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Master of Biological Sciences

In Vitro, Propagation of Strawberry (*Fragaria × annanasa* Duch.) Through Organogenesis via Runner Tips

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DECLARATION

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute, except where due acknowledgment has been mad in the text.

Emad Y. Youssief

DEDICATION

I dedicate this thesis to my parents, my wife, and to Youssief, Ahmed and Abed El Rahman, who always supported my career and helped me to achieve this degree.

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ABSTRACT

Using *in vitro* tissue culture technique through organogenesis, recognizing the most important problems during planting stages, and treating these problems. As a result of this, plants in large numbers and with high quality is produced. Furthermore, this technique can be applied on other kinds of plants. Growing strawberry with this technique is economically advantageous.

The cultivated strawberry (*Fragaria × ananassa* Duch.), a member of the Rosaceae, is the most important soft fruit worldwide. *In vitro* techniques are important for clonal multiplication. An efficient method for sterilization, browning, shoot regeneration and root formation from runner tips of strawberry plantlets cv. Sweet Charlie was developed. Runner tips of 1-2 cm long were used as source of explants. In sterilization method, when the runner tips were immersed in sodium hypochlorite solution (1.5%) containing two to three drops of Tween 20 per 100 ml for 20 minutes and 0.1% $HgCL_2$ for 10 minutes gave the maximum aseptic cultures in august, however, in October all the samples were failed to reduce explants contamination.

After surface sterilization apical meristems of 3-5 mm long were isolated and used as explants.

To study the effect of various pretreatments on explants browning and survival percentage, different sets of sterilized explants were dipped in three different concentrations of antioxidant solution (1, 1.5 and 2% PVPP) for 1-2 h prior to inoculation with addition of (0.2g/100 ml) PVPP in the MS free hormone medium and subjecting cultures to an initial ten days cold treatment before transfer the explants to growth chamber, the results indicated that percentage of survival explants when treatment with 1% and 2% PVPP were 20 and 60% respectively in August season.

However, when repeated the same experiment in October season, with addition to another set dipped in sterilized distilled water without antioxidant solution as a control, the lowest percentage of browning were observed in the control and 2% PVPP were 100 and 70 % respectively.

At multiplication stage results indicated that the highest auxiliary buds were observed when MS medium supplemented with 1 mg / l BA.

At rooting stage, it was clear visually that MS medium supplemented with 0.5 g/l activated charcoal and MS medium at full strength gave better results in enhanced the root number and length and shoot number and length per plantlet.

Effect of IBA on the root response, the results indicated that IBA with (0.5, 1.0, 1.5 mg/l) observed the lowest root response compared to all other treatments.

Because Israeli air force bombed the lab building at the Islamic university in 27-12-2008. We wrote down data visually in rooting stage.

This research paper is considered the first published in Gaza strip.

ملخص الدراسة

مخبريا استزراع نبات التوت الأرضي (فراجاريا أناناسا) من خلال إنتاج الأعضاء النباتية للقمم الخضرية للمدادات

استخدام تقنية زراعة الأنسجة مخبريا من خلال إنتاج الأعضاء النباتية و التعرف على أهم المشاكل التي تظهر من خلالها في مراحل الزراعة المختلفة و معالجتها بالطرق السليمة يؤدى إلى إنتاج أشتال بكميات كبيرة و بمواصفات عالية الجودة، مما يسهل تطبيق هذه التكنولوجيا الحديثة في معظم النباتات التي نحن بحاجة إليها.

يعتبر نبات التوت الأرضي من العائلة الوردية و هو من النباتات الهامة و المنتشرة عالميا لإنتاجه الفاكهـــة، كمـــا تعتبر تقنية إكثار التوت الأرضي بواسطة زراعة الأنسجة مخبريا من الأمور الهامة اقتصاديا.

لبدء تلك الدراسة تم استخدام القمم الخضرية للمدادات من نبات التوت الأرضي نوع سويت شارلى بطول 1-2 سم كمصدر للأجزاء النباتية المستخدمة للزراعة بالمعمل.

للتغلب على مشكلة التلوث تم معالجة الأنسجة بمادة الصوديوم هيبوكلورايت كمادة معقمه وذلك بتركيـــز (1.5%) لمدة 20 دقيقة تحتوى 2-3 نقاط لكل 100 ميليليتر مـــن مـــادة تـــوين 20 و بعـــد ذلـــك عولجـــت بمـــادة $HgCL_2$ بتركيز 0.1 % لمدة 10 دقائق وذلك في أوقات زمنية مختلفة (شهري أغسطس وأكتوبر).

