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

Standardization of protoplast isolation, purification and fusion in tomato hybrids

A Thesis

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

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B.Sc. in Biotechnology, Al-Nahrain University, 2003

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September **2008**

Ramadan **1429**

Acknowledgments

With great pleasure and deepest sense of gratitude, I would like to thank my supervisor Dr. Kadhim M. Ibrahim Al-Sumayday for his valuable advice, guidance and encouragement throughout the period of my study.

Grateful thanks should be going to all the staff of the biotechnology department, college of science, Al-Nahrain University, for their help encouragement throughout my study.

I would like to express my Special Grateful thanks to Mr. Oday Adnan Maqadsi, Mrs. Mayassa al-Romani and Mr. Hasan Hadi al-Janabi for their close friendship.

Grateful thanks should be expressed to all my Colleagues in biotechnology department, and Special thanks to my friend Hydar, Anas, Rajwan, Arwa, Mariam, Belal, Omar, Akram, Salam, Ali, Moyassar, Ahmed, and Hussam.

My Special, Deepest, Grateful thanks, should be expressed to my family.

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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Abstract

In an attempt to achieve optimum conditions for protoplast isolation, purification and fusion in two tomato (*Lycopersicon esculentum*) hybrids (Hatouf and Barakah). Several experiments were carried out.

Callus was induced on leaves explants and maintained on MS medium supplemented with (2.0) mg/l BAP and (0.5) mg/l NAA.

Optimum conditions for protoplasts isolation from leaf and callus were investigated. Results showed that the optimum conditions for protoplasts isolation was achieved using enzyme mixture (pectinase 0.5%, cellulase 2.0% and himecellulase 2.0%), at pH 5.4 and temperature 25°C for 24 hrs. and the number of isolated protoplasts from leaves under these conditions were 1950000 protoplast/ml and 3500000 protoplast/ml from callus.

Results showed that using several temperatures (20, 23, 28 or 30°C) gave protoplasts number isolated from leaves (600000, 1800000, 2150000 and 450000 protoplast/ml) respectively and (1500000, 3250000, 3750000 and 800000 protoplast/ml) from callus respectively. The use of several pH values (5, 5.2, 5.8 or 6) gave protoplasts number (900000, 1700000, 1550000 and 700000 protoplast/ml) from leaves and (1750000, 3100000, 2600000 and 1100000 protoplast/ml) from callus respectively. While the use of several incubation periods (18, 48 or 72hrs.) gave protoplasts number isolated from leaves (1500000, 1400000 and 600000 protoplast/ml) and (2100000, 2350000 and 950000 protoplast/ml) from callus.

The optimum conditions for protoplasts fusion between two tomato varieties (Hatouf and Barakah) were carried out. Results showed that adding deferent molecular weights of Polyethylene glycol (2000, 4000, 6000 or 8000 m.wt) gave fusion percentage (25, 35, 50 and 15%).

Results showed that the use of different chemical fusogens ($NaNO_3$ and Ca^{++} at high pH 10.5) gave fusion percent (10 and 35%) respectively.

Optimum conditions for protoplast fusion were achieved by adding Polyethylene glycol (PEG) 6000 (m.wt) + Ca^{++} at 10.5 pH, since such circumstances recorded the highest percentage of fused protoplast (60%).

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List of Abbreviations

Abbreviation	Full name
FDA	Flourescein diacetate
mpd	Minimum plating density
PEG	Polyethylene glycol
UV	Ultra violate light
°C	degree Celsius
NAA	1-naphthalene acetic acid
DDH_2O	Double distilled water
BAP	Benzyl adenine purine
TYLCV	Tomato yellow leaf curl virus
TOMV	Tomato mosaic virus

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1.1- Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the major vegetable crops grown in Iraq, member of the *Solanaceae* family, which includes about 1,500 tropical and subtropical species. Large-scale cultivation of tomato did not begin until about a century ago then it became generally cultivated only after the First World War. Now it is consumed all over the world and it is the second largest vegetable crop in terms of the economic value. The percentage of total world production of tomatoes was 15% and 0.62% for U.S.A and the Middle East, respectively (Thaman *et al*., 1994).

Tissue culture is a powerful tool that gives the possibility to grow millions of cell under controlled conditions, and to get preliminary physiological information about the behavior of the plant cells under stress conditions (Stefano and Edoardo, 2003).

Plant tissue culture and molecular genetics have opened new avenues in plant improvement. Screening and selection at the plant cell level has established plant clones with enhanced tolerance to virus environmental stresses like salt, heat, cold, drought, disease, insects, heavy metals and herbicides.

The development of protoplast systems has increased the versatility of plants for use in both biochemical and genetic research. They have become indispensable tools in genetic engineering and crop breeding. Of all the possible starting points for plant genetic manipulation, protoplasts offer the opportunity to take advantage of all the technologies now available (Maheshwari *et al.,* 1986).

The success of a protoplast culture system primarily lies with consistent yields of a large population of uniform and highly viable protoplasts. Several

protoplast isolation and purification protocols have been published to optimize the yield and reproducibility. They are often procedures of elaborate nature, labour-intensive involving too many explants or protoplast handling steps, and require extended exposure of explants to digestion environment. Further, the efficacy of such protocols or that of enzyme combinations used therein could be limited to a few plant species. These restrictions must be overcome by improvement of the existing conditions and methods. A number of commercial cellulases, pectinases and himecellulasae which allow protoplast release are available. By manipulating the source and concentrations of these, protoplasts may be released from most tissues, however, generalizations cannot be made (d'Utra Vaz *et al.,* 1992).

A simple method in which slightly elevated temperatures and a set of new enzymes that are efficiency at higher temperatures have functioned synergistically to release protoplasts with relative ease in a number of plant species (Sankara Rao and Srikantha 1986).

Protoplast fusion or somatic hybridization is one of the most important uses of protoplast culture. This is particularly significant for hybridization between species or genera, which can not be made to cross by conventional method of sexual hybridization.

Although somatic hybridization was successfully achieved first in animals, Kohler and Milstein reported a method to produce large quantities of specific antibody. The monoclonal B cell and myeloma tumor cell were found by utilization sendaivirus to produce hybridoma cell capable of producing significant amounts of an antibody specifically targeting the chosen antigen, and only later in plants, its significance has been realized fully in plants

because the hybrid cells can be induced to regenerate into whole plants (Kohler and Milstein, 2005).

Somatic hybridization is generally used for fusion of protoplasts either from two different species (interspecific fusion) or from two diverse sources belonging to the same species (intraspecific fusion). To achieve this objective, spontaneous fusion may be of no value, and induced fusion requiring a suitable agent as (fusogen) is necessary. In animals, inactivated Sendai virus is needed to induce fusion (Morgan and Sally, 2003).

The aim of this study was to standardize protoplast isolation, purification and chemical fusion in tomato hybrids.

The detailed aims of this work were:

1. To determine the optimum conditions for protoplasts isolated from two tomato hybrids by using many factors.
2. Purification, counting and viability testing of isolated protoplasts.
3. To determine the optimum conditions for protoplasts fusion method between two tomato hybrids.

1.2- Literature review

1.2.1- Tomato *(Lycopersicon esculentum)*

The word tomato is derived from a word in the Nahuatl language, the name Lycopersicon derived from the Greek word "Lycos" meaning wolf and the "persicon" meaning peach, it may refer to the tomato's inferiority to the peach (Cutler, 1998).

Lycopersicon esculentum common names are: Tomato, Giant Berry, Cherry tomato, Currant tomato and Pear tomato (Harlan, 1971).The tomato belongs to the genus *Lycopersicon* of Solanum or Night shade family, with 24 chromosomes, which is in the same family, as potatoes, most of them are important in economic terms (Rick *et al*., 1996).

Figure (1) Vegetative growth of *Lycopersicon esculentum* for hatouf hybrid. (Cultured in college nursery).

Tomato plant does not tolerate frost, grows as annual in cold regions. In warmer regions, tomatoes are perennial in protected agriculture, and flower

regardless of day length (Darwin, 2003).Their leaves are 10-25 cm long, the flower are 1-2 cm across, yellow, with five pointed lobes on the corolla (Peralta and Spooner, 2000).

Solanacae family is distributed in the Mediterranean and South America regions (Spooner *et al*., 2002). Geographically, the plant distribution is spread in Palestine, Lebanon, Syria, Turkey, Cyprus, Greece, China, Fiji and Northern Andes and in most countries (Beineke, 1989).

Carter *et al*., (1988) considered this plant indigenous to the Western Asia and Eastern Mediterranean region. The plant also found in South America, Southern Europe, Northern Africa and North America.

1.3- Plant Tissue Culture

Plant tissue culture techniques have many applications in life. It can be defined as the production of plants from very small parts (such as shoot tip, axillary's buds, nods or rhizomes) in sterile controlled environment (Stiff, 2006). Plant tissue culture is used to describe the *in vitro* and aseptic growth of any plant part on nutrient medium. This technology is based on three fundamental objectives:

1- The part or explant isolated from the rest of plant body.
2- The explant maintained in controlled physically (environment) and chemically defined (nutrient medium) conditions.
3- Aseptic must be maintained.

Whether the techniques are being used for simple propagation, as a method to study genetic, metabolic, or developmental changes in model system, or for the creation of new plant variations via genetic engineering.

The advantages of plant tissue culture techniques over traditional breeding methods are outlined by Gibbs, *et al*., (1989) and (Collin and Edwards, 1998) as follows:

a- Plant cells can grow in liquid culture on a large scale to provide convenient and profitable source of plant secondary products than the intact plants.

b- Time for production of dihaploid plants from haploid cultures reduced to achieve uniform homozygous line and varieties.

c- The crossing of distantly related species by protoplast isolation and somatic fusion increases the possibility for the transfer and expression of novel variation in domestic crops.

d- Cell selection increases the potential number of individuals in a screening program so that, in an *in vitro* or cell screen for salt tolerance or herbicide can be screened in a limited space very rapidly.

e- Micropropagation using meristem and shoot culture techniques allows the production of large numbers of uniform individuals of horticultural species from limited starting material.

f- Genetic transformation of cells enables very specific information to be introduced into single cells which can then be regenerated. In this way there is no major disturbance to the maternal genome, unlike the more conventional crossing of two individuals.

Anderson, 1980 reported that four sequential stages in plant tissue culture systems are: establishment, multiplication, rooting and acclimating.

Explants (starting point for all tissue culture) from any plant part, such as seeds, stems, roots, leaves or fruits can be excised, disinfested and placed on surface of culture medium to produce callus that is as a result of wounding

and in response to hormones, either endogenous hormone or supplied in the medium (Ramawat, 2008).

A number of different culture media have been used to initiate callus, but the most common is Murashige and Skoog, 1962 (MS) medium. This medium rich in macroelements, sucrose and certain vitamins (Purohit, 2003).

One major application of plant tissue culture is *in vitro* screening and selection of resistant plants which provide the variation required for a crop improvement program. The assumption is made in the selection at the cell level that the resistance to a particular set of cultural or environmental conditions is expressed in the same way in the cells as in the intact plant (Collin and Dix, 1990). Several screening and selection methods have been used to select tolerant hybrids. The efficiency of certain method depends on the effectiveness of selecting desired variants out of large number of individuals (Naik and Babu, 1988).

The risk that not all the cells in the callus aggregate may be uniformly exposed to the agent. Small pieces of callus may be used to overcome such problem. The new growth can be easily identified and subcultured (Collin and Dix, 1990).

