Rajam, 1995).

Somatic and zygotic embryos undergo basically the same stages of development, namely globular, heart-shaped, torpedo and cotyledonary. Somatic embryos allow the economical mass propagation of elite plant verities. They would also be a channel for new plant lines produced through biotechnological advances to be delivered directly to the greenhouse or field (Mullins and Srinivasan, 1976).

Many new plant varieties produced through cell culture techniques often cannot be propagated by seed because their meiotic instability leads to the loss of the desirable traits. To overcome this problem, the vegetative multiplication of shoots and buds through tissue culture (micropropagation) can be used but is often labour intensive and costly for most crops (Collin, and Edwards, 1998).

Greenhouse propagation through stem cuttings is also labour intensive with the multiplication factor limited by the size of the mother plant. These propagation limitations may be overcome by using artificial

seeds (synthetic seeds) produced through somatic embryogenesis. This rapid multiplication method can produce thousands of embryos, each with the potential to produce a plant, from a few grams of plant calli (Ferrie *et al.*, 1995). Somatic embryos are suitable materials for this purpose as large number of somatic embryos of same age can be produced in a bioreactor. This technology is developed to encapsulate embryos to produce artificial or synthetic seeds. An encapsulated embryo is protected against desiccation and mechanical injury by some types of gel (George, 1993).

Thus an artificial seed system would be a rapid, mass propagation method that maintains the genetic fidelity of the plants. In addition, artificial seeds would allow direct planting of plant propagules into the greenhouse or field, thus by passing many of the grow-out and acclimation steps normally associated with micropropagated plants (Keller, and Armstrong,1979).

Improvement methods may be compromised by natural lose of some important traits leading to lose of improved varieties, that's why *in vitro* anther culture stands up and is an increasingly powerful tool when integrated into breeding programs. This technique provides homozygous haploid or double haploid embryos within a short time.

Therefore, the aim of this work was the optimization of somatic embryogenesis in *Solanum melongena* L. using different types of plant explants and growth regulators.

1.2- Literature Review

1.2.1- *Solanum melongena* L.

Kingdom: Planta.

Phyllum : Gametophyta.

Division : Anthophyta.

Class : Dicotyledoneae.

Order : Solanales.

Family : Solanaceae.

Genus : *Solanum*.

Species : *melongena*.

Common name: Eggplant, Aubergene (Yamada *et al*., 1980).

1.2.2- Description

The common egg plant (*Solanum melongena* L.), is a member of nightshade family (Solanaceae). It is related to tomatoes, pepper and potatoes. It is non-tuberous solanaceous crop and grown primarily for its large oval fruit (Franklin, and Lakshmi, 2003).

Eggplant is a warm-season crop, does not tolerate frost, more sensitive to cold temperatures than peppers and tomatoes. It is a perennial but grown as annual with terminate growth. A long growing season of 80 days is required for the transplanted crop. Optimal temperatures for eggplant production are 26°C at day and 20°C at night. Plant growth slows, and pollination problems occur at temperatures below 17°C or above 35 °C. Flowering is not affected by day length (Anonymous, 1998).

The plant grows at vegetative status from May to October, in flowering from July to September, and seeds ripen from August to

October. The flowers are hermaphrodite (have both male and female organs) and are pollinated by insects, it grows in light (sandy) medium (loamy) and heavy (clay) soils but prefers well-drained soil. (Anonymous, 1998).

1.3- Plant tissue culture

The history of plant tissue culture begins with the concept of cell theory given independently by Scheilden 1838 and Schwann 1839 that established the cell as a functional unit. This implies that cells are autonomous. The concept was tested experimentally. Earlier attempts to grow plant cells met with failures. Success was achieved in growing animal cell culture at that time, earlier work involving plant tissue was mainly concerned with nutritional requirements of cells to make them divide and sustain growth. The work progressed rapidly after the discovery of auxins and other cell division factors. Early studies also frequently used complex nutritional factors like coconut water, yeast extract, malt extract and casein hydrolysate. The developments after World War II were rapid as many inventions developed for the war purposes and found their applications in biological sciences (Sagare *et al.*, 1995).

It was untiring efforts of Robbins, Kotte, White, Guathrete, Heller, Van Over-beek, Steward and Caplin, Miller, Nitsch, Reinert, Street, Morel, Skoog, Hildebrandt, Melcher, Cocking, La Rue and others that plant tissue culture, which was initiated as a tool has become a powerful technology later (Pierik, 1987).

With the better understanding of the technique of plant tissue culture and nutritional requirements, of plant cell, it was possible to develop newer technologies by culturing plant organs (anther, ovary, ovule, petal, leaf and meristem) leading to establishment of new

research lines, haploids, virus-free plants, *in-vitro* fertilization, embryo rescue and direct regeneration from leaf disc for genetic engineering (Newman *et al.*, 1996).

Subsequently, it has become possible to grow isolated epidermal cells, gland cells or even protoplasts to regenerate from such specialized cells or individual protoplasts, regeneration plants and produce useful metabolites. Demonstration of variability in cell cultures has given the concept of morphological variations and hence 'Somaclonal variation' (Misawa, 1985).

1.4- Calli cultures

Calli cultures are clumps of unorganized plant cells grown on solidified nutrient media or liquid medium. Generally he state of undifferentiated growth is maintained by the plant growth regulators balance, mainly auxins and cytokinins added to the culture medium, Calli tissue is formed after wounding and this cell mass close the wound rapidly. In *in vitro* culture, a tissue is wounded and the induced calli is further subcultured on nutrient media (Ramawat, 2008).

Sateesh (2003) divided calli growth to:

A- Lag phase, where cells prepare to divide.

B- Exponential phase, where the rate of cell division is increasing.

C- Liner phase, where cell division slowdown but the rate of cell expansion increase.

D- Deceleration phase, where the rate of cell division and elongation decrease.

E- Stationary phase, where the number and size of cells almost remain constant.

The optimum conditions for calli formation as well as the suitable sterilization procedures and nutrient media, have to be determined empirically (Haq, 1993).

The calli stock provides the material for the establishment of new suspensions, the degree of calli formation depends on the type of explants, plant species, type and level of plant growth regulators.

Exogenous plant growth regulators (type and concentration of auxin to cytokinin ratio) are crucial for calli formation and this may depend upon the endogenous hormone content of the tissues under investigation (Pierik, 1987; Ramawat, 2008).

1.4.1- Plant growth regulators

Growth regulators are required to induce calli tissues and to promote the growth of many cell lines. As an auxin, 2,4-Dichlorophenoxyacetic acid (2,4-D) or Naphthaleneacetic acid (NAA) are used. The concentration of auxins in the medium is generally between 0.1 to 5 mg/l. Cytokinins may also be added (Kuang and Cheng, 1981; Misawa, 1985). Since each plant species requires different kinds and levels of growth regulators for calli induction, to enhance its growth and metabolites production, it is important to select the most appropriate combination (Zhao *et al.*, 2001).

1.4.2- Physical factors

The effects of light, temperature, pH and oxygen and all other parameters should be examined in the studies of somatic embryogenesis. A temperature of 17– 25 ^{0}C is normally used for induction of calli tissue and growth of cultured cells; however, each plant species may favor a

different temperature. The pH of medium is usually adjusted to 5-6 before autoclaving and extremes of pH are avoided, the pH of the growth medium influences the production of phytochemicals in cultured cells. Each plant species has different optimized conditions for growth of the cells, so it is necessary to optimize the conditions in each case (Yamada *et al*., 1980).

1.5- Somatic embryogenesis

The capacity of flowering plants to produce embryos is not restricted to the development of fertilized egg; embryos (embryoids) can be induced to form in cultured plant tissues. This phenomenon was first observed in suspension cultures of carrot (*Daucus carota)*derived from calli was grown on an agar solidified medium (Reinert *et al*., 1975). Embryogenesis is a general phenomenon in higher plants, and experimental somatic embryos have been reported in tissues cultured from more than 30 plant families (Raghaven, 1976; Narayanaswamy and George**,** 1982; Ammirato, 1987).

Somatic embryoids may arise *in vitro* from three sources of cultured diploid cells: (1) vegetative cells of mature plants, (2) reproductive tissues other than zygotic, and (3) hypocotyls and cotyledons of embryos and young plantlets without any interfering calli development. Precisely how these adventives embryoids arise from these tissues have been the subject of numerous studies. According to Sharp *et al* (1980), somatic embryogenesis may be initiated in two different ways. In some cultures embryogenesis occurs directly in the absence of any calli production from "preembryonic determined cells" that are programmed for differentiation. The second type of development requires some prior calli proliferation, and embryos originate from "induced

embryogenic determined cells" within the calli (Ramawat, 2008). Although individual cells are totipotent and carry all the genetic templates necessary for the development of the whole plant, isolated single cells do not generally become transformed into embryos by repeated division. Embryoids are initiated from superficial clumps of cells associated with highly vacuolated cells that do not take part in embryogenesis.