كانت أفضل نتيجة تعقيم في شهر أغسطس، بينما في شهر أكتوبر فقد ظهر التلوث في جميع العينات.

للتغلب على مشكلة الأكسدة تم معالجة الأنسجة بمادة PVPP وذلك بعدة تراكيـــز (1، 1.5 ، 2 %) لمـــدة 1 – 2 ساعة بعد أن تم إضافة (0.2 جرام / 100 ميليليتر) من نفس المادة في بيئة الزراعـــة (موراشـــيج و ســـكوج) و تعريض الأنسجة المزروعة لدرجة حرارة 4 س لمدة عشر أيام قبل نقلها لغرفة النمو.

أما فيما يخص عملية الأكسدة والتي قيس نجاحها بمدى حيوية الأنسجة فقد أظهرت النتائج أن نسبة حيوية الأنسجة كانت 60 % للعينات المعالجة بتركيز 2% ونسبة 20 % للعينات المعالجة بتركيز 1% من مادة PVPP وذلك في شهر أغسطس.

عند إعادة نفس التجربة في شهر أكتوبر، و بإضافة مجموعة أخرى و هي معالجة الأنسجة المعقمة بالماء المقطـــر المعقم فقط بدون المعالجة بمادة ضد الأكسدة (كنترول). أظهرت النتائج أن أقل نسبة للأكسدة كانت فـــي العينـــات المعالجة بالماء المقطر المعقم (الكنترول) بنسبة 100 % و العينات المعالجة بتركيز 2 % بنسبة 70 %.

و في مرحلة التضاعف: و التي أجريت لدراسة تضاعف الأفرع و البراعم الناتجة من المرحلة السابقة، أثبتت النتائج أن بيئة موراشيج و سكوج المحتوية على 1 ملجم / لتر بنزيل أدينين أعطت معدل عال من البراعم الأبطية.

و في مرحلة التجذير: كان واضحا نظريا أن بيئة موراشيج و سكوج المحتوية على 0.5جم / لتر من الفحم المنشط و نفس البيئة التي تضمنت قوى أملاح موراشيج و سكوج الكاملة أعطت أفضل النتائج بالنسبة لطول الجذور و الأفرع مقارنة مع التراكيز الأخرى لكلا من الفحم المنشط وقوى أملاح موراشيج و سكوج.

عند إضافة هرمون اندول حمض البيوتيريك لبيئة موراشيج و سكوج بتركيز (0.5 ، 1 ، 1.5 ملجم / لتر)، لم يظهر أي نتائج على إنتاج الجذور.

لم يتم انجاز هذا البحث بالشكل المطلوب و هو إنتاج نباتات لها القدرة على النمو و الأقلمة خارج بيئة المختبر وذلك بسبب تعرض مبنى المختبرات في الجامعة الإسلامية للتدمير من قبل طائرات الجيش الأسرائيلى بتاريخ 27- 12- 2008 وتدمير غرفة النمو الخاصة بالبحث، فقد تم الوصول لمرحلة التجذير و لم نستطيع توثيق النتائج لذلك تم أخذ النتائج نظريا.

من الجدير بالملاحظة أن هذا البحث يعتبر الأول من نوعه على نطاق قطاع غزة.

الكلمات المفتاحية: الإكثار الدقيق، نبات التوت الأرضي، فراجاريا، تقنية زراعة الأنسجة، هرمون بنزايل أمينوبيورين، بولى فينيل بيرديزون، الفحم المنشط.

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ABBREVIATIONS

Abbreviation	Full form
AC	Activated charcoal
BA	N6 Benzylaminopurine
BM	Basal medium
CH	Casein hydrolysate
CV	Cultivar
°C	Degree Celcius (Temperature)
2,4-D	2,4-Dichlorophenoxyacetic acid
GA3	Gibberelic acid
2-iP	6-(g,g-dimethylallylamino)-purine or N6-(2-isopentyl)-adenine
IAA	Indolyl-3-acetic acid
IBA	Indolyl-3-butyric acid
KIN/K	Kinetin, 6-furfurylaminopurine
MS	Murashige and Skoog's Medium (1962)
µL	Microlitre
NAA	1-Naphthaleneacetic acid
ppm	Part Per million
PVP	Polyvinylpyrrolidone
RAPD	Random amplified polymorphic DNAs analysis
RFLP	Restriction fragment length polymorphism analysis
TDZ	Thidiazouron, N-phenyl-N'-1,2,3-thidiazol-5-ylurea
VAM	Vesicular - arbuscular mycorrhizae

CHAPTER 1

Intruduction

1.2 Cell Culture

The technique of plant tissue culture occupies a key role in the second green revolution in which gene modification and biotechnology are being used to improve crop yield and quality. Those working with plant cell tissue culture are still concerned with the details of the technique but are now more involved in its application to fundamental aspects of plant cell differentiation and development and to the problems of crop improvement [1].