A few attempts have been made to use cell culture systems to screen for variants with increased tolerance to a particular stress like salts, heat, cold, drought, disease, insects, heavy metals and herbicides. Tomato plants regenerated from callus tissues resistant to toxins secreted into media displayed resistance to *Pseudomonas solanacearum* (Toyoda *et al*., 1989).

Regeneration of shoots from poplar leaf explants exposed to glyphosate gave rise to glyphosate tolerant plant (Michler and Haissig, 1988). In pea, *in*

vitro sensitivity of some commercial cultivars showed some correlation with field sensitivity to glyphosate (Yenne *et al*., 1987).

Plant tissue culture is used widely in plant science; it also has a number of commercial applications including:

- Micropropagation is widely used in forestry and in floriculture. Micropropagation can also be used to conserve rare or endangered plant species (Ibrahim *et al.,* 1992).
- A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g herbicide resistance/tolerance.
- Large-scale growth of plant cells in liquid culture inside bioreactors as a source of secondary products, like recombinant proteins used as biopharmaceuticals **(**Bhojwani and Razdan, 1983).
- To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
- To cross-pollinate distantly related species and then culture the resulting embryo which would otherwise normally die (Embryo Rescue) (Brewer *et al*., 1999).
- For production of doubled monoploid plants from haploid cultures to achieve homozygous lines more rapidly in breeding programmes, usually by treatment with colchicine which causes doubling of the chromosome number (George, 1993).
- As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants (Rascio, 1977).
- Certain techniques such as meristem tip culture may be employed that can be used to produce clean plant material from virus free plant, such as potatoes and many species of soft fruit (Becerril *et al*., 2001).

1.4- Protoplasts

Breeding for crop improvement is highly dependant on available variability (the germplasm). The sources of resistance could not be found all the time among the cultivars, or from the same species, reliance on landraces, wild relatives, and other genus for source of desired trait is inevitable in most crops for crop improvement. However it is not always possible to attain successful hybrids through such wide crosses due to various factors which could be broadly accommodated into two classes (McCabe *et al.,* 1993).

1) Pre-embryogenic barriers

- Inability of the pollen to germinate on foreign stigma.
- Failure of pollen tube to reach the ovule in flowers with long style.
- Retarded growth of pollen tube.
- Bursting of the pollen tube in the style or before reaching embryo sac.

2) Post- embryogenic barriers

- Disintegration of the endosperms there by depraving the embryo of the source of the nutrients.
- Retarded growth of the endosperm and a deficiency in the vascular elements of the seed.
- Blockage in the transfer of nutrients from nurse tissues viz., nucleus and integuments to endosperm and embryo.
- Breakdown of the endosperm in the incompatible combination due to the disparity between genomic.

There are various in-vitro breeding techniques to overcome the species barriers. Protoplast fusion is one of the promising tools to attain asymmetric hybrids through para-sexual hybridization for crossing two distantly related species which could not fertilize to form embryo through normal sexual hybridization (Aitken, 1991).

Protoplasts are the biologically active and most significant material of the cell. Each living organism is capable of individual development when provided with suitable conditions (Amerson *et al*., 1985). The ability of a single cell to develop into an organism is called as Totipotency (Ammirato, 1987). This has been successfully proved by demonstrating that a single plant cell can develop into a whole plant (Bhojwani *et al*., 1977). Protoplast fusion is based on the property of protoplasm which is that it engulfs the cell materials which come into contact by means of some external forces to successfully fuse though (Anderson *et al*., 1987).

The important benefits gained from protoplast fusion are:

1. Protoplast fusion of intraspecific, interspecific, intergeneric and some times between plant and animal cells.

2. Mitochondrial and plastids transformation through protoplasts culture.

3. Uptake of beneficial genes of bacteria and viruses by protoplasts.

4. Transferring the genetic information into isolated protoplasts.

Intergeneric hybrids which are very difficult to achieve through sexual crosses have been successfully obtained using protoplast fusion, Potato + tomato, Datura + Atropa, barley + wheat, barley + rice, wheat + oat and sugarcane + sorghum are some of the examples of successful intergeneric hybrids (Attree and Fowke, 1991).

1.4.1- Protoplasts isolation

Isolation of protoplast refers to the removal of cell wall. Plant protoplasts were first isolated by Klercker in 1892 by slicing onion with thin knife, and isolation could be done in two ways (Becwar *et al*., 1988).

A- Mechanical isolation

Mechanical isolation is advantageous that the unknown side effects of the enzyme mixture on plasma membrane are reduced, the cells are subjected to plasmolysis, which causes the protoplast to shrink and recede away from the cell wall, and then a cut is made across the tissue pieces with a scalpel or a sharp knife. The tissue pieces are then deplasmolysed. The protoplasts within the damaged cell walls will swell making it easier to be squeezed out into the bathing culture medium. One major disadvantage of this method is that it yields very low protoplasts and needs highly skillful personnel (Beversdorf, 1990).

B- Enzymatic isolation

The enzymatic isolation of large number of protoplasts from the cells of higher plants was demonstrated by Cocking (1960). As plant cell wall is made up of cellulose, hemicellulose and pectins, Cocking used a concentrated mixture of enzymes such as cellulase and hemicellulase produced from the cultures of the fungus *Myrothecium verucarria* to digest the cell wall. However commercial preparations of enzymes for protoplast isolation were reported by Takebe *et al*., (1968). The disadvantage of this system is there could be some unknown side effects by the enzymes on plasma membrane

(Birch, 1993). The isolation of protoplasts using cell wall degrading enzymes is of two types:

1) Sequential method

The tissue from which the protoplast is to be isolated and treated with macerozyme and pectinase enzyme in mannitol. Once the pectin is digested, the cells are purified by filtration through a nylon mesh, the purified cells are then subjected to cellulase for 90 minutes at 25°C (Bonnet-Masimbert, 1987).

2) Simultaneous method

This method allows the enzymes to digest both pectin and cellulose simultaneously. The enzyme mixture contains pectinase + cellulase in sorbitol or mannitol at a pH 5.4 (Bonnet-Masimbert, 1987).

1.4.1.1- Sources of protoplast

Protoplasts can be isolated from a wide range of tissues and organs. These include leaves, petioles, petals, root, apices, microspores, pollen etc, however isolating protoplast from leaves is more preferred as it yields large number of uniform cells. It has to be taken in to consideration that each tissue type has its own physiological properties which are largely influenced by environmental conditions. Protoplast donor plants have to be grown under controlled conditions (Darwin *et al*., 2003).

The characteristics of each donor tissue are:

a- Leaves yield more uniform shape cells, but has some problems of surface sterilization. Cells are not uniform in size.

b- Shoot tip cultures are aseptic, juvenile, uniform and easy to maintain.

c- Callus and cell cultures, although they are aseptic but their yield is mainly influenced by the culture age (Evans, 1983).

1.4.1.2- Factors influencing protoplast yield

A- Pre-enzyme treatments

The donor tissue has to be subjected to some treatments prior to the enzymatic digestion of cell wall (Lorence *et al*., 2005).

● Surface sterilization

Surface sterilization of the leaf tissue is important to prevent contamination. It is usually done with 0.2% commercial bleach and followed by 10% alcohol (Narayanswamy, 1994).

● Pre-plasmolysis

The donor tissue has to be plasmolysed so that the protoplast shrinks and condenses away from cell wall. This minimizes the amount of enzyme intake by the protoplast avoiding any unwanted action by the enzymes. The plasmolysing treatment is for 10 minutes and the solution used for plasmolys´s should be of the same osmotic pressure as the enzyme solution (Vander and Nancy, 2003).

● Pre-digestion treatment

In order to facilitate the enzymes to penetrate into the intercellular spaces of the donor tissue. The lower epidermis of the donor tissue has to be peeled and or cut in to pieces, then placed in the enzyme solution where the peeled/cut surface comes in contact with the solution (Jogdand, 2001).

B- Enzyme treatment

Enzyme concentration is very important for protoplast isolation. Cellulase, pectinase and hemicellulase are three major enzymes used to digest the cell wall. These enzymes act on their substrates. Pectin digests the middle lamella. The commercial enzyme contains nucleases and proteases as impurities which could harm protoplast. The successful isolation of protoplasts by enzyme method is highly dependent on some factors such as: purity, concentration and volume of enzyme solution, pH, temperature, treatment duration (Saunders and Bates, 1987).

C- Osmotic treatment

The protoplasts suffering from plasmolysis or turgescence because of losing of the cell wall and the difference of medium solution concentration. When the cell is intact, the pressure exerted by the cell wall prevents protoplasts from bursting, but when they lose their cell walls they have to be supplemented with sugars and salts to maintain osmotic pressure. Mannitol and sorbitol are largely used as they do not metabolize the tissue they act on. Glucose and sucrose are of metabolizing nature and should be avoided. Concentration of the sugar is decided depending on the leaf cell osmotic pressure, usually a concentration of 0.23 to 0.90 M is used (Saunders and Bates, 1987).

1.4.2- Purification

1.4.2.1- Protoplast purification, viability and density

Protoplasts subjected to enzyme treatment may contain undigested cell walls, broken cells, vascular tissues, and other parts of the cell along with the intact protoplasts. This mixture has to be purified to obtain protoplasts for further use in fusion. Purification of protoplasts is carried out through filtration, centrifugation and washing. Protoplast mixture is passed through a filter of 50-100μM pore size which filters the undigested cell walls and other vascular tissues. When this filtrate is subjected to centrifugation, cell fragments will be eliminated from the separated protoplasts. The remaining protoplasts will be decanted and resuspended in the isolation solution. Repeated re-suspension in a solution of sucrose or sorbitol makes protoplasts float on the surface. Siphoning through Pasteur pipette, the intact protoplasts can be isolated (Evans, 1983).

A- Viability test

Viability of the freshly isolated protoplasts can be determined by one of the following staining methods (Topp, 2006).

1- Flourescein diacetate (FDA) method. FDA is fluorescing and non-polar substance and freely permeates into the plasma membrane. When it permeates inside the living cell, due to esterase activity, fluorescin is released giving green fluorescence when cells are observed under UV light (Tassin and Jacques, 2005).

2- Evan's blue stain. When cells are treated with a diluted (0.025 %) solution of Evan's blue. The damaged cells take up the stain but intact and viable cells exclude it and remain unstained (Swarbrick, 1997).

3- Phenolsafranine stain. Phenolsafranine (0.1%) is used to detect dead protoplasts. Dead cells turn red when treated with phenolsafranine and living ones remain unstined (Rick *et al*., 1990).

B- Plating density

Successful protoplast culture, depends on the number of protoplasts per unit volume of media. There should be a minimum density for plating termed as minimum plating density (mpd). It is important to maintain an optimum concentration. A haemocytometer is used to count the protoplasts and determine the density **(**Jones**,** 1999).

1.4.3- Culture of protoplasts

Johnson and Veilleux, 2001 stated that methods employed for culturing protoplasts are the same as those for any tissue/cell culture. Protoplasts can be cultured on solid or liquid medium depending upon the specific requirements (figure 2).

A. Liquid cultures

Protoplasts are placed in liquid medium in a petridish, and then sealed with parafilm to prevent drying. This method gives rapid proliferation, however it is only suitable for large number of protoplasts (Grosser *et al*., 2004).

B. Agar Medium

Protoplasts are cultured in agar medium in a petridish and sealed with parafilm (Grosser *et al*., 2002).

C. Agarose or Alginate as gelling agents

Protoplasts are cultured in a solid medium, but the solidifying agent used is agarose, which is devoid of contaminants and is relatively neutral (Guo and Deng, 2001).