Initially it was observed that these embryos originated from cell aggregates, however, the "young embryoids have always a clearly defined outline indicating discontinuity with the surrounding cells". The aggregates are found because cells fail to separate following cell division (Magioli *et al.*, 2001).

After the initial observation of somatic embryos, there were considerable efforts devoted to define the medium conditions, which permitted embryogenesis, and characterizing the morphological and developmental events leading to embryo formation. Embryogenesis is a two step process, the first stage is the induction of embryogenesis and the second step is the development of embryo, ultimately leading to germination. The requirements for embryo induction and embryo development are different, and thus separate media are used for each step (Hutchinson *et al.*, 1996).

Ammirato (1987) described four stages viz., induction, early growth, embryo maturation and germination or conversion, these stages not only differ in their morphological structure but also in their physico-chemical requirements, carrot somatic embryogenesis is the most extensively studied system for various aspects of somatic embryogenesis.

Usually an exogenously supplied auxin is required in appropriate concentration for the induction of somatic embryogenesis from calli or

explant (Ho and Vasil, 1983).

However, in some cases like carrot, exogenous supply of auxin may not be required for embryogenesis to occur, it is proposed that in case of direct embryogenesis cells of explanted tissue are already determined for embryogenic development and termed as pro-embryogenic determined cells (PEDC's). In case of indirect embryogenesis cells required redetermination through a period in culture and termed as Induced Embryogenic Determined Cells (IEDC's), it is postulated that this phenomemnon is determined by epigenetic factors (Sharma and Rajam, 1995).

Embryogenesis occurs from a single cell or from a group of cells; the earlier cell divisions in embryogenetically determined cells follow various patterns, but finally produce embryos of similar shape. Embryogenic cells are small, isodiametric in shape, filled with dense cytoplasma and have conspicuous nucleus. In comparison to this, non-embryogenic cells are relatively large, vacuolated and lack dense cytoplasm. Embryogenic cells from an auxin-containing medium when transferred onto a medium containing low auxin concentration or without auxin, proembryos develop. Generally media containing 2,4-D, 2,4,5-T and picloram are used as embryo induction medium. Other auxins like IAA and IBA in combination with a cytokinin have also been found suitable for embryo induction in many dicotyledonous species. Several species of monocotyledons and dicotyledons have been regenerated though somatic embryo formation by this method (Matsuoka and Hinata, 1979).

When somatic embryos, at early stage or developed, are transferred to induction medium, they give rise to secondary somatic embryos. This method of obtaining embryos recurrently is known as repetitive or

cyclic embryogenesis, this method is useful for continuously obtaining embryos in large numbers, e.g., in *Atropa belladonna*, carrot, Ranunculus, *Pennisetum purpurium* and *Panicum maximum* (Hutchinson *et al*., 1996).

During somatic embryogenesis in cell suspension cultures, embryos of different sizes are produced, for any experimental or micropropagation method, embryos of uniform size are required; this can be achieved by sieving or fractionation of suspension with appropriate sieve size. Such cultures may be fully synchronized for their growth. The development and maturation of somatic embryos is similar to zygotic embryos, cell differention is most evident in the formation of vascular tissues especially visible in the hypocotyls region due to cell vaculation and in cotyledon (Ramawat, 2008).

1.6- Somatic embryogenesis in *S. melongena* L.

Somatic embryogenesis was induced from leaf explants and cell suspension cultures in the presence of 54 µM NAA (Gleddie *et al*., 1983).

On the other hand, Rao and Singh (1991) cultivated leaf explants in medium supplemented with 0.5 and 1.8 µM (NAA) and failed to induce embryogenesis with the same concentrations. In their work, embryogenic calli were obtained in the presence of higher (NAA) concentrations (27 and 64 µM), although best results were achieved when (NAA) was combined with 0.5 µM (Kin), higher embryogenesis rate in cotyledons cultured in the presence of 50µM 2,4-D was obtained by Saito and Nishimura (1994), as compared to 54 µM NAA.

Conversion of somatic embryos into plantlets is usually limited due to abnormalities such as hyperdricity, lack of apical meristem, cotyledon

fusion and inefficient maturation (Gleddie *et al.*, 1983; Saito and Nishimura, 1994; Magioli *et al.*, 2001). Nevertheless, conversion rates can reach up to 92% by culturing mature embryos on MS medium solidified with 1% phytagel (Saito and Nishimura 1994; Magioli *et al.*, 2001).

1.6.1- Factors affecting somatic embryogenesis

Almost every aspect of the cultural environment has been shown to affect embryogenesis(Ramawat, 2008), i.e.:

- the nutrient medium.
- exogenous plant growth regulators.
- evolved gases and light.
- The composition of culture medium is critical particularly the levels of sucrose and nitrogen, especially reduced nitrogen. Reduced nitrogen (ammonium or amino acids) is not required for the induction and oxide nitrogen (nitrate nitrogen) alone in high amount is sufficient for induction of somatic embryogenesis. However, reduced nitrogen in the embryo development medium supports embryo development, beneficial effect of certain amino acids like praline and glutamine has established.
- Increasing the osmotic concentration by increasing sucrose levels or by addition of mannitol or sorbitol, has shown to affect the embryo development.
- Auxins, especially the synthetic auxin (2,4-D) appears to be required for embryo induction but adversely affect embryo development.
- Cytokinins, except zeatin, suppress embryogenesis, ethylene development of embryos, whereas, gibberellic acid and zeatin produce

an increased frequency of abnormal embryo development, ABA imparts dormancy and helps in the formation of cotyledonary stage somatic embryo suppresses embryogenesis, abscisic acid (ABA) suppresses abnormal.

- In liquid cultures, dissolved oxygen can play a critical role in differentiation, Carrot cells produce somatic embryos below 16% dissolved oxygen while above 16% oxygen levels, roots are produced (Ramawat, 2008).

1.6.2- Advantages of Somatic embryogenesis

Somatic embryogenesis is a versatile technique for micropropagation of plant species. A large number of herbaceous dicots and monocots have been regenerated through somatic embryogenesis, however, woody trees are still difficult materials to regenerate via somatic embryogenesis. This method of micropropagation offers several advantages over organogenesis and clonal propagation through explants.

- Rapid multiplication through production of somatic embryos in cell cultures and use of bioreactors for scale-up technology.
- Presence of bipolar structures in the same unit (presence of both root and shoot) avoids the rooting step required in organogenesis.
- Somatic embryos grow individually making the system easy to manipulate (to sub-culture) and develop scaling-up methods.
- It is possible to induce dormancy and store cultures for long duration.
- Possibilities of encapsulation and store the embryos for long

duration.

- Provides an important resource for the analysis of molecular and biochemical events that occur during induction and maturation of embryo.
- Isolation of specific storage proteins is possible.
- It shortens the breeding cycle of deciduous trees and increases the germination of hybrid embryos where delayed germination of seeds is a significant handicap in rooting of the plants of horticultural interest.
- Avoidance of somaclonal variation via direct-embryogenesis.

1.7- Synthetic seeds technology

Synthetic seeds offer a potential technology towards the use of somatic embryogenesis for large-scale propagation of plants through automation, the micropropagation industry started with orchids and ferns are still largely restricted to horticulture, plantation and ornamental plants. During the past decades production of bulb and tuber plants, such as lilies, Potato, gladiolus and cassava have become quite common, large-scale propagation requires planting material in suitable age, shape and of germination percentage. Somatic embryos are suitable materials for this purpose as large number of somatic embryos of same age can be produced in a bioreactor.

Technology is developed to encapsulate them to produce artificial or synthetic seeds. Encapsulated embryos are protected against desiccation and mechanical injury by the gel. It was Murashige and Skoog (1977) who conceived the idea of developing synthetic seeds.

The first construction of synthetic embryos was achieved by single embryo encapsulation of hydrated alfalfa somatic embryos. The necessary components of synthetic seeds depend on whether they are hydrated or desiccated (Ramawat, 2008).

1.8- Advantages of haploids

Haploid production includes the regeneration of haploid embryos from male or female gametes and the production of haploid and double haploid (DH) plants. The spontaneous occurrence of haploid in natural populations is very rare, and it is strongly genotype-dependent. Therefore, these restricted possibilities limit the exploitation of this system. In 1964, Guha and Maheshwari (1964) reported a new finding. They observed numerous embryos from *in vitro* culture of anthers in *Datura innoxia* Mill. Further, they confirmed that these embryos and regenerated plants had originated from immature pollen grains which were haploids (Guha and Maheshwari, 1966).