Plant tissue culture is the science or art of growing plant cells, tissues or organs on artificial media by isolating them from the mother plant [2].

Historically, the science of tissue culture development is linked to the discovery of cell and subsequent cell theory, which states that the cell is the basic structural unit of all living things. Plant tissue culture is based on the cell doctrine that states a cell is capable of autonomy and is potentially totipotent. In 1902, the German botanist Gottlieb Haberlandt developed the concept of *in vitro* cell culture. He isolated single cells from palisade tissue of leaves, pith parenchyma, epidermis and epidermal hair of various plants and cultured on Knop's salt solution containing glucose and peptone. In his cultures, cells that synthesized starch and increased in size survived for several weeks though none of them divided. He predicted the requirements for cell division under experimental conditions that have been proved through time. Therefore, Haberlandt is considered as the father of plant tissue culture. Following Haberlandt, many workers continued working on plant tissue cultures. In 1939, Gautheret cultivated cambial tissues of carrot root, Nobecourt (carrot), and White (tobacco) for prolonged periods of time. In strict sense, these were the first true plant tissue cultures [3].

By using plant tissue culture techniques, complete new plants can be obtained from different explants through direct or indirect morphogenesis and through somatic embryogenesis. Direct morphogenesis is the production of shoots from explants without passing through callus (unorganised tissue) phase while indirect morphogenesis refers to induction of shoots through callus phase. The culture in which an organised form of growth can be continuously obtained is referred to as organ culture [2]. The most important kinds of organ cultures used for micropropagation are meristem cultures, shoot cultures, embryo cultures and isolated root cultures. Callus cultures, suspension or cell cultures, protoplast cultures or anther cultures are grouped as unorganised tissue cultures. Although

the basic nutritional requirements of *in vitro* cultured plant cells are very similar to those utilized by plants, the nutritional composition varies depending on the type of cells, tissues, organs, protoplasts and the plant species. There is also a difference in nutritional requirement among genotypes or cultivars of the same species.

A nutrient medium is defined by its composition of mineral salts, carbon source, vitamins, plant growth regulators and other organic supplements. A particular medium is identified by its salt composition unless otherwise specified.

Other additives such as amino acids, vitamins, growth regulators or organic supplements can be added in any concentrations to a given salt composition to get the desired results. Although several media have been developed, the MS medium [4] is very widely used in different plant tissue culture systems. Organic additives and the type and concentrations of growth regulators that are added to the basic media are generally considered to be the most important factors that affect the response of plant tissues in culture. However, other additives such as activated charcoal and polyamines and exogenous factors such as temperature, light intensity and quality, are important in determining the responses of some tissues in culture [5]. If cultures are maintained for long time, abnormal phenotypes could be observed. Most of those changes are physiological and temporary, but some are genetic and affect the manner in which the plants grow, flower and fruit so that changing the economic and aesthetic value. Therefore, due to these changes it is important to carefully evaluate the performance of tissue cultured plants as they grow to maturity in greenhouse, garden or field [6].

Plant cell and tissue culture are used for clonal propagation, production of disease-free plants, haploid production, triploid production, *in vitro* pollination and fertilization, embryo rescue, somatic hybridization and cybridization, somaclonal and gametoclonal variant selection, germplasm conservation, secondary metabolite production, and genetic transformation. Although living cells are considered potentially totipotent, only some cells that are competent divide and give rise to complete plant in tissue culture. Furthermore, not all plant species are equally amenable to tissue culture. Although production or improvement of perennial plants, both woody and herbaceous, using tissue culture especially for cloning and genetic engineering seem very attractive, the complex seasonal

cycles and life cycles of those plants complicate the control of their growth in tissue culture [7].