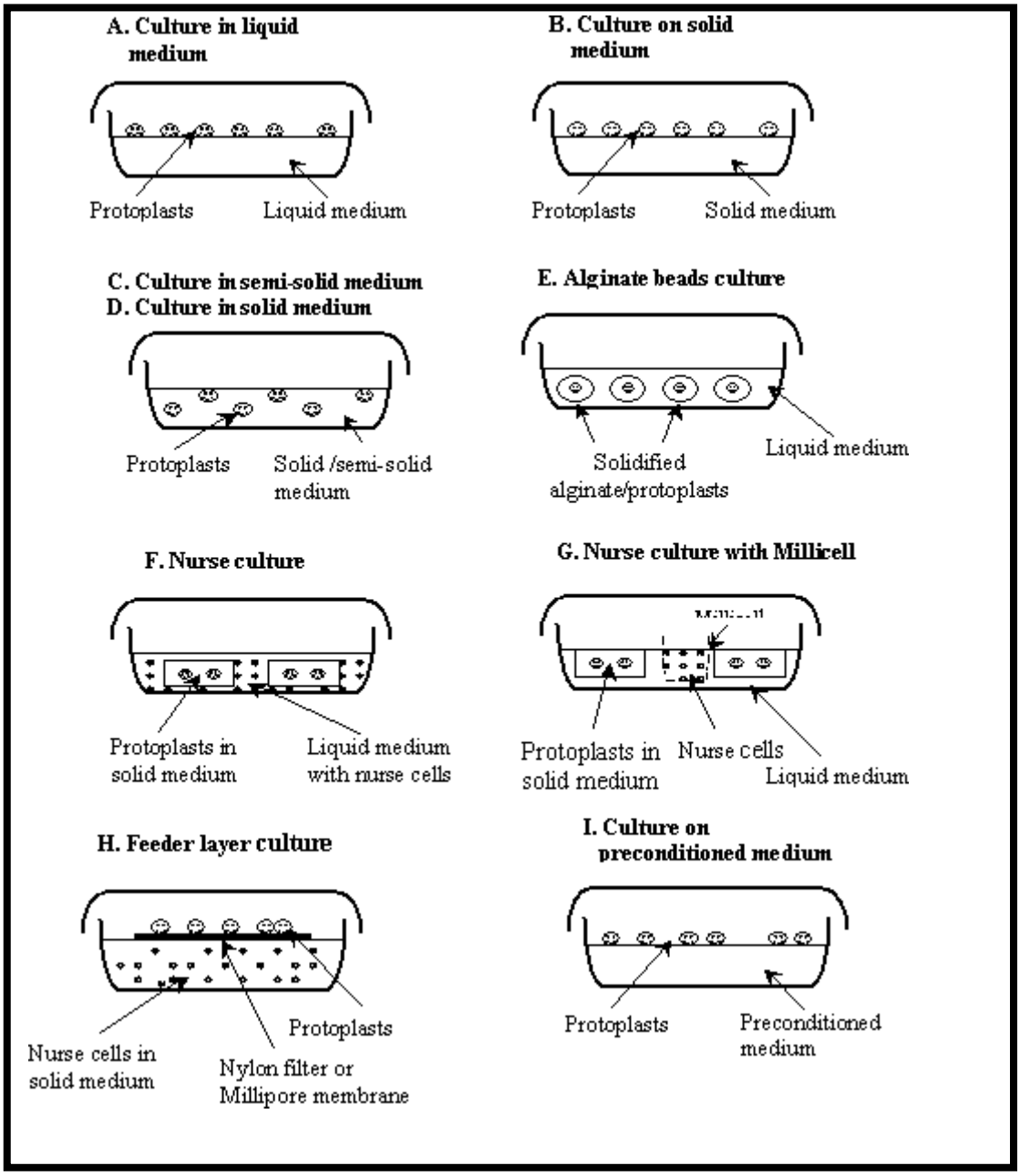

Figure (2) Culture of protoplasts using various methods (Peberdy, 1980).

D. Nurse cultures

Single cells are picked-up from cell suspension cultures under the microscope with the help of micropipette. Small sterile pieces of filter papers are placed aseptically on the top of callus of the same or different species, filter paper placed on nurse callus tissue is wetted by liquid and nutrients

released by the cultures. The single cell (that placed on filter paper) divides and produces a small colony of cells within a few days or weeks. This small colony of cells or microcalli can be transferred to fresh medium, where it survives (Ramawat, 2008).

E. Feeder layers

Grosser demonstrated that the inactivation of the feeder layer by irradiation, and he can obtain hybridization in citrus by using this method (Grosser *et al*., 2001).

1.4.4- Protoplast fusion

Withers and Cocking (1972) laid foundation for the protoplast fusion technique. Protoplast fusion can be affected by several ways as described below:

A- Spontaneous fusion

Fusion of protoplasts could be spontaneous after the cell wall is enzymatically digested. The underlying principle behind this fusion is the expansion of the plasmodesmata (Moriguchi *et al*., 1996).

B- Induced fusion

Induced through fusogens or fusogenic agents (Moreira *et al*., 2000). The most popular methods are listed below:

1- Treatment with $NaNO_3$

Protoplast fusion between oats (*Avena sativa*) X Maize (*Zea mays*) was achieved by Paull *et al*., (1979) by using $NaNO_3$ as a fusogenic agent. This

method however has some drawbacks such as deterioration of protoplasts by the action of $NaNO_3$ and non-reproducibility.

2- High pH and high calcium treatment

Amerson *et al*., 1976 demonstrated the efficient fusion of protoplasts through a combination of conditions which are alkaline (High pH) 10.5 with high Calcium at 37^0C for 30 minutes. This method proved to be reproducible and the frequency of fusion was between 20-50%.

3- Polyethylene glycol (PEG) method

Various aqueous solutions have been used to induce chemical fusion of protoplasts (Hancock and Henderson, 1988). PEG is a high molecular weight (1500- 8000) compound. Inducing fusion by PEG is one of the preferred methods, and fusion using PEG is usually associated with high pH and high Calcium concentration. About 25-33 % W/V of PEG is used. PEG binds to the protoplast and agglutinates them. Too low concentration of PEG will end up adhering and no fusion, over addition might cause massive agglutination.

4- Electro fusion

Topp, 1988 was able to fuse protoplasts exposed to an alternating non-uniform electric field of low strength (e.g., 10 KVm-1, 2MH2). The protoplast surface is usually negatively charged, the same charged materials tend to repel each other. Alternating electric current causes dipole generation in protoplasts leading to a pearl chain arrangement of protoplasts between electrodes. The number of protoplasts within a pearl chain is dependent on the distance between the electrodes. This method is considered the best because it is controllable and the absence of adverse effects on protoplast viability**.**

1.4.4.1- Mechanism of protoplast fusion

The events involved in protoplast fusion are

- Agglutination between protoplasts.
- Fusion of plasmalemmae to form cytoplasmic bridge.
- Rounding of the fused protoplasts due to the expansion of the cytoplasmic bridges.
- Formation of heterokaryon or homokaryon depending upon the genetic constitution of the protoplasts (Guo and Deng, 2000).

1.4.4.2- Fusion products

Deng *et al*., (2000) showed that when two or more isolated protoplasts are fused together, there is always a coalescence of the cytoplasms of the various protoplasts (figure 3). The nuclei of the fused protoplasts may fuse together, or they may remain separate. Cells containing nonidentical nuclei are referred to as heterokaryons or heterokaryocytes. The fusion of nuclei in a binucleate heterokaryon results in the formation of the true hybrid protoplast or synkaryocyte. The fusion of two protoplasts from the same culture results in a homokaryon. Frequently genetic information is lost from one of the nuclei. If one nucleus completely disappears, the cytoplasms of the two parental protoplasts are still hybridized, and the fusion product is known as a cybrid (cytoplasmic hybrid; heteroplast).

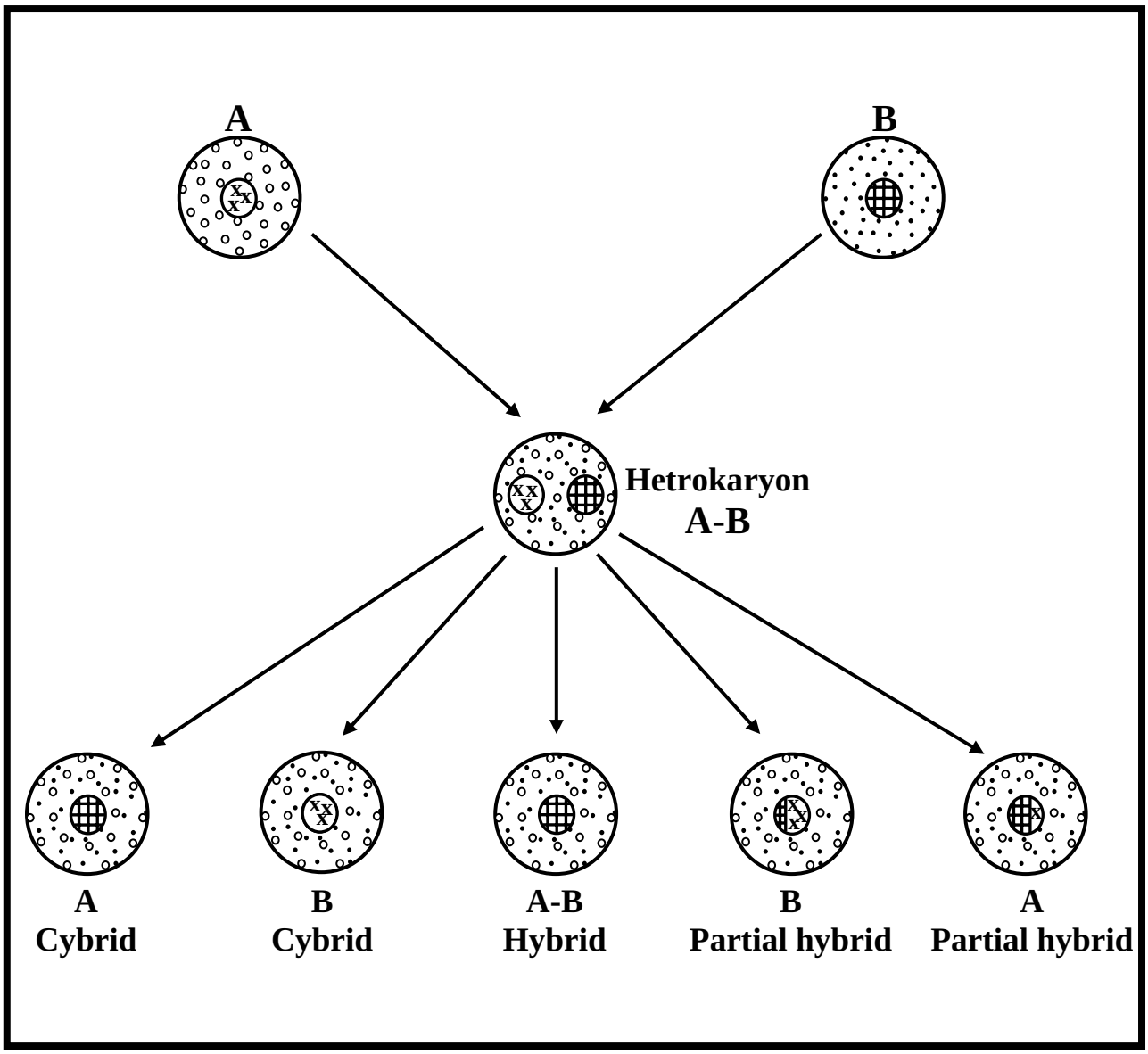

Figure (3) Hybrids or cybrids resulting from protoplast fusion (Leaver, 1992).