This discovery has demonstrated that the male gametophytic cell has totipotency. The immature pollen grains could be stimulated to sporophytic division, which afterwards leads to the production of embryos and complete plants.

Haploidy could be obtained from unfertilized ovule or ovary culture. This approach was unsuccessful until 1960s. Haploid production by ovule or ovary culture brought out of obscurity in 1976. The first gynogenetic haploid plant obtained from barley (Uchimiya *et al.*, 1971; Mullins and Srinivasan, 1976).

1.8.1- Anther culture

This technique is considered one of the most popular ways to

produce haploid plants. In 1953 Tuleck observed for the first time that mature pollen grains of a gymnosperm *Ginkgo biloba* can be induced to proliferate in culture to form haploid calli (Ramawat, 2008).

Successful development of morphogenic calli was obtained from isolated microspores. These calli were differentiated spontaneously to doubled haploid plants (Miyoshi, 1996).

1.8.2-Initiation of embryoids and calli from vegetative cells

Most commonly, embryoids and calli are formed in cultured anthers. This takes place by segmentation of the normally quiescent vegetative cell that loses its morphogenetic individuality and is partitioned by a series of internal walls. These divided cells produce a mass of cells within the confines of the pollen wall. After the rupture of wall, the cellular mass is liberating into the anther locule. Then passes through typical globular, heart-shaped, torpedo-shaped and cotyledonary stages. The generative cell either remains undivided or undergoes few divisions then destroyed at the beginning of the growth of the vegetative cell. The final fate of generative cell is degeneration without contribution to the formation of the embryoid (Sunderland and Wicks, 1971).

This pathway of embryogenesis was described in cultivars of *N. tabacum* (Bernard, 1971) in *Datura metel* L. (Iyer and Raina, 1972) and in *C. annuum* L. (George and Narayanaswamy, 1973).

During calli initiation, the vegetative cell of binuclear pollen grains generates a mass of cells. These cells are apparently disposed to continue unorganized growth as a calli rather than differentiate into embryoids. However, the generative cell as a result of competition for space within the pollen wall probably disintegrates completely (Clapham, 1971).This pathway was investigated in cultivars of *Hordium vulgare* L. (Zhou

and Yang, 1980) as well as in cultured anthers of *Triticum aestivum* L. (Wang *et al*., 1973; Pan *et al*., 1983), *Oryza sativa* L. (Yang and Zhou, 1979) and *Zea mays* L. (Miao *et al*., 1978).

1.8.3- Initiation of embryoids and calli from generative cells

Early cytological observations on the division patterns of the pollen grains in cultured anthers of *N. tabacum* L. provided suggestive evidence for the participation of the generative cell (Nitsch, 1972; Rashid and Street, 1974).

Anthers of (*Hyoscyamus niger* L.) cultured in a mineral salt medium formed a good proportion of embryoids by the division of the generative cell. Variable planes of divisions of these cells give rise to multicellular embryoids. The vegetative cell might be countered various fates, one of which is to remain undivided and passive, or divides to form a two to many cell entities, or its division products contribute with those of the generative cell to form chimeric embryoids at the cellular level. This pathway was investigated in cultivars of maize (Miao *et al*., 1978) and barley (Sunderland *et al*., 1979).

1.8.4- Initiation of embryoids and calli from both vegetative and generative cells

This type of pollen embryogenesis involves both the generative and vegetative cells and their nuclei. Although nuclear divisions are invariably followed by cytokinesis, in extreme cases, the vegetative or the generative cells may go through a free nuclear phase. This situation might occur before their incorporation into an embryoid or a calli (Sunderland

et al., 1979).

After the first haploid mitosis, chromosome complements of the two unlike cells fuse and enter into mitosis simultaneously. This division occurs on a common spindle. The fusion might occur between one or two haploid vegetative nuclei and a haploid or an endopolyploid generative nucleus. Sometimes, the fusion occurs between an endodiploid vegetative nucleus and an endopolyploid generative nucleus. It is believed that this accounts for the frequent occurrence of embryoids with levels of ploidy higher than the expected haploid level. This pathway documented in cultured anthers of *D. innoxia* L.(Sunderland, 1974).

Chapter Two

Materials and Methods

2.1- Materials

2.1.1- Apparatus and equipments

The following apparatus and equipments were used through this experimental work

Apparatus	Company
Autoclave	Karl / Germany
Distillator	GFL / Germany
Electric balance	Mettler (Switzerland)
Incubator	Sanyo / Japan
Laminar air flow cabinet	ESCO /
Micropipettes	Brand / Germany
Oven	Gallenkamp / England
pH-meter	Metter Gmbh-Teledo / England
Refrigerator	Concord / Lebanon
Sensitive balance	Delta range /Switzerland
Light microscope	Olympus/Japan
Digital camera	Sony/Japan
Fluorescence Microscope	Olympus/Japan
Neubauer slide	Metter Gmbh-Teledo / England
Shaker incubator	Sanyo / Japan

2.1.2- Chemicals

Chemicals	Company
2, 4-Dichlorophenoxy acetic acid	BDH
Acetone	Mall
Acetic acid	Mall
Aceto-carmine stain	BDH
Agar-Agar	BDH
Ammonium nitrate	BDH
Boric acid	BDH
Benzyl adenine purine (BA)	BDH
Calcium chloride anhydrate	Mall
Cobalt chloride.$6H_2O$	Mall
Cupric sulphate.$5H_2O$	Mall
Ethanol	Merk
Ferrus sulfite.$7H_2O$	BDH
Flourescein diactate	BDH
Glacial acetic acid	Fluka
Glycine	BDH
Hidrocloric acid	BDH
Kinetin (kin)	BDH
Magnesium sulphate anhydrate	Fluka
Manganese sulphate.$4H_2O$	BDH
Molybdic acid (sodium salt).$2H_2O$	BDH
Myo-inositol	BDH
Naphthalene acetic acid (NAA)	BDH
Sodium hydroxide	BDH
Nicotinic acid (free acid)	Kochligh
Pectenase	BDH
Potassium iodide	BDH
Potassium nitrate	Fluka
Potassium phosphate monobasic	BDH

Pyridoxin. HCl	Tetanal
Sodium ethylene diamin tetracetate	Fluka
Thiamine.HCl	BDH
Zinc sulphate.$7H_2O$	BDH

2.2- Methods

The project was carried out in the plant tissue culture laboratory, Biotechnology Department, College of Science / Al-Nahrain University.

2.2.1- Plant material

Ishtar F1 hybrid eggplant seeds were kindly supplied by Petoseeds Company. Its characteristics are:

- A certified hybrid by the Iraqi ministry of agriculture since 2003.
- Heavy yield and grows in open fields.
- Plants height is 20-45cm (Fig. 1).
- Tolerant to high temperatures.
- Fruit length is 18-23cm.
- Fruits are shiny black, cylindrical and have no bitter taste.
- Fruits color remain black even at high temperatures.
- Leaves cover eggplant fruit.
- Main branches count 3-4 branches.
- Continuous flowering even at high temperatures.

Figure (1): ***Solanum melongena*** L., Ishtar F1 hybrid grown in the garden of the college of science Al-Nahrain University, (photographed by the researcher).

2.2.2- Preparation of culture medium

Two types of culture media, MS and R were used. MS medium was prepared and used as in Table (1). Sucrose 20 g/l, myoinositol 100 mg/l, plant growth regulators (NAA, kin and BA) at different concentrations were added.

The second medium was R medium which was supplemented with sucrose 20g/l, myo-inositol 50.3mg/l, kin and 2,4-D were added at different concentrations. The pH was adjusted to 5.8 using NaOH or HCl (1N), and then 8g/l of the agar type (Agar-Agar) was added to the medium, placed on a hotplate magnetic stirrer till boiling. Aliquots of 20 ml were dispensed into (5x12cm) culture vessels. Autoclaved at 121°C under 1.04 Kg/cm^2 pressure for 15 min..

Table 1: Composition of the two media used in the experimental work.