The early establishment of shoot cultures for these perennial plants is one of the important approaches. Stabilized shoot cultures are excellent sources of cells, tissues and organs that can be used in further complex procedures such as protoplast generation [8, 9] gene insertion and transclone recovery. If shoot cultures cannot be readily established, these advantages cannot be realized. Therefore, a major cause of recalcitrance in perennial plants is the inability to establish fully stabilized shoot cultures.

It is difficult to generate stabilized shoot cultures for plants that have seasonal growth dynamics dominated by strong episodic or determinant shoot growth. The relatively slow growth rate of perennials in culture also complicates the tissue culture procedures as many perennial tissues release high content of phenolic compounds into a culture medium. In some cases, some technical approaches can overcome those limitations in tissue culture. However, development of a deeper understanding of physiological bases of such genetically predetermined phenomena is important [7].

When Haberlandt 1839 attempted the first plant cell culture, his intentions were to develop a more versatile tool to explore morphogenesis and to demonstrate totipotentiality of plant cells. He probably did not suspect that the cell culture technique would become a valuable aid in economically oriented activitie [10].

The goal of asexual propagation is to produce uniform plants of a selected genotype. Vegetative propagation usually assures that the desired characteristic of the selected plant is retained throughout its clone. In contrast, there is no certainty that seedlings will reproduce even some of the parental characteristics. Nevertheless, seed propagation has been used in many instances for economic reasons, and major characteristics of a variety have been reproduced with sufficient consistency e.g. many flowers and vegetables [10].

Asexual multiplication of both hardwood and softwood using tissue culture methods can be achieved by axillary bud breaking, production of adventitious buds and somatic embryogenesis. Plantlets via axillary buds were produced from about 45% of the angiosperm, 13% from adventitious buds, 25% indirectly from callus and 17% from

somatic embryogenesis, for gymnosperm, the percentages were 28, 55, 5, and 12%, respectively. Therefore, for angiosperms the most successful method was axillary bud multiplication, and for gymnosperms, direct adventitious bud formation [11].

Tissue culture and micropropagation have become important elements in the plant propagation industry. The total volume of micropropagated plants annually in the world is estimated to be hundreds of millions of plants. It is reported that around some 50,000 varieties   of plants are propagated in the world using the tissue culture method. The business of plant propagation involves approximately 600 companies and accounts for more than 500 million units, the majority of which are ornamental species [12].

1.2 Application of Tissue Culture

 During the last 30 years it has become possible to regenerate plantlets from explants and/or callus from all types of plants. As a result, laboratory-scale micropropagation protocols are available for a wide range of species and at present micropropagation is the widest use of plant tissue-culture technology. There were over 300 commercial operators World-wide in 1990. In Europe, there were 172 micropropagation firms and about 1800 different tissue lines (species and varieties) in culture amongst the 501 plant tissue-culture laboratories identified in 1993. For example, of the 88 European laboratories using potato in tissue culture, 58 were listed as using *in-vitro* multiplication, 49 were involved in the elimination of pathogens, 45 were using tissue-culture simply to store germplasm, 44 were involved in genetic modification and 26 had plant-breeding programmes [13].

There are four areas in which applications of plant tissue culture are possible, either presently or in the near future:
  a. production of pharmaceuticals and other natural products;
  b. the genetic improvement of crops;
  c. The recovery of disease-free clones and preservation of valuable   germ plasm; and d. rapid clonal multiplication of selected varieties. Although there has been substantial research on the use of cell and organ cultures as sources of pharmaceuticals, a successful commercial application remains unrealized [10].

1.3 Stages of the Tissue Culture Method

While it has been recognized that the propagation of a plant through tissue culture must proceed through a sequence of steps, it has not been the general practice to systematically explore specific requirements of each step. As a commercial procedure, the sequential steps must be identified and virtually optimum conditions of each should be established. In principle, there are three major steps, hereafter referred to as stages for the purpose of emphasis, each with a different objective and possibly different requirements [10].

1.3.1 Stage I: Establishment of the Aseptic Culture

The objective of Stage I is simply to attain an aseptic tissue culture of the plant in question. The culture may result as enlarging shoot tips, rooted shoot tips, callus, etc. It is only necessary that the culture be free from obvious infection, that a suitable proportion of explants survive culture, and that there is rapid growth among the explants [10].