1.4.5- Plant regeneration

Protoplasts after fusion regenerate the cell wall. (when protoplasts loose their characteristic spherical shape, it indicates the cell wall formation) Cell wall formation occurs due to the deposition of cellulose as microfibrills either directly on plasmalemma or in-between plasmalemma and multilamellar wall of the cell is loosely arranged first **(**George, 1993). The

microfibrills later become organized to form a typical cell wall. Normal cell walls undergo mitosis and produce daughter cells, the multinucleate cells then develop cell wall size increase and after a lag phase they develop into callus which is then developed into plantlets under normal tissue culture protocols (Demain and Solomon, 1986).

1.4.6- Applications of protoplast fusion in somatic hybridization

There are many examples of the applications of protoplast fusion in plant improvement.

Solanum is one of the most well studied plants using protoplast fusion Hassanein *et al*., (1998) studied the nuclear/chloroplast interactions through cybrids developed from *S. nigrum* genome and a *S. tuberosum* plastome. Resistance to Colarado beetle was studied in the fusion products of diploid solanum (Jansky *et al*., 1999). Successful development of somatic hybrids in other crops was also reported. Rice (*O. sativa*) and barley (*Hordeum vulgare*) (Kisaka *et al*., 1998), sunflower (*Helianthus annuus*) and *H. maximiliani* (Binsfeld and Schnabl, 2002), peppermint (*M. piperita* cv. Black Mitcham) with those of spearmint (*M. spicata* cv.Nature Spearmint)…etc.

The best example of successful protoplast fusion involving two related species but sexually incompatible partners is that of the domestic potato, *Solanum tuberosum* with that of the wild potato, *S. brevidens*. The purpose of this cross was to introduce resistance to potato leaf roll virus and potato virus into the domestic potato from the wild relative. The chromosome number of the potato 48 and that of *S. brevidens* is 24. To make the two partners more compatible, dihaploid plants were produced from *S. tuberosum* with a

chromosome number of 24. Protoplasts from these plants were then fused with protoplasts from *S. brevidens*. The heterokaryons were regenerated and the plants were found to be tetraploid of 48 chromosome. The latter were a result of three protoplasts fusing at the same time. When the hybrid plants were tested for resistance to both virus diseases, the hybrids showed the same levels of resistance as the *S. brevidens* and many female were fertile. This meant that they could be used as a source of compatible germplasm that could be crossed in standard way with other potato varieties in order to introduce the virus resistance characters (Blackhall *et al*., 1994).

Another example of successful cross which has potential for the production of hybrid seed is the induction of cytoplasmic male sterility. Where one partner in a cross is male sterile there will be no self pollination in these glowers. Any seeds obtained from crossing into a cytoplasmic male sterile female parent will be 100% hybrid seed **(**Gould**,** 1983). The expression of cytoplasmic sterility resides in the mitochondria and chloroplasts of the cytoplasm. Somatic hybridization by protoplast fusion provides a mechanism for transferring the cytoplasm of a species with cytoplasmic male sterility to one that would benefit from this character. Tomato is an example where this has been achieved (Blackhall *et al*., 1994).

2.1- Materials

2.1.1- Apparatus and equipments

The following equipments and apparatus and their origin used throughout this experimental work:

Apparatus/Equipment	Company and origin
Autoclave	Karl / Germany
Distillator	GFL / Germany
Centrifuge	Harrier / U.K.
Incubator	Sanyo / Japan
Laminar air flow cabinet	ESCO / Japan
Micropipettes	Brand / Germany
pH-meter	Metter Gmbh-Teledo / England
Refrigerator	Concord / Korea
Digital sensitive balance	Mettler / Switzerland
Oven	Gallenkamp / England
Hot plate with magnetic stirrer	Gallenkamp / England
Water bath	Gallenkamp / England
Fluorescence phase contrast microscope	Moti / Japan
Hemocytometer	Neubaure / Germany
Compound microscope	Olympus / Japan

2.1.2- Chemicals

The following chemicals and their origin used in the experimental work:

Chemicals	Company
Agar-Agar	Sleeze
Ammonium nitrate	Mall
Benzyle Adenine Purine (BAP)	BDH
Boric acid	Merk
Calcium chloride anhydrate	Fluka
Calcium ions	BDH
Cellulase	Aldrich /Germany
Cobalt chloride.6H2O	BDH
Cupric sulphate.5H_2O	BDH
Ethanol	BDH
Ferrous sulfate.7H_2O	BDH
Floercent diacitate	Aldrich
Glycine	BDH
Himecellulase	Aldrich / Germany
Magnesium sulphate anhydrate	Fluka
Manganese sulphate.4H_2O	BDH
Molybdic acid (sodium salt).2H_2O	BDH
Myoinositol	BDH
Naphthaleneacetc acid (NAA)	BDH

Chemicals	Company
Nicotinic acid(free acid)	Kochling
Pactinase	BDH / England
Poly ethylene glycol	Merk
Potassium iodide	Telanal
Potassium nitrate	BDH
Potassium phosphate monobasic	Fluka
Pyrodoxine.HCL	BDH
Sodium ethylene diamine tetraacetate	Fluka
Sodium hypochlrite	Alwazer
Sodium nitrate	Fluka
Sucrose	BDH
Thiamine.HCL	BDH
Zinc sulphate.7H_2O	BDH

2.2- Methods

This study was carried out in the plant tissue culture laboratory, Biotechnology Department/ College of Science/ Al-Nahrain University during the period 1/12/2006 to 1/7/2008.

2.2.1- Plant material

The two tomato seed hybrids, Hatouf and Barakah F1 hybrids were kindly supplied by Petoseeds Company Iraqi representative.

The seed of the two hybrids were sown in 12 cm in diameter clay pots, then the leaves samples were taken after 14 days of culturing when plant was 15 cm height.

2.2.2- Sterilization of explants (Pierik, 1987)

Leaf discs (1.5) cm in diameter were excised from hatouf and barakah hybrids, rinsed with tap water for 10 minutes then transferred to laminar air flow-cabinet where submerged in 70% ethanol for 1 minute, washed with sterilized DDH_2O, then rinsed with sodium hypochlorite at different concentrations (0, 1.5, 3.0 or 6.0%) for 3 or 5 minutes. Explants then rinsed with sterilized DDH_2O for three times. For each concentration 12 explants were used and the ends of each explant were cut to remove tissues affected by sterilization solution. The final diameter of the leaf discs was 1 cm.

2.2.3- Preparation of culture medium

Murashige and Skoog, 1962 (MS) medium was prepared and used (table 1). Sucrose (30 g/l), myoinositol (100 mg/l) and the plant growth regulators (NAA and BAP) at different concentrations were added. The pH

was adjusted to 5.8 using NaOH or HCl (1N), then 8g/l of the agar type (Agar-Agar) was added to the medium, placed on a hot plate magnetic stirrer till boiling. Aliquots of 20 ml were dispensed into (8 ×2.5) cm culture vessels. Culture media were sterilized by autoclaving at 121ºC under (1.04 Kg/cm^2) pressure, for 15 minutes. The medium was left at room temperature to cool and became ready to culture explants.

Table (1) Murashige and Skoog, 1962 (MS) culture medium components

Macronutrients		
Components	**Chemical formula**	**Weight (mg/l)**
Ammonium nitrate	**NH_4NO_3**	**1650**
Potassium nitrate	**KNO_3**	**1900**
Calcium chloride anhydrate	**$CaCl_2.2H_2O$**	**440**
Magnesium sulphate anhydrate	**$MgSO_4.7H_2O$**	**370**
Potassium phosphate monobasic	**KH_2PO_4**	**170**
Micronutrients		
Boric acid	**H_3BO_3**	**6.20**
Potassium iodide	**KI**	**0.83**
Manganese sulphate.$4H_2O$	**$MnSO_4.H_2O$**	**15.60**
Zinc sulphate.$7H_2O$	**$ZnSO_4.7H_2O$**	**8.60**
Molybdic acid (sodium salt).$2H_2O$	**$Na_2MoO_4.2H_2O$**	**0.25**
Cupric sulphate.$5H_2O$	**$CuSO_4.5H_2O$**	**0.025**
Cobalt chloride.$6H_2O$	**$CoCl_2.6H_2O$**	**0.025**
Chelated Iron		
Sodium ethylene diamine tetraacetate	**Na2-EDTA**	**37.3**
Ferrous sulfate.7 H_2O	**$FeSO_4.7H_2O$**	**27.8**
Vitamins		
Thiamine.HCl	**$Cl_2H_{17}C_1N_4OS.HCl$**	**0.5**
Nicotinic acid(free acid)	**$C_8H_{11}NO_3.HCl$**	**0.05**
Pyrodoxine.HCl	**$C_6H_5NO_2$**	**0.5**
Glycine(free base)	**$C_2H_5NO_2$**	**100**
Meso-insitol	**$C_6H_{12}O_6$**	**100**

2.2.4- Plant growth regulators

Different concentrations of the auxin NAA (0.0, 0.1, 0.2 or 0.3) mg/l and the cytokinin BAP (0.0, 0.5, 1.0, 2.0 or 3.0) mg/l were prepared and added to the culture media as required before autoclaving.

2.2.5- Media and instruments sterilization (Cappuecino and Sherman, 1987)

The Culture media was sterilized by autoclaving at 121°C under (1.04 Kg/cm²) pressure, for 15 minutes, while glassware and other instruments were sterilized either by autoclaving or using electric oven (180-200) °C for 2 hrs.

2.2.6- Incubation of cultures

Surface sterilized leaf disc explants (1) cm in diameter were inoculated into the culture vessels under aseptic conditions, placed in the incubator at 25°C for 16/8 hrs. Light/dark photoperiod using day light inflorescent and light intensity of 1000 lux.

2.2.7- Initiation of callus cultures

Different combinations of plant growth regulators were examined to determine the most effective one for callus induction. Explants were placed onto MS medium containing NAA and BAP as in 2.2.4. Cultures were placed in the incubator. The response of these explants to auxin and cytokinin combinations was evaluated after 21 days in culture.

2.2.8- Maintenance of callus cultures (Bos, 1997).

The initiated callus was removed from the explants using forceps and scalpel, and then pieces weighting 50 mg were subcultured onto fresh medium supplemented with the same combinations of NAA and BAP as in 2.2.4.

2.2.9- Protoplast isolation (Fish *et al*., 1988)

Leaf discs (1.5 cm in diameter) were excised from fully expanded young leaves. They were surface sterilized by dipping them into 70% ethanol for one minute and then rinsed with 2% solution of sodium hypochlorite for 20 minute. The leaves then washed three times with sterile distilled water to remove the traces of the disinfectant.

Lower epidermis of sterilized leaves was carefully peeled off and the stripped leaves were cut into small pieces, then peeled leaf pieces were placed with lower surface down into 20 ml solution of 13% mannitol and 1% Sodium chloride in Petridish (90 mm) for one hr. Mannitol solution was removed by using Pasteur pipette, then, enzyme mixture (20 ml) and mannitol (0.5% w/v pectinase with 2% w/v cellulase and 2% w/v hemicellulase in 13% w/v mannitol at pH 5.4) were added then incubated overnight at 20 to 25°c.

Petri plates were gently agitated to facilitate the release of the protoplasts, then, larger pieces of leaf-material were moved to one side with a sterile forceps keeping the petridish at an angle of 15°. After 5 minutes, protoplasts settled down in the petridish, then, protoplasts-enzyme mixture was transferred to screw- capped centrifuge tube by using Pasteur pipette. The material was centrifuged at (100 rpm) for one minute. This process was repeated 2-3 times, then, protoplasts were washed with 13% mannitol solution.