Components	Chemical formula	MS	R
Macronutrients		**mg/l**	
Ammonium nitrate	NH_4NO_3	**1650**	**1238**
Potassium nitrate	KNO_3	**1900**	**2150**
Calcium chloride.2H_2O	$CaCl_2.2H_2O$	**440**	**313**
Magnesium sulphate.7H_2O	$MgSO_4.7H_2O$	**370**	**412**
Potassium phosphate monobasic	KH_2PO_4	**170**	**142**
Potassium chloride	KCl	**170**	**7**
Sodium di hydrogen phosphate	$NaH_2PO_4.H_2O$	**–**	**38**
Ammonium sulphate	$(NH_4)_2.SO_4$	**–**	**34**
Calcium nitrate.4H_2O	$Ca(NO_3)_2.4H_2O$	**–**	**50**
Micronutrients		**mg/l**	
Boric acid	H_3BO_3	**6.2**	**1.55**

Potassium iodid	**KI**	**0.83**	**0.33**
Manganese sulphate.4H_2O	**$MnSO_4.4H_2O$**	**22.3**	**26.56**
Zinc sulphate.7H_2O	**$ZnSO_4.7H_2O$**	**8.6**	**3.225**
Sodium molybdate. 2H_2O	**$Na_2MoO_4.2H_2O$**	**0.25**	**0.138**
Cupric sulphate.5H_2O	**$CuSO_4.5H_2O$**	**0.025**	**0.011**
Cobalt chloride.6H_2O	**$CoCl_2.6H_2O$**	**0.025**	**0.011**
Chelated Iron		**mg/l**	
Sodium ethylene diamine tetra acetate	**Na_2-EDTA**	**_**	**18.56**
Ferrous sulphate.7H_2O	**$FeSO_4.7H_2O$**	**27.8**	**13.9**
Vitamins and Organics		**mg/l**	
Thiamine. HCl	**$C_{12}H_{17}ClN_4OS.$ HCl**	**0.1**	**0.6**
Nicotinic acid	**$C_8H_{11}NO_3.HCl$**	**0.5**	**0.7**
Pyridoxin.HCl	**$C_6H_5NO_2$**	**0.5**	**5.5**
Ca pantothenate	**$C_{18}H_{32}CaN_2O_{10}$**	**_**	**0.5**
Biotin	**$C_{10}H_{16}N_2O_3S$**	**_**	**0.005**

Glycine (free base)	**$C_2H_5NO_2$**	**2.0**	**0.1**
Myo-inositol	**$C_6H_{12}O_6$**	**100**	**50.3**
Cyanocobalamine (B_{12})	**$C_{63}H_{88}CoN_{14}O_{14}P$**	**–**	**0.03**

2.2.3- Preparation of Carnoy's-I fixative solution

This solution was freshly prepared by mixing 3 parts of absolute alcohol and 1 part of glacial acetic acid as mentioned by (Devi, 2003) sited from (Avers, 1982).

2.2.4- Preparation of Aceto-Carmine stain 2% (w/v)

This stain was prepared by dissolving 2g of Carmine in 100ml of 45% (v/v) acetic acid and refluxed for 2 hrs with gentle boiling then kept in a brown dropper bottle in a refrigerator as mentioned by (Devi, 2003) sited from (Avers, 1982).

2.2.5- Plant growth regulators

Different concentrations of NAA (2.0, 3.5, 5.5 or 8)mg/l, Kin (0.01, 0.02, 0.1, 0.2 or 0.3)mg/l, BA (0.1, 0.2 or 0.5)mg/l and 2,4-D (0.01, 0.02, 1, 2, 5, 8 or 10)mg/l were prepared and added to the culture media as required before autoclaving.

Different concentrations of kin (0.00, 0.01, 0.002)mg/l and 2,4-D (0.00, 0.01,0.02)mg/l were added to R medium.

2.2.6- Sterilization of media and instruments

Culture media were sterilized by autoclaving at 121°C under 1.04Kg/cm^2 pressure, for 15min, while glassware and other instruments were sterilized either by autoclaving or using electric oven (180-200)°C for 2hrs.

2.2.7- Sterilization of seeds

Seeds were rinsed in tap water for 15min. then transferred to the laminar air flow-cabinet where submerged in NaOCl at different concentrations (1,2 and 3)% for 5 or 10min. Seeds were then rinsed with sterilized deionized water 3 times before culturing on MS medium free of plant growth regulators. The resulted seedlings are already sterile and ready for taking explants.

2.2.8- Incubation of cultures

Surface sterilized seeds were transferred into culture vessels (5x12)cm under aseptic conditions, placed in the incubator at 25°C for 16/8 hrs light/dark photoperiod using day light inflorescents at light intensity of 1000 lux.

After 3 weeks, seedlings of about 3-4cm in height were obtained. Stem explants and roots were dissected at 1cm long, while leaves were dissected as discs with 1cm in diameter and wounded with sterile razor. Those explants were instantly cultured on MS medium containing different combinations of plant growth regulators.

2.2.9- Initiation of callus cultures

Different combinations of plant growth regulators were examined to determine the most effective one for callus initiation. Leaf and stem segments were placed onto MS medium containing NAA, kin and 2,4-D.

Cultures were then placed in the incubator at 25^0C for 16/8 hrs light/dark photoperiod. The response of these explants to auxin and cytokinin combinations was evaluated after 21 days in culture to determine the proper combination for callus initiation. Callus cultures were transferred to a plant growth regulator free medium for shoot initiation, for 3 weeks. The percentage of callus induction from stem segments was calculated by dividing the number of explants that formed callus by the total count of the explants multiplied by 100 percentage. The percentage of embryos induced by from cell suspension cultures of anther was calculated by dividing the number of embryos over the total embryo number (callus cells and embryos) then multiplied by 100.

2.3- Maintenance medium

The maintenance medium for callus culture was MS medium free of plant growth regulators.

2.4- Root formation

Roots developed on excised shoots (3 cm in length), transferred to half-strength MS medium containing different concentrations of NAA (0.1, 0.19 or 0.5)mg/l and BA (0.1, 0.2 or 0.5)mg/l.

2.5- Examination of embryos

The embryos are characterized by dense cytoplasm contents, large starch grains, and a relatively large nucleus with a darkly stained nucleolus (Dodds and Roberts, 1995). The following procedure was used for distinguishing embryos from callus cells:

Fixation : A small piece of the embryonic calli was cut and immersed in Carnoy's fixative I solution (3 parts of absolute alcohol to one part of glacial acetic acid) for 24hrs. Embryonic calli were transferred to 70% ethanol and stored in a refrigerator at 4^{o} C for use as required (Dodds and Roberts, 1995).

Staining and Squash preparation: Embryonic calli (that have been fixed and stored) were placed in a watch glass. Callus clump was transferred onto a filter paper in order to remove the acid mixture and then placed on a clean slide. The surface of the embryonic calli which is known to contain the highest percentage of embryos was scratched (periphyral) and placed on a slide. One drop of 2% aceto-carmine was added and the cover slip was fixed. One edge of the cover slip was hold down to prevent any movement. Then a folded filter paper was placed over the cover slip and gentle pressure was applied with the thumb, this helps in separating the embryos.

Embryos were observed in young embryonic calli under light microscope at low and high magnification. The slide was sealed and labeled for further studies.

2.6- Examination of embryos from calli cultures of anthers

The procedure was slightly different with anther culture, since the embryos are single or in small aggregates, as below:

Fixation: A well dispersed drop of the cells suspension was added to Carnoy's fixative I solution (3 parts of absolute alcohol to one part of glacial acetic acid) for about 24hrs, then transferred into 70% ethanol, and was stored in the refrigerator at 4°C for use as required as mentioned by (Devi, 2003) sited from (Avers, 1982).

Staining and Squash preparation: Embryos in liquid medium that was fixed, placed in a watch glass. Nine parts of 2% aceto-carmine stain and 1 part of 1N HCl were mixed, heated gently for 5-10 seconds for three to four times and then was left covered for 10-15min.

The squash was transferred onto a filter paper in order to get rid of the acid mixture and was placed on a clean slide. aliquot of 2% aceto-carmine was added and covered with a cover slip. One edge of the cover slip was held down to prevent movement. Folded filter paper was placed over the cover slip and gentle pressure was applied with the thumb. The thumb was rolled over the folded filter paper, to make embryos in a single layer as mentioned by as mentioned by (Devi, 2003) sited from (Avers, 1982).

Different stages of embryogenesis were observed under the light microscope at x1000. The slides were sealed and labeled for further observations.