1.3.2 Stage II: Multiplication of Propagula

In Stage II a rapid increase of organs and other structures which can ultimately give rise to plants is the aim. The increase can be achieved in most instances by either inducing adventitious organ or embryo formation or by enhancing axillary shoot initiation. With most species the more likely method of multiplication would be adventitious organogenesis, either of shoots or of asexual embryos. In many cases an intermediary callus has been involved. The method of adventitious organogenesis may enable a substantially faster increase in propagula, it can have serious disadvantages. For example, a high incidence of genetically aberrant plants has not been uncommon with this method. The method of axillary shoot multiplication may be slower, but genetically deviant plants have been virtually absent when it has been used, e.g. Asparagus officinalis and Gerbera jamesonii [10].

1.3.3 Stage III: Preparation for Reestablishment of Plants in Soil

A successful tissue culture method of propagation must result in reestablishment in soil of a high frequency of the tissue culture derived plants. Stage III is intended to prepare the propagula for their successful transfer to soil. Research on Stage III has been largely neglected. This stage involves the rooting of shoot cuttings, hardening of plants to impart some tolerance to moisture stress, conferring of a degree of resistance to certain pathogens,

and conversion of plants from the heterotrophic to the autotrophic state. With certain plants, e.g. bulbs and others adapted to the temperate climate, it may be necessary to satisfy dormancy-associated requirements.

Bulbs, corms, and tubers may undergo dormancy when transferred from test tube to soil without having fulfilled their chilling requirements.

Common conditions with respect to nutrient medium and culture environment may sometimes be applicable to two stages. Thus, it has been possible to proceed through Stages I and II by utilizing nutrient media of the same composition and form and the same light and temperature provisions. For maximum success stage III conditions should be distinct from the other two [10].

1.4 Micropropagation Methods

1.4.1 Organogenesis

Organogenesis referred to the process whereby explants, tissue or cell can be induced to form root and shoot and even whole plantlets [14].

Embryos are not classified as organs because these structures have an independent existence that is embryos do not have vascular connections with the parent plant body [15].

Organogenesis *in vitro* can follow either of the two paths:
Indirect organogenesis by Primary explant is induced to form callus prior to undergoing de novo organogenesis. The resultant plants may show variations due to the involvement of the callus phase and direct organogenesis without passing through callus phase is involved and the regenerants may be identical to the parent plant [16].

The key feature of *de novo* organogenesis is the formation of a 'meristemoid' arising from vacuolated parenchyma cells. The cells of the meristemoid are small, isodiametric, and thin-walled with prominent nuclei and with extensive network of plasmodesmata. This is the first step of 'Cellular Differentiation'. It is followed by 'Acquisition of Competence', 'Cellular Determination' and finally 'Cellular Differentiation' giving rise to organs [16].

Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. There are three methods of plant regeneration via organogenesis:

The firest two methods depends on adventitious organs arising either from a callus culture or directly from an explant, alternatively, axillary bud formation and growth can also be used to regenerate whole plants from some types of tissue culture [17].

1.4.1.1 Multiplication by Apical and Axillary Shoots

Micropragation through apical and axillary shoot proliferation is the most reliable technique for mass multiplication since it ensures genetic stability of clones. Apical and axillary shoots contain active meristems. Shoot tips cultured on basal medium containing no growth hormones typically develop into single seedling like shoot with strong apical dominance. On the contrary when the shoots of the same explant material are grown on culture medium containing cytokinins or other growth adjuvants, axillary shoots develop clusters of secondary and tertiary shoots. These clusters can be further subdivided into smaller clumps of shoots or separate shoots which, in turn, will form similar clusters when subcultured on a fresh medium. This subdivision process may continue indefinitely provided the basic nutrient formulations are adequate for normal growth [18].

1.4.1.2 Multiplication by Adventitious Shoots

Adventitious shoots arise naturally on plant tissues located in sites other than at the normal leaf axil regions. Many ornamental and horticultural species have been successfully propagated *in vitro* by adventitious shoot initiation. New adventitious shoots can develop directly from the explants like root, stem, petiole, leaf lamina, flower parts or indirectly from the callus cultures obtained from these explants. The initiation of adventitious shoots is dependent on two factors a) choice of explant; b) hormone regime to which plant is subjected [18].

1.4.2 Initiation of Callus

A callus consists of an amorphous mass of loosely arranged thin walled parenchyma cells arising from the proliferating cells of the cultured explants. Frequently, as a result of wounding, a callus is formed at the cut end of a stem or root. Using tissue culture techniques, callus formation can be induced in numerous plant tissue and organs that do not usually develop callus in response to an injury. Plant material typically cultured

includes vascular cambia, storage parenchyma, pericycle of roots cotyledons, leaf mesophyll, and pro-vascular tissue. In fact, all multicellular plants are potential sources of explants for callus initiation [15].