For the final wash, mannitol was replaced with 20% sucrose solution, and centrifuged at (200 rpm) for one minute. The cleaned protoplasts float and debris settled down, then the floated protoplasts were carefully pipetted with a Pasteur pipette leaving the remains of mesophyll cells.

2.2.10- Purification of isolated protoplasts (Evans, 1983)

The medium contained protoplasts, cell debris and broken cell organelles. The crude protoplasts suspension in osmotically adjusted medium was decanted into a centrifuge tubes, then centrifuged at low speed (50-100 rpm) for five minutes. Under these conditions the intact protoplasts accumulated in the form of soft pellet in the bottom of the tube. The supernatant containing broken cell debris, cell wall and cell organelles were pipetted off. The pellet was gently resuspended in fresh culture medium containing mannitol, and then rewashed three times by using culture medium.

2.2.11- Protoplast counting (Klebe and Mancuso, 1982)

The cover slip was placed over the surface of a counting chamber (Neubauer hemocytometer), then the protoplast samples were mixed gently to insure a uniform distribution of cells, and then, the sample was withdrawn with a Pasteur pipette. The tip of the pipette was placed at the edge of the cover slip and both chambers were filled by capillary action, then the cells were allowed to settle for five minutes. Examination done at the appropriate magnification (x100) and the number of protoplasts was counted in five grids in each chamber (including all cells touching the middle line along the left or top margin of the square). The number of protoplasts that counted in five squares was divided by the volume of the squires and multiplied by 1000.

2.2.12- Protoplast viability test (Kao and Wetter, 1977)

The most frequently used method for the estimation of protoplast viability is the use of Fluorescein diacetate (FDA) at 0.01% (w/v). The (FDA) accumulates within the plasma membrane, viable protoplasts stianed greenish white under UV illumination. Protoplasts that were treated with (FDA) were observed within 5 to 15 minutes.

2.2.13- Standardizations of protoplast isolation

In order to optimize conditions for protoplast isolation from leaves and callus by using the following factors, type of enzyme, temperature, incubation period and pH were examined.

• Type of enzyme

Three different enzymes (pectinase, cellulase and hemicellulase) were tested. These enzymes were added at a concentration of (0.5% w/v) for pectinase, and (2% w/v) for cellulase and hemicellulase respectively.

• Temperature

In order to determine the optimum temperature for protoplast isolation five different temperatures (20, 23, 25, 28 or 30°C) were experimented for this purpose at medium pH 5.4 for 24 hrs.

• pH

In order to determine the optimum pH of enzyme mixture for protoplast isolation, the following pH values were examined for this purpose (5.0, 5.2, 5.4, 5.8 or 6.0) at 25°C for 24 hrs.

• Incubation period

In order to determine the optimum incubation time for protoplast isolation, four incubation periods (18, 24, 48 or 72 hrs.) were used for this

purpose at 25°C, pH 5.4 and (0.5 pectinase, 2.0 cellulase and 2.0 himecellulase w/v) enzyme mixture.

2.2.14- Protoplast fusion (Senda *et al*., 1979)

Many fusion agents polyethylene glycol (PEG), high Ca++ at high pH 10.5 at high temperature (30ºC) and sodium (5.5 w/v) nitrate were used for fusion of protoplasts.

• (PEG)

One drop of suspension containing protoplasts was placed in sterile Petri plate. The plate was gently shaken to ensure proper mixing. The number of protoplasts for each variety was about 1 x 10^6 / ml before mixing (1:1).

After five minutes (to allow the protoplasts to settle to the bottom of Petri dish), 300-450 µl of 56% (w/v) PEG solution was added to the edge of the protoplast suspension, then, in the centre of the droplet, after waiting for 15 minutes, 1 ml of 13% mannitol solution was added to dilute the (PEG) solution.

One side of the plate was raised and the protoplasts were washed with 9 ml of mannitol. The PEG and mannitol residues were removed from the Petri dish, and then a few drops of mannitol solution were added to the fused cells. Fusion products were observed by using inverted microscope at magnification power of (x100).

The above procedure was repeated by using several types of PEG at different molecular weights (8000, 6000, 4000 or 2000).

• Treatment with (Ca++) at high pH (10.5)

The density of protoplast suspension was diluted to $1x10^6$ protoplast/ ml, then 1ml of the suspension of each variety was mixed using centrifuge tube, then centrifuged at 50 (rpm) for 5 minutes to settle the protoplast mixture and the supernatant was removed. The protoplasts were re-suspended in 5ml solution of high pH Ca++ (0.05 M $CaCl_2.2H_2O$ at a pH of 10.5) and centrifuged at a very slow speed 50 (rpm) to settle the protoplasts then incubated in water bath at 30ºC for 20 minutes. The supernatant was gently removed without disturbing the protoplasts pellet, then 8-10 ml of 13% mannitol solution were added without disturbing the pellet and incubated at room temperature for 30-40 minutes. Mannitol solution was removed and washed with the culture medium.

• PEG- Ca++ with high pH

Protoplast suspension (0.5 ml) at a density of $1x10^6$ /ml was taken from each variety, and then mixed in centrifuge tube. The protoplast mixture was centrifuged at 50 (rpm) for 5 minutes, and then most of the supernatant was removed leaving (0.5 ml) of protoplast suspension.

The protoplast suspension was removed and placed in 90 mm sterile Petri dish, then the dish was agitated gently to allow protoplast assemble in the center of the drops and allowed to settle down for 5 minutes.

0.2 ml (1 drop) of PEG- Ca++ at high pH (10.5) solution was added around the droplet containing protoplast, incubated at room temperature for 10-12 minutes, then 0.2 ml/drop stabilizer solution (10% sucrose) was added slowly, incubated for 5 minutes, then the stabilizer solution was removed along with some fusogen. This process was repeated 2-3 times without further incubation.

• Sodium nitrate (NaNO3)

Isolated protoplasts from leave and callus were suspended in a mixture of 5.5% sodium nitrate in 10% sucrose solution. The solution containing the protoplasts was incubated in water bath at 35ºC for 5 minutes and then centrifuged for 5 minutes at 200 (rpm). After centrifugation, most of the supernatant was decanted and the protoplast pellet was transferred to a water bath at 30ºC for 30 minutes, then culture medium supplemented with 0.1% $NaNO_3$ was added, then the protoplasts were left for 5 minutes before washing twice with culture medium.

3.1- Results and discussion

3.2- Sterilization of the explants

Commercial bleach was used for explants sterilization (leaves) of *Lycopersicon esculentum*. Figure (4) shows that the most effective concentration of commercial bleach was 3% for (3) minutes that gave the highest percent (100%) of survival (number of explants showed no contamination). Increasing time to (5) minutes caused damage to plant tissues and reduced the survival (36%), whereas lowering the concentration of commercial bleach led to high percentage of contamination. Most concentrations of commercial bleach used for 5 minutes reduced survival.

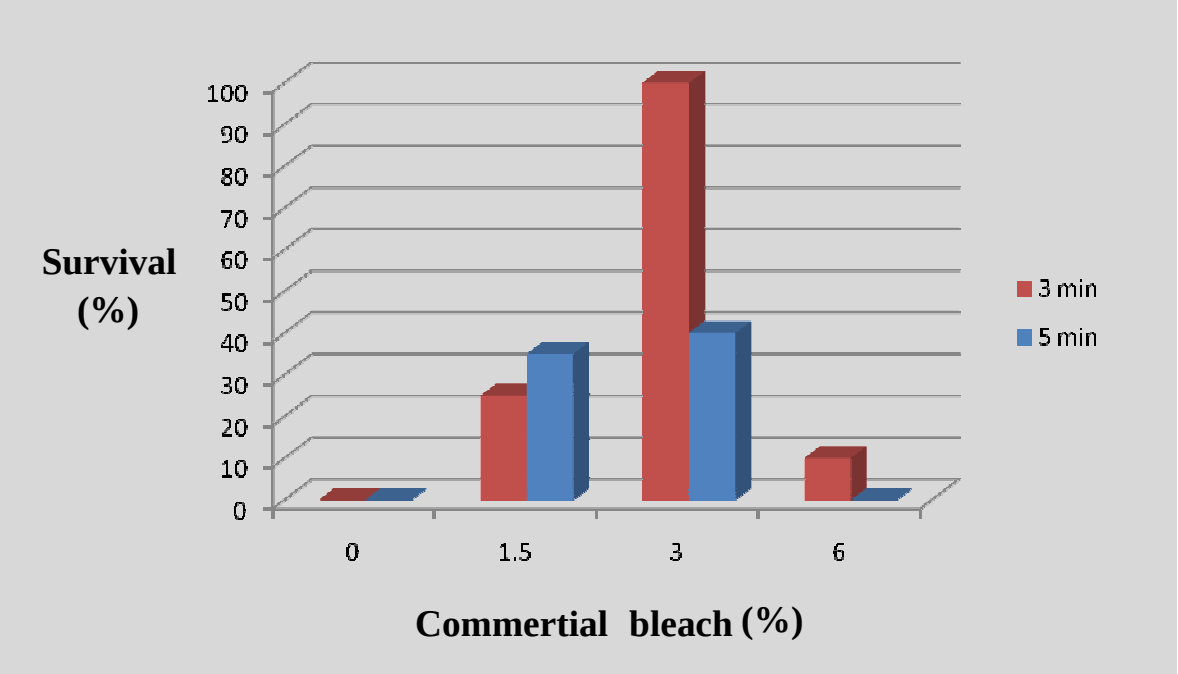

Figure (4) Effect of different concentrations of commercial bleach on (Hatouf and Barakah) explants tomato hybrids survival at sterilization periods of 3 or 5 min. n= 12.

Using commercial bleach was important to eliminate the contaminations. It's used widely for tissue sterilization. The selection of sterilization materials depends on the source of the explants, roughness of its surface and other factors. The sterilization material should be easily removed from explants when washed with sterilized DDH_2O (Yeoman and Macleod, 1997; Sateesh, 2003). The results are in agreement with (Pierik, 1987) who referred to the importance of sodium hypochlorite in explants sterilization. Increasing the surface sterilization period and concentration often lead to serious reduction in survival rate. Optimization experiment is therefore necessary to achieve maximum survival rate with minimum concentrations and time exposure.

3.3- Induction of callus cultures

Table (2) shows the effect of different concentrations of BAP and NAA on the response (%) of callus induction on leaf explants. Maximum percentage of callus induction occurred at a combination of 2.0 mg/l BAP and 0.1 mg/l NAA reaching 100% after 21 days. This percentage decreased to 22.9% in the combination of (3.0 mg/l BAP and 0.1 mg/l NAA).

Table (2) Percentages of tomato hybrids leaf explants showed callus induction cultured on MS medium supplemented with different concentrations of BAP and NAA after 21 days (n= 12)

NAA (mg/l)	BAP (mg/l)					**Mean**
	0.0	0.5	1.0	2.0	3.0	
0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	22.9	68.7	100	22.9	43.32
0.2	0.0	35.4	52.0	35.4	22.9	28.32
0.3	0.0	0.0	14.5	16.6	0.0	4.98
Mean	0.0	14.57	31.22	37.47	12.5	

These percentages fluctuated with increasing NAA concentrations which were (35.4, 52.0, 35.4, 22.9, 8.3 and 16.6) % for combination of (0.2 mg/l NAA and 0.5 mg/l BAP), (0.2 mg/l NAA and 1.0 mg/l BAP), (0.2 mg/l NAA and 2.0 mg/l BAP), (0.2 mg/l NAA and 3.0 mg/l BAP), (0.3 mg\l NAA and 1.0 mg\l BAP) and (0.3 mg\l NAA and 2.0 mg\l BAP) respectively. No callus induction was reported on untreated leaf explants, and in the interaction between low concentrations of NAA and BAP.