2.7-Embryo counting

In order to count embryos, they should be fresh (1-2 weeks old), Pectinase added (to disperse the clumps). Pectinase concentration was 0.025g/ml added to 5 clumps of fresh callus which was macerated by a glass rod. The pH of the solution was adjusted to 5.4 and stored for 72 hrs. One drop was applied to a Neubauer chamber and all squares were counted. The following equation is embryo counting as mentioned by (Devi, 2003) sited from (Avers, 1982).

$$\text{Embryo count} = 5000 \times \text{embryo count average} \times \frac{\text{Total macerated volume}}{\text{Number of callus clumps}}$$

Counting the haploid embryos of the cell suspension was easier than embryos from callus clumps, since single embryos were present. The total count of haploid cells was divided by 0.02 (which represents the volume of the counting area including five grids, top and left border lines). Thus was multiplied by 1000 to obtain the volume in ml. The equation for embryo count/ml is shown below (Klebe and Mancuso, 1982):

$$\textbf{Embryo count} = \frac{\textbf{Embryo total count (5 grids)}}{\textbf{0.02ml (size of the liquid in 5 grids)}} \times 1000$$

2.8- Embryogenesis in cell suspension derived from anther cultures

Callus induced on anthers was cultured on liquid R medium for embryogenesis induction. Each jar (150ml capacity) was containing 20ml of the liquid R medium with about 2g of the fresh callus inoculum and different concentrations of kin (0.00, 0.01, 0.002)mg/l and (0.00, 0.01,0.02) mg/l 2,4-D. The jars were placed in the shaker incubator at 100 rpm. in the dark for 2 weeks for further tests (photography, counting and viability test).

2.9- Viability test

Viability test is much like the counting test using a counting chamber (Neubauer haemocytometer) plus adding Flourescein diactate (FDA) to view and count the viable embryos using UV light. The (FDA) is fluorescing, non-polar substance that penetrates living cell membrane only. When embryos were exposed to UV light they emitted a green color while dead ones were invisible (they remained in a dark background).

The viability percentage was calculated by dividing the number of viable embryos by the total embryo count.

A drop from the fresh cell suspension was added to another drop of 0.01% mg/l Flourescein (0.1g of the Flourescein was defused in 10ml of Acetone), and then a drop was placed on the counting chamber, cover slip was then placed on the surface of the counting chamber. After 5-10min, embryos were examined under light microscope at high magnification (x1000) for observation and counting the viable embryos in each chamber of the five grids. Counting started from the top of the square to the bottom (Klebe and Mancuso, 1982) and (Tassin and Jacques, 2005). The viability percentage was recorded according to the following equation:

Viability (%) = (Viable embryo count/Total embryo count) x 100

2.10- Number of replicates

Twelve replicates representing the number of cultured explants were used. The standard errors of the mean percentages were calculated. Five replicates were used for the initiation of roots on shoots. For the induction of embryogenesis on anther calli, 10 replicates were used.

Chapter Three

Results

and

Discussion

3.1- Results and Discussion

3.1.1- Sterilization of seeds

Different concentrations of NaOCl (1, 2 or 3%) were used for seeds surface sterilization at two periods 5 or 10min.

Seeds which surface sterilized with 1% of NaOCl for 10min gave 70% of non-contaminated seedlings (Fig. 2).

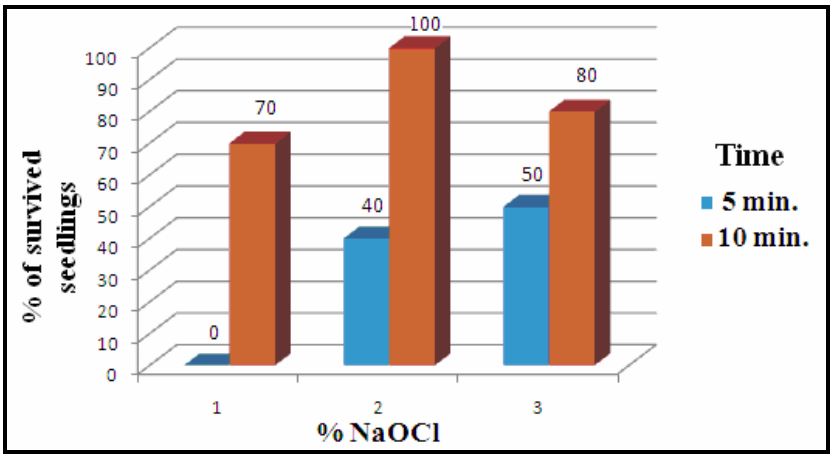

Figure (2): Effect of different concentrations of NaOCl on percentages of survived seedlings at sterilization periods of 5 and 10 min.

Seeds that surface sterilized at (1% NaOCl) for 5min showed 0% of seedlings survival. Maximum percentage (100%) of non-contaminated seedlings was obtained with 2% of NaOCl for 10min while (40%) of survived seedlings occurred with the same concentration at 5 min The percentage of survived seedlings was (80%) with 3% of NaOCl for 10min and 50% with the same concentration for 5min. Percentages of germinated seeds that were surface sterilized with NaOCl are displayed in (Fig. 3).

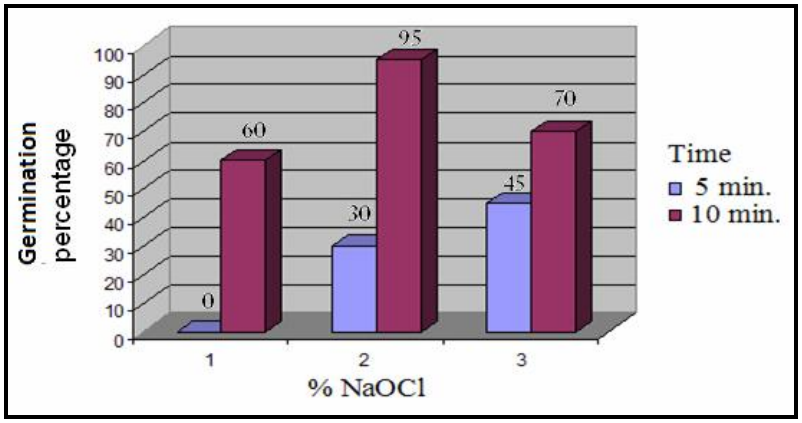

Figure (3): Effect of different concentrations of NaOCl on seed germination percentage at surface sterilization periods of 5 and 10min.

The percentages of germinated seeds that were surface sterilized with 1% NaOCl were 0 or 60% at 5min and 10min. respectively.

Maximum percentage (95%) of germinated seeds was obtained with 2% NaOCl for 10min, while reduced to 30% using the same concentration for 5min. Germination reached 70% in seeds that were surface sterilized with 3% NaOCl for 10min, whereas 45% of seeds showed germination at the same concentration for 5min.

The sterilization material should be easy to be removed from seeds when washed with distilled water. Pierik, (1987) referred to the importance of using NaOCl for seeds surface sterilization.

Increasing the surface sterilization period and concentration often lead to serious reduction in germination rate. Optimization experiments are therefore necessary to achieve maximum germination rate with minimum contamination.

The type, concentration, plant material surface roughness and duration of exposure of the particular sterilant to be used are dependent on the plant material under use. A drop of the detergent or wetting agent (Tween-80) enhances the penetration and effectiveness of the surface sterilization.

Chlorine combines with water to form hypo chlorous acid (HOCl), a strong oxidizing agent:

$$Cl_2 + H_2O = HOCL$$

$$Cl_2 + 2NaOH = NaOCl + H_2O$$

Eliminating contamination by using NaOCl was important. It's used widely for tissue surface sterilization (Pierik, 1987).

3.1.2- Induction of somatic embryogenesis via calli cultures (indirect)

The effect of different levels of kin and NAA on calli induction on stem segments is shown in (table 2).

The highest mean of calli induction was initiated on MS medium supplemented with 0.1mg/l kin (63.36%). Lower percentages (38.68, 11.44 and 20.88%) of calli induction were recorded on explants grown on MS medium supplemented with 0.2, 0.3 and control treatments.

Table (2): Percentages of explants induced calli on stem segments grown on MS medium containing 7g/l agar supplemented with different concentrations of NAA and kin and their interactions ± SE (n=12).

kin (mg/l)	NAA (mg/l)					Mean
	0.0	**2.0**	**3.5**	**5.5**	**8.0**	
0.0	0.0±0.0	10.8±0.9	21.6±0.9	32.4±0.9	39.6±0.9	20.88
0.1	32.4±1.2	50.4±1.2	64.8±1.2	79.2±1.2	90.0±1.2	63.36
0.2	18.0±1.1	25.2±1.1	35.0±1.1	50.4±1.1	64.8±1.1	38.68
0.3	10.8±0.6	18.0±0.6	28.4±0.6	0.0±0.0	0.0±0.0	11.44
Mean	15.3	26.1	37.45	40.5	48.6	

Maximum mean of calli initiated occurred on MS medium supplemented with 8mg/l NAA (48.6%). The percentages (40.5, 37.45, 26.1 and 15.3%) were recorded at the levels 5.5, 3.5, 2.0mg/l and control respectively.

The interaction between the two growth regulators gave maximum response (90%) of calli induction at the combination of 0.1mg/l kin and 8.0mg/l NAA. The percentages (79.2, 64.8 and 50.4%) were obtained at the combinations of 0.1mg/l kin and (5.5, 3.5 and 2.0)mg/l of NAA respectively.

In other combinations of kin and NAA, the percentage of explants showed calli induction were varied from low values to zero.

Calli induced on stem segments is shown in (Fig. 4).

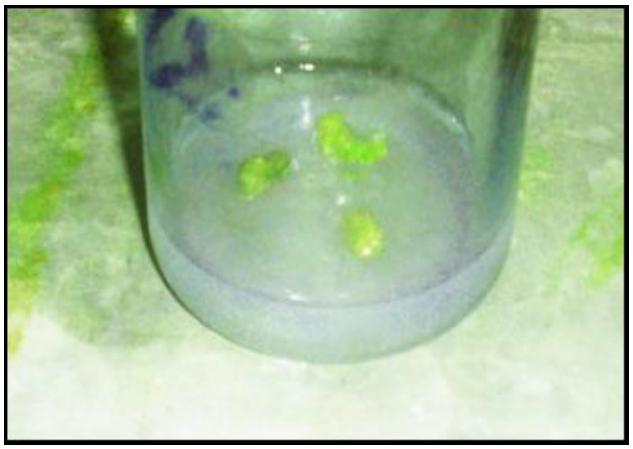

Figure (4): Calli initiation on stem segment explants grown on MS medium containing 7g/l agar supplemented with 8mg/l NAA and 0.1mg/l kin after 2 weeks of culture.

It is well known that cytokinins induce cell division, enlargement and differentiation. Cytokinins probably act at the molecular level causing cell division (Street, 1977). NAA as an auxin has a role in increasing the plasticity of the cell wall. When extensibility of the wall is increased, the turgor pressure caused by osmotic force in the cell sap causes water to enter the cell resulting in cell enlargement. Plasticity is

not reversible as a result of breaking the cross-links between the cellulose microfibrils of the cell wall (Roberts and Hooley, 1988).

The combinations of 2,4-D and kin for induction of embryogenesis resulted in a low induction percentage, the results are as shown in (table 3).

Table (3): Percentages of explants induced calli on stem segments grown on MS medium containing 7g/l agar supplemented with different concentrations of 2,4-D and kin and their interactions ±SE (n=10).

kin (mg/l)	2,4-D (mg/l)						Mean
	0.0	**1.0**	**2.0**	**5.0**	**8.0**	**10.0**	
0.0	0.0±0.0	0.0±0.0	0.0±0.0	6.0±0.7	9.0±0.7	15.0±0.7	6.0
0.1	0.0±0.0	12.0±0.5	18.0±0.5	21.0±0.5	27.0±0.5	30.0±0.5	21.6
0.2	0.0±0.0	0.0±0.0	9.0±0.7	12.0±0.7	18.0±0.7	24.0±0.7	12.6
0.3	0.0±0.0	0.0±0.0	6.0±0.5	9.0±0.5	12.0±0.5	18.0±0.5	9.0
Mean	0.0	3.0	8.25	12.0	16.6	21.75	

Stem segments grown on MS medium containing 0.1mg/l kin recorded (21.6) mean value of calli induction.

The mean (9.0, 12.0, and 6.0) were obtained when the explants grown in the presence of (0.3, 0.2 and 0.0)mg/l kin respectively.

Maximum mean of calli induction obtained on explants initiated on MS medium supplemented with 10mg/l 2,4-D was (21.75%) followed by (16.6, 12.0, 8.25, 3.0 and 0.0%) at concentrations (8, 5, 2, 1 and 0)mg/l respectively.

The combination between the two growth regulators gave maximum response (30.0%) of calli induction at 0.1mg/l kin and 10mg/l 2,4-D (Fig. 5). Other values varied according to the level of 2,4-D in the

medium. The inclusion of the auxin 2,4-D in the culture media may regulate cell elongation, tissue swelling, cell division, formation of adventitious roots, inhibition of adventitious and auxiliary shoots formation, calli inhibition and induction of embryogenesis (Collin and Edwards, 1998). Establishment of calli from the explants was illustrated by Dodd's and Roberts (1995) who divided the process into three developmental stages: induction, cell division and differentiation. The length of these phases depends mainly on the physiological status of the explants cells as well as the cultural conditions including the appropriate combination of plant growth regulators.

Figure (5): Calli initiated on stem segment explants grown on MS medium supplemented with 2,4-D at 10mg/l and 0.1mg/ l kin after 2 weeks in culture.

Calli cultures derived from anthers were kindly supplied by Mr. Ahmad K. Ali (post graduate student in the Biotechnology Department, College of Science, Al-Nahrain University). Calli cultures grown on C medium (Dumas de Vaulx *et al.*, 1981) are shown in (Fig. 6).

Figure (6): Calli initiated on anthers grown on C medium containing 5mg/l of 2, 4-D and kin under continuous dark then transferred to R medium containing 0.1mg/l kin after 40 days.

3.1.3- The effect of decreasing agar concentration on calli induction

Agar was reduced from 7g/l to 5 g/l (table 4). The highest mean (73.44%) of calli induction was recorded on explants grown on MS medium containing 0.1mg/l kin.

Table (4): Percentages of stem explants induced calli grown on MS containing 5g/l agar, medium supplemented with different concentrations of NAA, kin and their interaction ± SE(n=12).

kin (mg/l)	NAA (mg/l)					Mean
	0.0	2.0	3.5	5.5	8.0	
0.0	10.8±2.0	18.0±2.0	32.4±2.0	43.2±2.0	50.4±2.0	30.96
0.1	43.2±1.2	61.2±1.2	75.6±1.2	90.0±1.2	97.2±1.2	73.44
0.2	21.6±1.1	36.0±1.1	46.8±1.1	61.2±1.1	75.6±1.1	48.76
0.3	18.0±0.6	28.8±0.6	39.6±0.6	14.4±0.6	10.8±0.6	21.32
Mean	23.40	36.00	48.60	52.20	58.50	

Nutrient uptake by explants and thereafter calli is higher when they are grown in liquid or semi-solid medium compared to explants grown on solid medium. Water uptake by plant cells is ruled by the relative potential values of water between the vacuolar sap and the medium. Agar is one of the major components of the nutrient medium that influence water availability. The disadvantage of semi-liquid medium is that its rapidly exhausted and evaporated, that makes it suitable only for short term cultures, it is recommended to use agaros in such sensitive cultures (Dodds and Roberts, 1995).

3.1.3- Induction of somatic embryogenesis

Embryogenic calli were obtained from calli cultures at high rate after transfer from the induction medium to MS medium lacking plant growth regulators. Shoots appeared on embryogenic calli after five weeks in culture (Fig. 7).

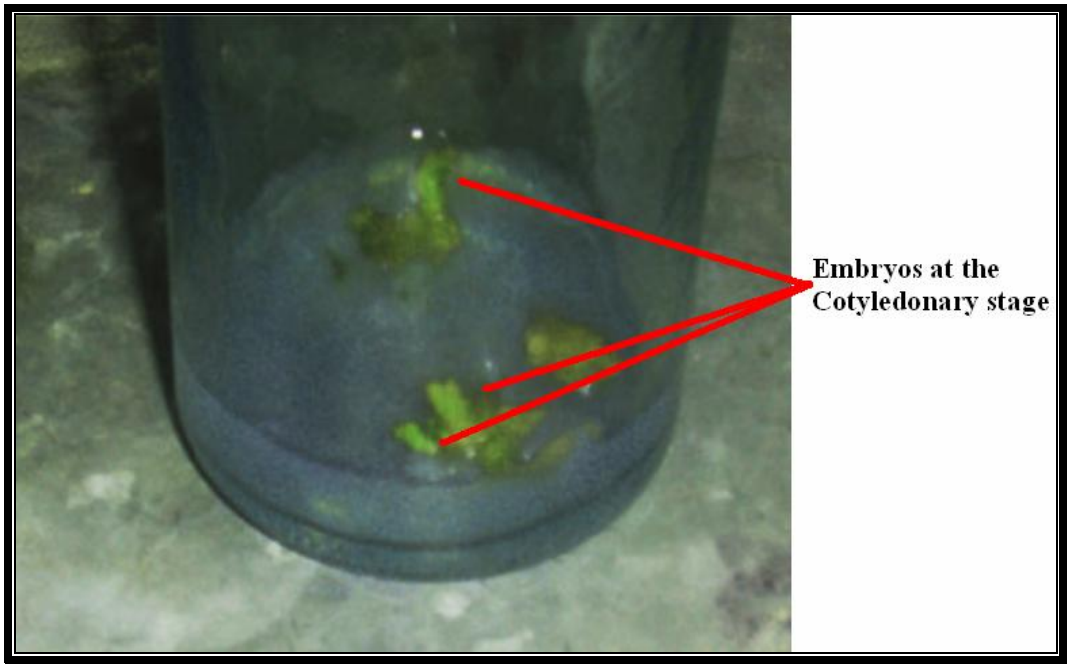

Figure (7): Embryos appearance on calli grown on MS medium free of PGRs after 5 weeks in culture.