Figure (5) Callus induction on tomato hybrids leaf explants grown on MS medium containing a combination of 2.0 mg/l BAP and 0.1 mg/l NAA, 21 days after culture.

This result is in agreement with Hssain, (2005) who reported that callus of *L. esculentum* was established from leaf explants on (MS) medium supplemented with 2.0 mg/l BAP and 0.1mg/l NAA. When BAP concentration increased over 2.0 mg/l and NAA over 0.1 mg/l, resulted in lower responses for callus induction.

Callus induction requires a balanced ratio between auxin(s) and cytokinin(s) as reported by Skooge and Miller (1957). Establishment of a callus from the explants divided into three developmental stages: induction, cell division and differentiation. The length of these phases depends mainly on the physiological status of the explant's cells as well as the cultural conditions including the appropriate combination of plant growth regulators (Dodds and Roberts, 1995).

3.4- Maintenance of callus cultures

Callus cultures induced on leaf explants from the best combination of (2.0 mg/l) BAP and (0.1 mg/l) NAA respectively, were inoculated into the same combinations of plant growth regulators used for callus induction to determine the appropriate concentration for callus maintenance (Table 3).

Table (3) Callus fresh weight (mg) initiated on (Hatouf and Barakah) leaf explants of *L. esculentum* grown on different concentrations of NAA and BAP. Initial weight was 50 mg after 21 days. (n= 12).

NAA (mg/l)	BAP (mg/l)					**Mean**
	0.0	0.5	1.0	2.0	3.0	
0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	91.2	392.4	721.3	627.9	366.56
0.2	0.0	168.5	112.5	515.2	393.4	237.92
0.3	0.0	0.0	107.9	67.2	0.0	35.02
Mean	0.0	94.92	153.2	325.92	225.32	

A combination of 0.1 mg/l NAA and 2.0 mg/l BAP produced more callus fresh weight (721.3mg) than any other combinations (Figure 6). Increasing both NAA and BAP levels reduced callus fresh weight recording 627.9 mg for the combination 3.0 mg/l BAP and 0.1 mg/l NAA, and reached 515.2 mg and 393.4 mg for (0.2 mg/l NAA and 2.0 mg/l BAP), (0.2 mg/l NAA and 3.0mg/l BAP) respectively. Thus, the combination of 0.1 mg/l NAA and 2.0 mg/l BAP was chosen to maintain callus cultures.

According to the results stated above, callus was induced on leaf explants then maintained for many subcultures on MS medium containing 2.0 mg/l BAP and 0.1 mg/l NAA. Induction and maintenance of callus cultures in *L. esculentum* seems to favor high levels of BAP and lower level of NAA

Callus induction and maintenance may be influenced by many factors: medium components, type and concentration of plant growth regulators, plant physiology, source of plant explant and environmental conditions (Torbert *et al*., 1988). Smith (2000) considered genetic content and nutrient components as main factors affect responses of callus induction and maintenance.

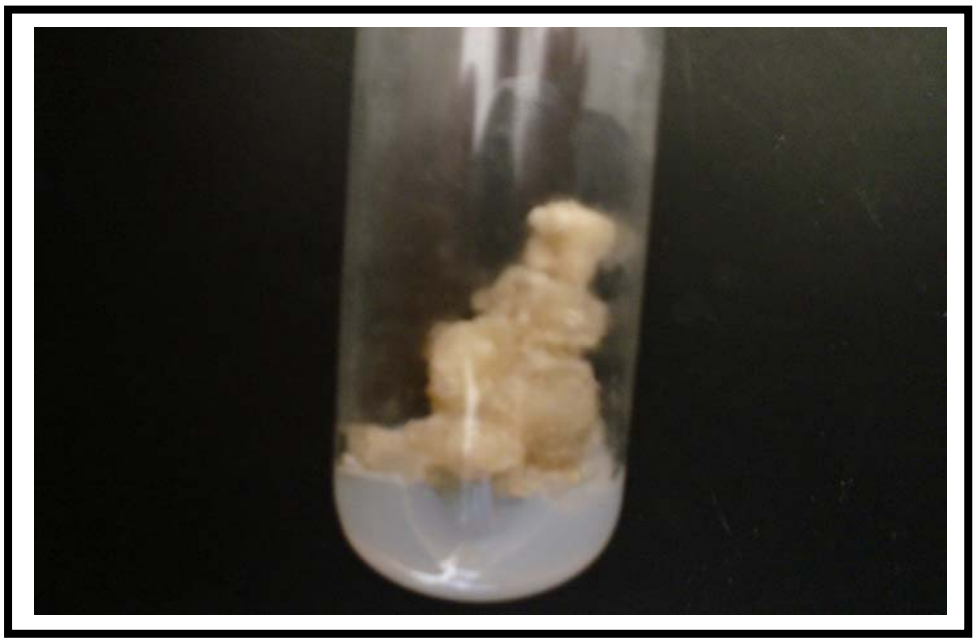

Figure (6) Callus cultures originated from (Hatouf and Barakah) leaf explants grown on maintenance medium containing 2.0 mg/l BAP and 0.1 mg/l NAA. Cultures were continuously cultured on fresh medium at 21 days intervals.

3.5- Optimization of protoplasts isolation

3.5.1- Enzyme mixture

Three types of enzymes (pectinase, cellulase and himecellulase) were used to determine the optimum enzyme mixture for high yield production of isolated protoplasts. These enzymes were used at a concentration of (0.5% pectinase, 2% cellulase and 2% hemicellulase in 13% mannitol at pH 5.4).

Results in table (4 and 5) showed that maximum number of isolated protoplasts from leaves and callus obtained when the enzyme mixture included the three types of enzyme. Using this enzyme mixture, the numbers of isolated protoplasts were (1950000 protoplast/ml) with

viability (54%) from leaves and (3500000 protoplast/ml) with viability (61%) form callus, while the number of isolated protoplasts by using other single or combination of enzyme (pectinase only, pectinase + cellulase, cellulase only, pectinase + himecellulase and himecellulase + cellulose, himecellulase only) was (0, 1700000 protoplast/ml with viability 50%, 0, 1600000 protoplast/ml with viability 47%, 0 and 0) from leaves and (0, 3000000 protoplast/ml with viability 58%, 0, 2800000 protoplast/ml with viability 55%, 0 and 0) from callus respectively.

Table (4) Number of isolated protoplasts from leaves using single or combination of enzymes and their viability by at pH 5.4 and 25°C after 24 hrs.

Enzyme % (w/v)	No. of isolated protoplasts (protoplast/ml)	Viability (%)
Pectinase (0.5)	0	0
Pectinase (0.5) + cellulase (2)	1700000	50
Cellulase (2)	0	0
Pectinase (0.5) + cellulase(2) + himecellulase (2)	1950000	54
Pectinase (0.5) + himecellulase (2)	1600000	47
Himecellulase (2) + cellulase (2)	0	0
Himecellulase (2)	0	0

Table (5) Number of isolated protoplasts from callus using single or combination of enzymes and their viability at pH 5.4 and 25°C after 24 hrs.

Enzyme % (w/v)	No. of isolated protoplasts (protoplast/ml)	Viability (%)
Pectinase (0.5)	0	0
Pectinase (0.5) + cellulase (2)	3000000	58
Cellulase (2)	0	0
Pectinase (0.5) + cellulase (2) + himecellulase (2)	3500000	61
Pectinase (0.5) + himecellulase (2)	2800000	55
Himecellulase (2) + cellulase (2)	0	0
Himecellulase (2)	0	0

It is concluded from these results that a mixture of pectinase, cellulase and himecellulase was efficient for protoplast isolation since the pectinase works on degrading the galacturonic acid residues of pectins that confer the cell to cell adhesion, and macerate the tissue to single cells. While cellulase works on digesting the cellulose component, conferring the spherical shape to protoplast. Himecellulase assists in the breakdown of xylans (Butt, 1985).

This optimum enzyme mixture (pectinase, cellulase and himecellulase) was used in the next experiments for optimization of protoplasts isolation from explants and callus.

3.5.2- Temperature

As shown in tables (6 and 7) it was found that maximum number of protoplasts was obtained at (28ºC). In this temperature, the number of isolated protoplasts was (2150000 protoplast/ml) with a viability reached (35%) from leaves and (3750000 protoplast/ml) with a viability reached (37%) from callus. This temperature may be the optimum for enzymes work and therefore better isolation of protoplasts. High temperature (30ºC) led to a decrease in protoplasts number which made conditions unsuitable for enzymes to work efficiently. Maheshwari *et al*., (1986) found that the optimum temperature for protoplasts isolation from potato plant (*Solanum tuberosum*) was (25ºC). While the number of isolated protoplasts by using other temperatures (20, 23, 25 or 30°C) was (600000 protoplast/ml with viability 12%, 1800000

Table (6) Number of isolated protoplasts from leaves using different temperatures and their viability by using (pectinase, cellulase and himecellulase) enzyme mixture at pH 5.4 after 24 hrs.

Temperature ºC	No. of isolated protoplasts (protoplast/ml)	Viability (%)
20	600000	12
23	1800000	28
25	1950000	54
28	2150000	35
30	450000	22

protoplast/ml with viability 28%, 1950000 protoplast/ml with viability 54% and 450000 protoplast/ml with viability 22%) from leaves and (1500000 protoplast/ml with viability 10%, 3250000 protoplast/ml with viability 33%, 3500000 protoplast/ml with viability 61% and 800000 protoplast/ml with viability 25%) from callus respectively.

Table (7) Number of isolated protoplasts from callus using different temperatures and their viability by using (pectinase, cellulase and himecellulase) enzyme mixture at pH 5.4 after 24 hrs.

Temperature °C	No. of isolated protoplasts (protoplast/ml)	Viability %
20	1500000	10
23	3250000	33
25	3500000	61
28	3750000	37
30	800000	25

Temperature can affect enzyme activity and its effect is very complex. It affects the speeds of molecules, the activation energy of the catalytic reaction and the thermal stability of the enzyme and substrate. At low temperatures the rate of enzyme reaction is very slow. The molecules have low kinetic energy and collisions between them are less frequent and even if they do collide the molecules do not posses the minimum activation energy required for the reaction to occur. The enzymes are deactivated at low temperatures. An increase in temperature increases the enzyme activity since the molecules now possess greater

kinetic energy. After 30ºC the rate of reaction starts to decrease. This is because the increase in temperature does not increase the kinetic energy of the enzyme but instead disrupts the forces maintaining the shape of the molecule. The enzyme molecules are gradually denatured causing the shape of the active site to change (Porter, 1984).

This optimum temperature (25°C) was used in the next experiments for optimization of protoplast isolation.

3.5.3- pH

Maximum number of isolated protoplasts was obtained when the pH value of the enzyme mixture was adjusted to 5.4 (table 8 and 9).

Table (8) Number of isolated protoplasts from leaves using different pH and their viability by using (pectinase, cellulase and himecellulase) enzyme mixture at 25°C after 24 hrs.

pH	No. of isolated protoplasts (protolast/ml)	Viability (%)
5	900000	33
5.2	1700000	44
5.4	1950000	54
5,8	1550000	41
6	700000	28

Table (9) Number of isolated protoplasts from callus using different pH and their viability by using (pectinase, cellulase and himecellulase) enzyme mixture at 25°C after 24 hrs.

pH	No. of isolated protoplasts (protoplast/ml)	Viability %
5	1750000	34
5.2	3100000	40
5.4	3500000	61
5.8	2600000	44
6	1100000	23

The number of isolated protoplasts from leaves was (1950000 protoplast/ml) with viability (54%) while from callus was (3500000 protoplast/ml) with viability (61%). This pH value may be ideal for enzymes to work properly, resulting in higher rate of protoplast yield (Niedz *et al*., 1985). The increase or decrease of pH above or below this value caused a decrease in protoplast number. This may due to the alteration in the activity of enzyme(s) responsible for cell wall degradation.

Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This will affect the total net charge of the enzymes and the distribution of charge on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighborhood of the active sites. Taken

together, the changes in charges with pH affect the activity, structural stability and solubility of the enzyme (Monteiro *et al*., 1991).

Since enzymes are proteins, they are very sensitive to changes in pH. Each enzyme has its own optimum range for pH where it will be most active. This is the result of the effect of pH on a combination of factors: (1) the binding of the enzyme to substrate, (2) the catalytic activity of the enzyme, (3) the ionization of the substrate, and (4) the variation of protein structure.

This optimum pH value (5.4) was used in the next experiments for optimization of protoplast isolation.

3.5.4- Incubation time

It is clear from the data presented in table (10 and 11) that the maximum number of isolated protoplasts was obtained when the enzyme mixture incubated for 24 hours, after this period the number of isolated protoplast was (1950000 protoplast/ml) with viability (54%) from leaves while from callus was (3500000 protoplast/ml) with viability (61%). On other hand, results showed that the increase of the incubation period above the optimum period (42 and72 hours) causes a decrease in number of isolated protoplasts (1400000 and 600000 protoplast/ml with viability 39 and 9% from leaves while 2350000 and 950000 protoplast/ml with viability 40 and10.5% from callus). This may due to the exhausting of the enzyme in the mixture in addition to entrance the death phase, while the decrease of the incubation period under the optimum (18) also causes a decrease in number of protoplasts (1500000 protoplast/ml with viability 50% from leaves while 2100000 protoplast/ml with viability 59% from callus), and this may due to period were insufficient to utilize all the tissue as it was mentioned by (Sankara Rao and Prakash, 1995).

Table (10) Number of isolated protoplasts from leaves using different incubation periods and their viability by using (pectinase, cellulase and himecellulase) enzyme mixture at pH 5.4 and 25°C.

Incubation period (hr.)	No. of isolated protoplasts (protoplast/ml)	Viability (%)
18	1500000	50
24	1950000	54
48	1400000	39
72	600000	9

Table (11) Number of isolated protoplasts from callus using different incubation periods and their viability by using (pectinase, cellulase and himecellulase) enzyme mixture at pH 5.4 and 25°C.

Incubation period (hr.)	**No. of isolated protoplasts (protoplast/ml)**	**Viability (%)**
18	2100000	59
24	3500000	61
48	2350000	40
72	950000	10.5

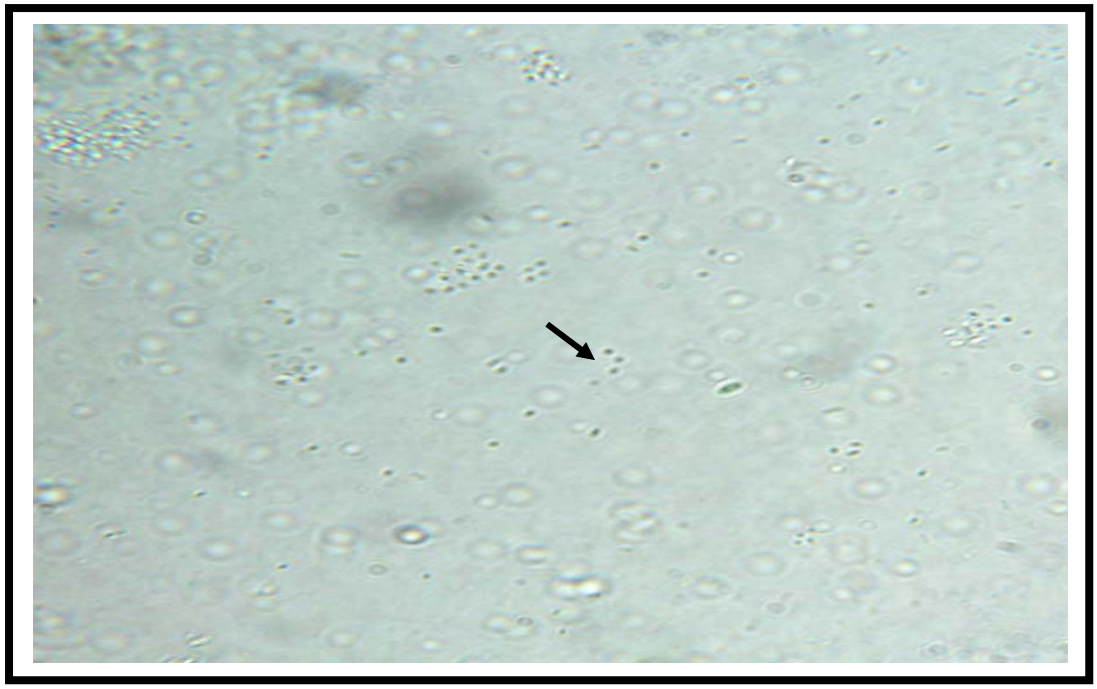

Figure (7) Isolated protoplasts from *Lycopersicon esculentum* tomatos leaves (x100).

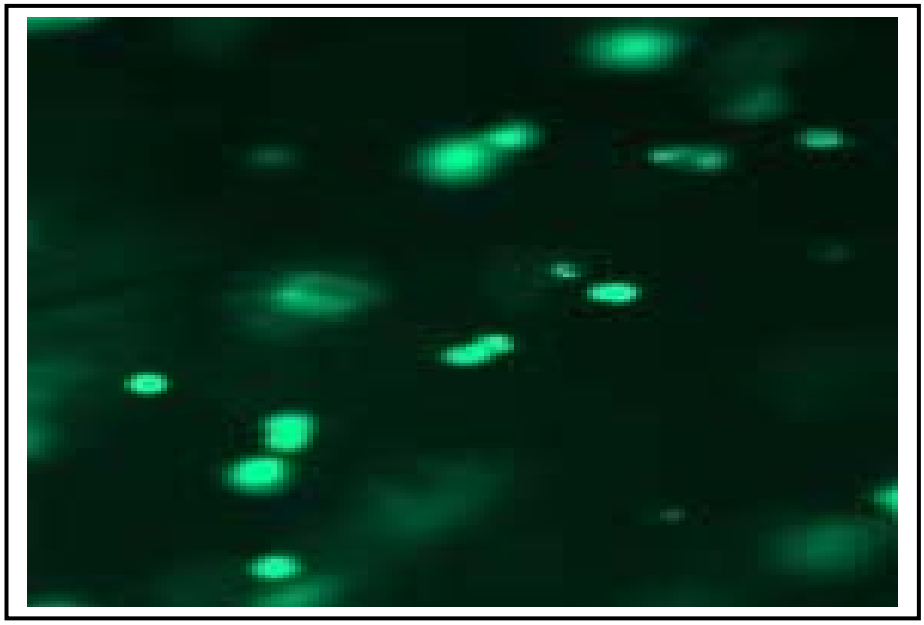

Figure (8) Isolated protoplasts from *Lycopersicon esculentum* tomatos leaves stained by viability stain (FDA) under UV microscope (x100).

3.6- Protoplast fusion

3.6.1- Poly ethylene glycol (PEG)

Four different molecular weights of Poly ethylene glycol (PEG) (2000, 4000, 6000 and 8000 m.wt) were used as chemical fusing agent to determine the optimum in protoplasts fusion that isolated from two tomato hybrids.

Results in figure (9) showed that the maximum percentage of fused protoplasts was obtained when the PEG of (6000 m.wt) was used as chemical fusing agent. Using this fusing agent, the fusion percentage of isolated protoplasts was (50%), while the fusion percentage of protoplasts by using other molecular weights of PEG (2000, 4000 and 8000 m.wt) were (25, 35 and 15%) respectively.

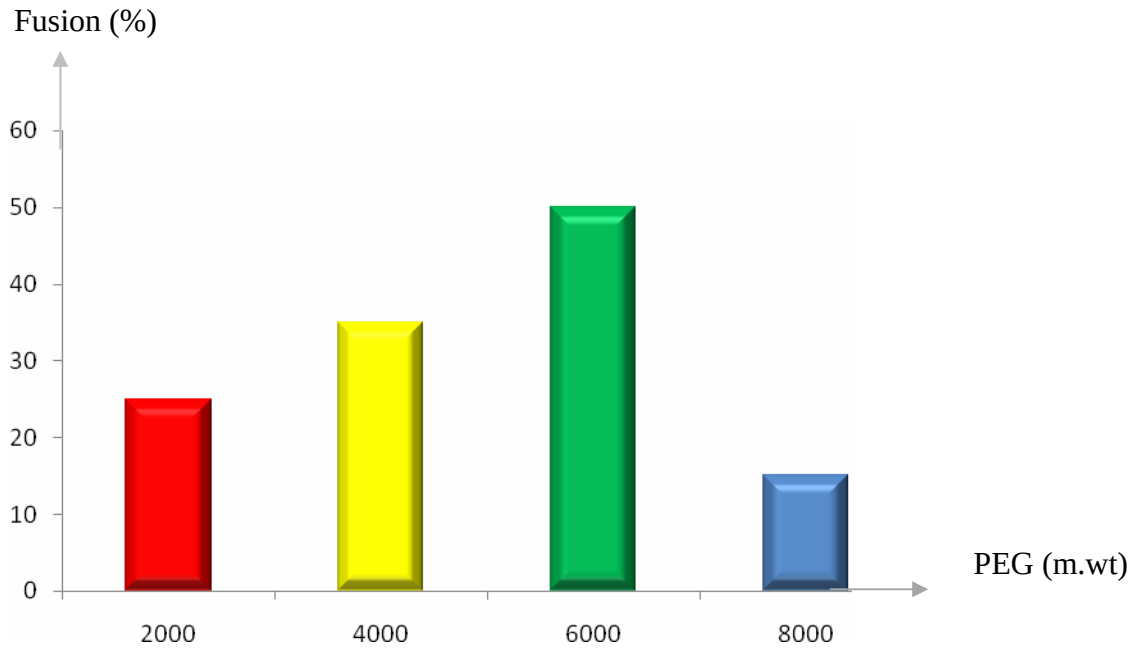

Figure (9) Effect of different (m.wt) of PEG on protoplasts fusion percentage of *L. esculentum*.

From these results we conclude that both, the concentration and molecular weights of PEG are important in relation to fusion. PEG, whose general formula is HOCH2-(CH2-O-CH2)-CH2OH is a water soluble compound whose other linkage make the molecule slightly negative in charge. The high molecular weight of the polymer acts as abridges connecting the protoplasts together. A strong affinity of PEG for water causes local membrane dehydration and increased fluidity. This in combination with the reduction of an exclusion volume between adjacent protoplasts causes diminishing mutual membrane electrostatic repulsion. The reduction of glycoprotein and glycocalyx macromolecules, causes fusion (Chen *et al*., 2002).

PEG of molecular weight (4000 – 6000) was found to be active in fusion, where as PEG 1500 and 10000 m.wt was almost inactive (Chen and Imanishi, 1991). This optimum molecular weight of PEG (6000 m.wt) was used in the next experiments of optimization for protoplast fusion.

3.6.2- Chemical fusogen solutions

Four types of fusogens (PEG, Ca^{++} at high pH, PEG + Ca^{++} at high pH and sodium nitrate) were used as chemical fusogen to determine the optimum in protoplasts fusion that isolated from two tomato hybrids.

Results in figure (10) showed that the maximum percentage of fused protoplasts was obtained when the PEG + Ca^{++} at high pH was used as a chemical fusogen. Using this fusing agent, the fusion percentage of isolated protoplasts was (60%), while the fusion percentage of protoplasts by using (PEG, Ca^{++} at high pH or sodium nitrate) was 50%, 35% and 10% respectively.

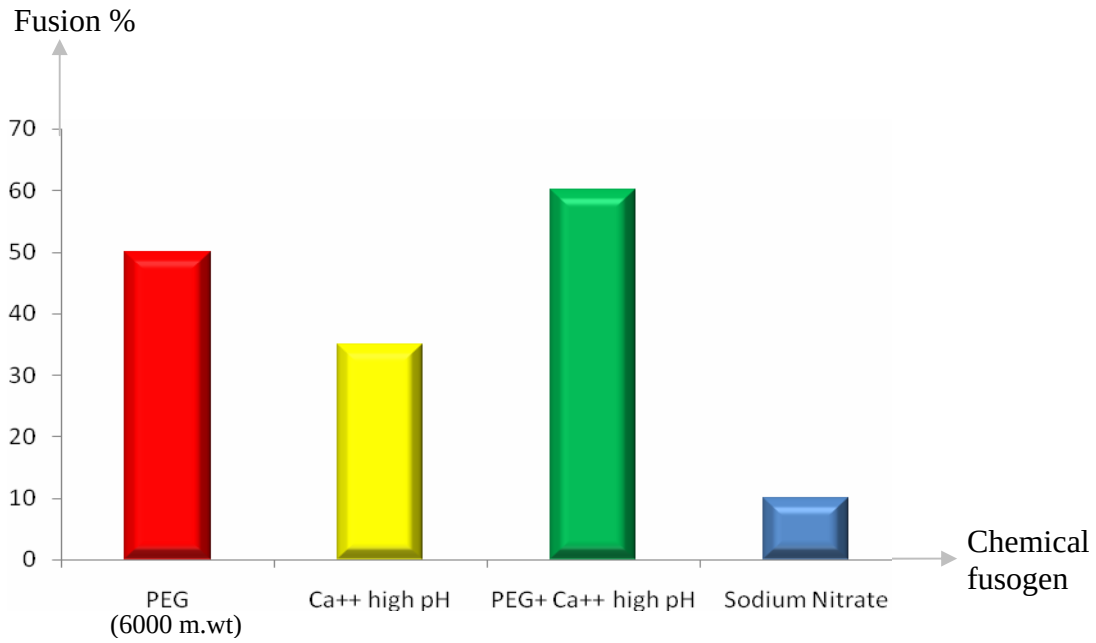

Figure (10) Effect of different chemical fusing agent on protoplasts fusion percentage of *L. esculentum*.

From these results we can conclude that PEG + Ca^{++} at high pH was the best chemical fusogen because the addition of Ca^{++} to the PEG causes the potential of the surface negative charge on protoplasts to be reduced, facilitating protoplast adhesion. The high alkalinity (pH 10.4) induces the formation of intramembranous lysophospholipids such as lysolecithin and lysophosphatidyl-ethanolamine that increase membrane fluidity that results in fusion (Jourdon *et al*., 1993).

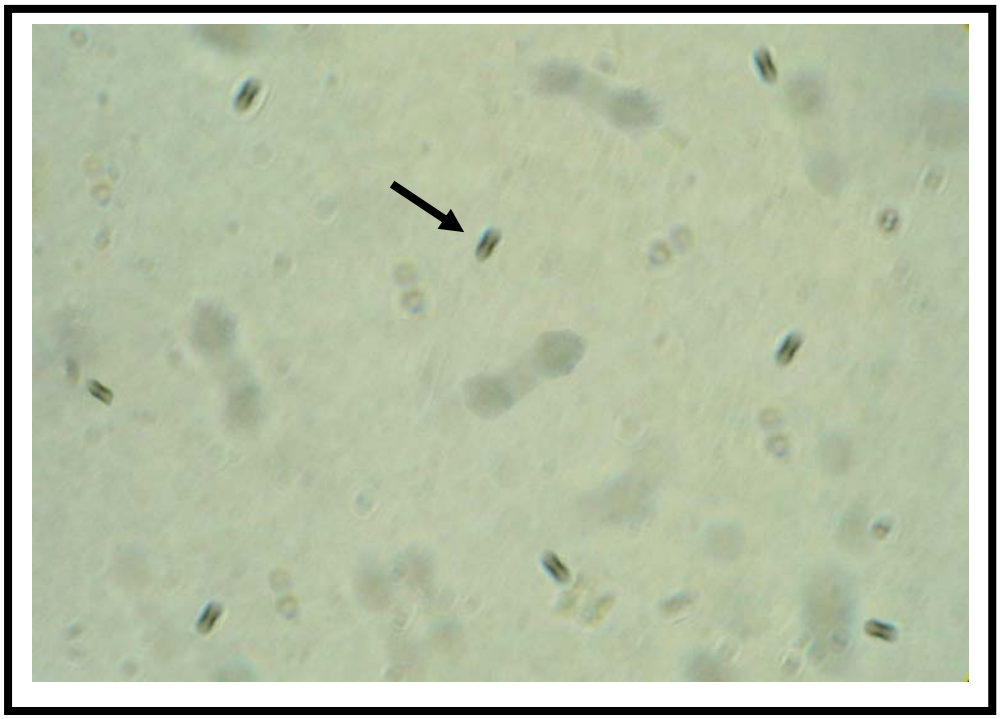

Figure (11) Protoplast fusion between two hybrids (Hatouf and Barakah) of *L. esculentum*.

Conclusions

1) The best concentration for *Lycopersicon esculentum* leaf surface sterilization was 3% of NaOCl for 3 min.
2) Callus cultures can be induced and maintained on MS medium supplemented with 2.0 mg/l BAP and 0.1 NAA using leaves as a source of explants.
3) Isolation of protoplasts from callus was easer and gave high yield compared with these isolated from leaves.
4) Optimum conditions for protoplasts isolation (leaf or callus) from the two tomato hybrids was 1950000 protoplast/ml from leaves and 3500000 protoplast/ml from callus when the enzyme mixture consisted of (0.5% w/v pectinase, 2% w/v cellulase and 2% w/v hemicellulase), temperature 25ºC, pH 5.4 and incubation time 24 hrs.
5) Optimum conditions for protoplasts fusion were achieved when PEG (6000 m.wt) + Ca^{++} at high pH was used as chemical fusing agent.

Recommendations

1) Investigating the effect of using different plant organs (root, leaves and flowers) on the efficiency of isolated protoplast yield.
2) Investigating the effect of various enzyme mixtures/types (pectinase, cellulase, himecellulase) on the efficiency of protoplast yield.
3) Investigating the effect of initial protoplast concentration on the percentage of protoplasts which fuse.
4) Regeneration of *L. esculentum* plants result from protoplast fusion, then use of PCR to examine the regenerated progeny for their resistance to viruses.

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الملخص

درستْ إمكانية تَحقيق الظروف المُثلى في عَزل ودَمج ألبروتوبلاست لِصنفي هتوف وبركة لِنبات ألطماطم *Lycopersicon esculentum* من الأوراق ومزارعها النسيجية.

أذ أُستحثَ الكالس وأُديم على وسط MS المجهز بـ (2.0) ملغم/لتر من BAP و (0.1) ملغم/ لتر من NAA بأستعمال الأوراق َمصدراً لِنشوء الكالس.

درستْ إمكانية تَحقيق الظروف المُثلى في عزل البروتوبلاست من الأوراق والكالس. أظهرت النتائج إن إضافة خليط مكوّنْ من إنزيمات ألبكتنيز، السليليز والهميسليليز بالمقادير 0.5، 2.0 و 2.0 %(وزن/حجم) على التوالي وعلى درجة حرارة 25°م و pH مقداره 5.4 بعد الحضن لِمدة 24 ساعة، كانت ألأفضل من حيث إنتاج أعداد البروتوبلاست المعزولة. بلغتْ إنتاجية البروتوبلاست تحت هذه ألظروف 1950000 بروتوبلاست/مل من ألأوراق و 3500000 بروتوبلاست/مل من الكالس. أظهرتْ النتائج إن اعداد البروتوبلاست المعزولة من الاوراق بأستخدام درجاتْ حرارة مُختلفة (20 و 23 و 28 و 30°م) بلغتْ (600000 و 1800000 و 2150000 و450000) بروتوبلاست/مل، فيما بلغت الاعداد المعزولة من الكالس (1500000 و 3250000 و 3750000 و 800000) بوتوبلاست/مل. كما اَظهرتً عند استخدام قيم مُختلفة من الـ pH (5 و 5.2 و 5.8 و 6) ان اعداد البروتوبلاست المعزولة من الأوراق (900000 و 1700000 و 1550000 و700000) بروتوبلاست/مل بيما بلغتْ (1750000 و 3100000 و 2600000 و 1100000) بروتوبلاست/مل من الكالس. اما عند استخدام مدد حضن مختلفة (18 و 48 و 72) ساعة بلغتْ اعداد البروتوبلاست المَعزولة من الأوراق (1500000 و 1400000 و 600000) بروتوبلاست/مل فيما بلغت اعدادها من الكالس (2100000 و 2350000 و 950000) بروتوبلاست/مل.

تَضمنت الدراسة أيضاً مُحاولة أمثلة ظروف دَمج البروتوبلاست المَعزول من صنفي الطماطم هتوف وبركة إذ أظهرت النتائج إن أضافة كلايكول متعدد الأثيلين وبأوزان جُزيئية مُختلفة (PEG) (2000 و 4000 و6000 و8000) أدى ألى تَحقيق نسب دَمج بلغتْ (25 و 35 و 50 و 15%) على التوالي.

اختبرت توليفات مُختلفة من مُدمجات كيميائية (Chemical fusogens) أدت إلى تَحقيق نسب دَمج بلغتْ 10% عند اضافة نترات الصوديوم و 35% عند اضافة آيونات ألكالسيوم في pH 10.5، وفي حالة إستخدام (PEG) ذو وزن جزيئي 6000 و آيونات ألكالسيوم في pH 10.5 بلغتْ نسبة دَمج 60%.

الإهداء

إلى رمز الشموخ الذي علمني الاصرار على التفوق...

والدي الحبيب

إلى القلب الذي اتسع لكل الحنان...

والدتي الحبيبة

إلى سندي الدائم...

اخوتي

إلى من اعز واقرب إلى نفسي..

اختي

اهدي جهدي المتواضع

آزاد

بِسم لله لرحمن لرحيِم

نرفَع درجاتٍ من نـشاءُ

وفَـــوق كُـــل ذ ي

علمٍ عليم

صدق لله لعظيم

سورة يوسف

الاية 76

جمهورية ألعراق
وزارة ألتعليم ألعالي وألبحث ألعلمي
جامعة ألنهرين
كلية ألعلوم
قسم ألتقانة الأحيائية

عزلْ وتنقية ودمج ألبروتوبلاست بينَ هجينين من ألطماطم

رسالة

مُقدمة إلى كُلية ألعلوم - جامعة ألنهرين وهي جزء من مُتطلبات نيل دَرجة ماجستير عُلوم في ألتقانة ألأحيائية

مِنْ قبَل

آزاد أسعد عبد ألكريم خيلانيْ

بَكالوريوس تقانة أحيائية – جامعة ألنهرين –2003

تحت إشراف

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أيلول 2008

رمضان 1429