Two distinctly different types of media are needed for induction of somatic embryogenesis; the first medium of initiation of the embryogenic cells must contains auxin, the second medium generally lack of auxin or a lower concentration of the same auxin (Ammirato, 1987).

Well developed shoots were formed on the same cultures mentioned above when left for further 2 weeks (total 7 weeks) as shown in (Fig. 8).

Figure (8): Shoot formation after 7 weeks on PGRs free MS medium.

Shoot formation does not occur on the induction medium, but can initiated only after transfer of the calli to a PGRs free medium (Walker *el al.*, 1979).

Roots were developed at the bases of the shoots when transferred to half-strength MS medium containing different concentrations (0.0, 0.1, 0.2 or 0.5)mg/l of BA and (0.0, 0.1, 0.2 or 0.5)mg/l NAA after 4 weeks in culture (Table 5).

Table (5): Percentages of shoots developed roots grown on half-strength MS medium containing 7g/l agar supplemented with different concentrations of NAA and BA and their interactions ±SE (n=5).

BA (mg/l)	NAA (mg/l)				Mean
	0.0	**0.1**	**0.2**	**0.5**	
0.0	0.0±0.0	4.5±4.7	16.5±4.7	19.5±4.7	10.1
0.1	6.0±3.5	12.0±3.5	40.5±3.5	30.0±3.5	22.1
0.2	7.5±2.4	25.5±2.4	31.5±2.4	28.5±2.4	23.25
0.5	10.5±1.8	19.5±1.8	30.0±1.8	22.5±1.8	20.6
Mean	6.0	15.5	29.6	25.1	

The highest mean of root formation obtained on shoots grown on MS medium supplemented with 0.2mg/l BA was 23.25%, while 20.6, 22.1 and 10.1% obtained at the concentrations 0.5mg/l, 0.1mg/l BA and non-treated ones respectively.

The highest mean of rooting was recorded at 0.2mg/l NAA (29.69%). Other percentages were 25.1, 15.5 and 6.0% occurred at 0.5, 0.1mg/l NAA and 0.0 respectively.

The interaction of the two growth regulators resulted in 40.5% rooting when the medium was supplemented with 0.1mg/l BA and 0.2mg/l NAA as shown in table (5) and (fig. 9). The percentages obtained from the interaction between 0.1mg/l BA and (0.1 or 0.5)mg/l NAA were 12.0 and 30.0% respectively.

In other combinations of BA and NAA which were (0.2, 0.1; 0.2, 0.2; 0.2, 0.5; 0.5, 0.1; 0.5, 0.2 or 0.5, 0.5)mg/l, the percentages of rooting

induction were (25.5, 31.5, 28.5, 19.5, 30.0 and 22.5%) respectively, as shown in table (5) and (Fig. 9).

Figure (9): Root development on shoots grown on half strength MS medium containing 0.2mg/l NAA and 0.1mg/l BA after 4 weeks.

Root initiation occurs frequently after cultured tissues had produced shoots, that had alter the endogenous hormones within the culture (Gresshoff, 1978).

Auxins, in some cultures, promotes root formation (Thomas and street, 1970).

3.1.4- Effect of different levels of 2,4-D and kin on calli cultures initiated on anthers

Fresh anther calli was used for the induction of embryogenesis in liquid medium. Different concentrations of kin (0.00, 0.01 or 0.02mg/l) and 2,4-D (0.00, 0.01 or 0.02mg/l) were supplied to the liquid medium and kept in a shaker incubator at 100rpm for 2 weeks in the dark.

Table (6): Percentages of embryogenesis in anther calli grown on R medium supplemented with different concentrations of 2,4-D and kin and their interactions ±SE (n=10).

kin (mg/l)	2,4-D (mg/l)			Mean
	0.00	**0.01**	**0.02**	
0.00	0.0±0.0	28.0±3.3	44.0±3.3	24.0
0.01	9.0±5.2	60.0±5.2	81.0±5.2	50.0
0.02	18.0±3.2	39.0±3.2	63.0±3.2	40.0
Mean	9.0	42.3	62.6	

Table (6) shows maximum mean of embryogenesis was obtained from cell suspension cultures of anther calli grown on R medium supplemented with 0.01mg/l kin 50.0% while, 24.0 and 40.0% obtained at the concentrations 0.02mg/l kin and control treatment respectively.

Maximum mean of embryogenesis induction was recorded at 0.02mg/l 2,4-D 62.6, while other means were 9.0 and 42.3. The highest percentage obtained from the interactions of the two growth regulators was 81.0% at 0.02mg/l 2,4-D and 0.01mg/l kin. The percentages obtained from the interaction between 0.01mg/l kin and 0.01mg/l 2,4-D was 60%.

In other combinations of kin and 2,4-D (0.02, 0.01 and 0.02, 0.02)mg/l, the percentages of embryogenesis induction were 39.0 and 63.0% respectively.

Embryogenesis may be affected by many factors like the nutrient composition, exogenous plant growth regulators, evolved gases and light. The composition greatly affects somatic embryogenesis induction. Reduced nitrogen in the embryo development medium supports embryo development, beneficial effect of certain amino acids like proline and glutamine have established. Increasing the osmotic concentration by increasing sucrose levels or by addition of mannitol or sorbitol, have

shown to affect the embryo development. Auxins, especially the synthetic auxin (NAA) appears to be required for embryo induction but adversely affect embryo development. Cytokinins generally suppress embryogenesis(Ramawat, 2008).

3.1.4- Embryo photography

Two types of embryos were photographed. The first one was obtained from calli clumps initiated on stem segments, that grown on MS medium supplemented with 8mg/l NAA, 0.1mg/l kin and 5g/l agar after one week of establishment on PGRs free medium (Fig. 10, A) The second one was obtained from anther cell suspension culture grown on R medium supplemented with 0.02mg/l 2,4-D and 0.01mg/l kin after two weeks of establishment on germination medium (Fig. 10, B).

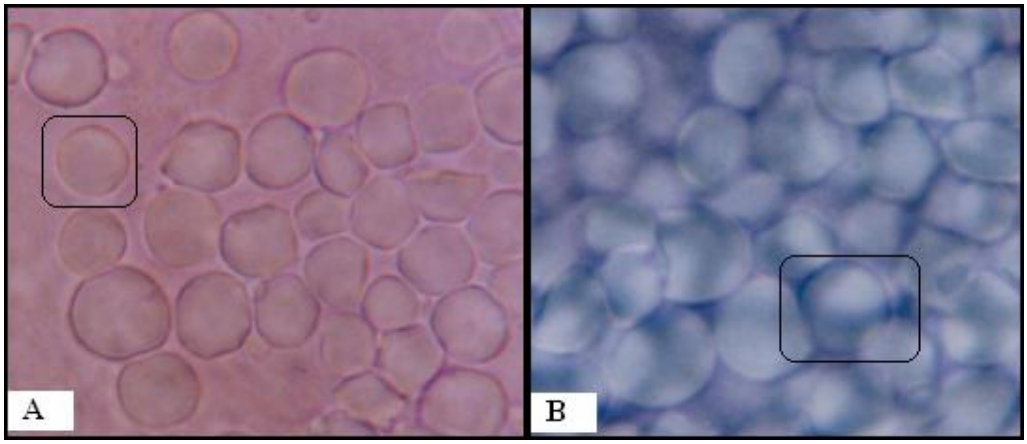

Figure (10): Embryos at the globular stage under light-microscope (x1000). (A). from calli clumps (B). from anther cell suspension cultures.

Photographed embryos showed small uniform isodiametric (embryos), results agree with those of (Ramawat, 2008), who stated that embryogenesis occurs from a single cell or a group of cells, their division ultimately produce embryos of similar shape, small, isodiametric in

shape, cytoplasmically dense and have a conspicuous nucleus. In comparison to embryos, non-embryogenic cells are relatively large, vacuolated and lack dense cytoplasm.

3.1.5- Embryo count

Embryo count from calli clumps was conducted. Embryos were counted from the equation:

$$\textbf{Embryo count} = 5000 \times 229 \times \frac{13}{5} = 2977000 \text{ embryo/ml.}$$

Counting of anther culture embryos was conducted according to the equation below:

$$\textbf{Embryi count} = \frac{71}{0.02} \times 1000 = 3550000 \text{ embryo/ml.}$$

According to (Street, 1977), cell suspension cultures embryo count must be equivalent or more than $3x10^3$embryo/ml. and the above results agree with these findings.

3.1.6- Viability test

The benefit of the viability test is to obtain the percentage of viable embryos within a population. The dead embryos percentage may also be an indicator of the lost embryos that may lead to designate the problem within the technique.

The percentage of viable embryos is an indication of the number of embryus that can be introduced for synthetic seed and their approximate germination in the field, as in the equation below:

Viability (%) = 2560000 / 3550000 X 100 = 72% viable embryos.

The photographed viable embryos are shown in (Fig. 11).

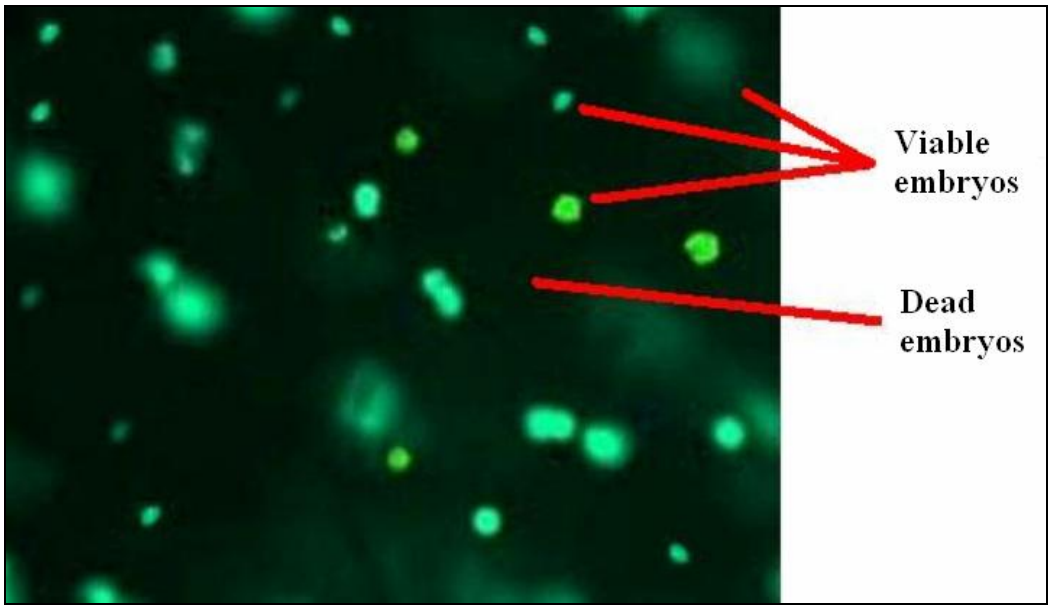

Figure (11): Viable embryos under fluorescence-microscope (X1000)**.**

Embryo count indicated that 72% of embryos were viable (2560000/3550000).

The above result of viable embryos was an indicator of how much embryos are viable and that 72% of the embryos can be coated as a synthetic seed and introduced as artificial seeds.

The above work demonstrated that somatic embryos could be derived either from calli clumps or anther calli cultures.

Conclusions

and

Recommendations

Conclusions

1- High percentage 91% of stem segment calli initiated on MS medium supplemented with 8mg/l NAA and 0.1mg/l kin.

2- Somatic embryogenesis was induced on calli initiated on stem segments using free PGRs MS medium. Shoots developed from embryogenic calli after 30 days further subculture on MS medium free of PGRs. Regenerated shoots developed roots after transfer to a half strength MS medium supplemented with 0.2mg/l NAA and 0.1mg/l BA.

3- Decreasing the Agar-Agar concentration from 7 g/l to 5g/l increased calli induction by 8% and improved embryogenesis.

4- Somatic embryogenesis was induced from anther calli on R medium supplemented with 0.02mg/l 2,4-D and 0.01mg/l kin.

5- Globular stage was obtained from calli initiated on stem segments and anther cell suspension cultures.

6- Embryos count was 2977000embryo/ml, in calli initiated on stem segments.

7- Embryos count in anther cell suspension culture was 2560000embryo/ml.

8- Percentage of viable embryos was 72%, in suspension cultures of anther calli.

Recommendations

1- Investigation of other physiological and nutritional factors such as pH, liquid medium, gelling agents, nitrogen sources and natural nutritional additives to improve somatic embryogenesis.

2- Investigation of all developmental stages that undergo embryogenesis in S. melongena and other crops

3- production and encapsulation of somatic embryos to produce synthetic seeds for the economical advantages.

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الملخص

هذا المشروع تم تصميمه لتحفيز تكوين الاجنه ألجسميه من مختلف أجزاء النبات التي تم تقطيعها من الباذنجان *Solanum melongena* L. .

في هذه الدراسة، تم تحفيز الكالس من قطع الساق على وسط MS المزود بـ8 غم/لتر من NAA و0.1 ملغم/لتر من الكاينتين.أدى تقليل الآكار إلى 5 غم/لتر إلى زيادة تحفيز الكالس من خلال زيادة نسبة التحفيز و المدة المطلوبة للتحفيز.صور الكالس الناتج من قطع الساق تحت المجهر الضوئي بعد أسبوع من زراعته على وسط MS الخالي من منظمات النمو بعد تحفيز تكوين الاجنه ألجسميه, ظهرت اجنه جسميه بالمرحلة Globular من التطور ألجنيني, أدى تقليل تركيز الأكار إلى تسريع ورفع نسبة تحفيز تكوين الاجنه ألجسميه. تطورت السيقان و الأوراق من الكالس ألجنيني بعد 30 يوما على وسط MS الخالي من منظمات النمو، والذي نقل إلى وسط MS مزود بـ0.02 ملغم/لتر من NAA و 0.01 ملغم/لتر من BA. تطورت الجذور عن الأفرع على هذا الوسط بعد 30 يوما. أعيدت زراعة مزارع ألمتك على وسط R السائل في معلق خلوي مزود بـ0.02 ملغم/لتر من 2,4-D و 0.01 ملغم/لتر من الكاينتين في الظلام في حاضنه هزازه على 100 دوره/دقيقه .صورت تحت المجهر الضوئي بعد أسبوعين و أظهرت نشوء أجنه جسميه في مرحله Globular من التطور. تم عد الاجنه في الكالس ألجنيني ووصل العدد الى 2977000 جنين/مل بينما كان عدد الأجنة في المعلق الخلوي 3350000 جنين/مل. جرى اختبار الحيوية على معلق الخلايا ألجنينيه وكانت نسبة الخلايا ألحيه 72%.

الإهداء

إلى قدوتي الذي علمني الاصرار على التفوق...

والدي الحبيب

إلى القلب الذي اتسع لكل الحنان...

والدتي الحبيبة

إلى سندي الدائم...

اخي العزيز

إلى من اعز واقرب إلى نفسي..

نينوس و نوئيل

اهدي جهدي المتواضع

حسام

**قُل لَّو كَانَ البَحرُ مِدَاداً لِّكَلَمِـٰتِ رَبّي لَنَفِدَ البَحرُ قبلَ أن تَنفَـدَ كَلَمِـٰتُ رَبّـي وَلَـو جِئنَـا بمثلِـهِ مَـدَدا*قُـل إنَّمَـا أنَابَشَرُمِّثلُكُم يُوحَىٰ إلىَّ أنَّمَـا إلـٰهكم إلـٰهٌ واٰحِدٌ فمَن كَـانَ يَرجوٌاْ لِقَاءَ رَبهِ فليَعمَل عَمَلاً صَـٰلِحاً وَلَا يُشرِك بعِبَـادَةِ رَبّهِ أحَدا*

صدق الله العظيم

سورة الكهف
الآية (109، 110)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانه الأحيائيه

تحفيز تكوين الأجنه الجسميه في نبات الباذنجان

Solanum melongena L.

رساله
مقدمه الى كلية العلوم - جامعة النهرين
وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانه الأحيائيه

من قبل
حسام احمد محمود
بكلوريوس تقانه أحيائيه جامعة النهرين 2005

باشراف

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محرم 1430
كانون الثاني 2009

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سنة التخرج : 2005-2006

عنوان الاطروحه : تحفيز تكوين الأجنه الجسميه في نبات الباذنجان Solanum melongena L.

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تأريخ المناقشه : 2009/1/21