Many parts of a whole plant may have an ultimate potential to proliferate *in vitro*, but it is frequently found that callus cultures are more easily established from some organs than others. Young meristematic tissues are most suitable, but meristematic areas in older parts of a plant, such as the cambium, can give rise to callus. The callus formed on an original explant is called 'primary callus'. Secondary callus cultures are initiated from pieces of tissue dissected from primary callus. Subculture can then often be continued over many years, but the longer callus is maintained, the greater is the risk that the cells thereof will suffer genetic change [19].

1.4.3 Zygotic and Somatic Embryo Development

Somatic embryos may be developed from different vegetative tissues. The term somatic is an adjective meaning "of the body", but is broadly applied to dividing (living) tissue. Embryogenesis refers to the generation of embryos. Somatic embryogenesis may then be interpreted as the generation of embryos from plant organs or tissue. In common vernacular, somatic embryogenesis is the "cloning" of embryos and refers to dividing tissue. After the initial stages, somatic embryos develop through a sequence of stages that resemble those observed for developing zygotic embryos (embryos derived from sexual reproduction) [20].

In theory, embryogenic tissue may be developed from any plant explant. An explant may be a mature plant organ, such as a root, stem, leaf or bud, or it may be a cell or group of cells. In practice, tissues from the cotyledon, the meristem, or the zygotic embryo are preferred.

Somatic embryogenesis (SE) is a tissue culture approach where proliferative embryo suspensor masses are established from non-meristematic cells and subsequently cultured to produce organized bipolar structures possessing shoot and root meristems (that is, somatic embryos) [20].

Somatic embryogenesis can be initiated directly from "preembryonic determined cells" that are programmed for embryonic differentiation, where plants are genetically identical (clonation) or indirectly from unorganized tissues (callus) and embryos originate from "induced embryogenic cells" within the callus. Propagation by indirect embryogenesis carries the risk of producing plants that may differ genetically from each other and from the parental plant [21].

1.4.4 Cell Suspension Culture

A suspension culture originates with a "random critical event "occurring during the early exposure of the plant cells to the liquid medium. Cells undergoing this transition in metabolism and growth rate produce a "cell line". Cell suspension cultures are generally initiated by transferring fragments of undifferentiated callus to a liquid medium, which is then agitated during the culture period. Although a longer time is required, suspension cultures can be started by inoculating the liquid medium with explants of differentiated plant material (e. g, a fragment of hypocotyls or cotyledon) [15].

The growth of plant cells is more rapid in suspension than in callus culture and is also more readily controlled because the culture medium can be easily amended or changed. Organs can be induced to develop in cell suspensions: root and shoot initiation usually commences in cell aggregates. Somatic embryos may arise from single cells. Cells from suspensions can also be plated onto solid media where single cells and/or cell aggregates grow into callus colonies from which plants can often be regenerated. For these reasons suspension cultures might be expected to provide a means of very rapid plant multiplication [19].

1.5 Background on Strawberry Farming in Gaza Strip

Growing strawberry in Palestine started in 1976. The area in which strawberry was grown, 1-2 dunums. The growing of strawberry aimed to:
   a- Studying how successful is planting strawberry according to climate, soil and irrigation water.
   b- Studying how acceptable is the crop to the farmer.

The situation continued as it as was tell 1971. At that time, planting strawberry was mainly planted in the north Gaza where water (less than 250 ppm Chloride) and soft sandy soil are available. The crop continued to be planted in the north of Gaza till the arrival of the Palestinian authority in Gaza Strip, 1993. After the arrival of Palestinian authority, nearly 30 dunums were planted with strawberry in Rafah and Khan Yunus, especially in Al-Mawasi area. The thing continued for two years. However, because of the Israeli actions, planting strawberry in the Rafah and Khan Yunus stopped. Farmers were unable to export or market their crops [22].

So far, four main organizations and other some private exporters have been responsible for exporting strawberry abroad. Lately, EURO GAP is used in planting strawberry to secure high quality in all the processes. In addition, environment and saving environments quality caution procedures are put under consideration. Strawberry is now exported within the Palestine group called Coral [22].

(Table 1): Following timetable shows further details related to the years where strawberry is planted and the amount of production in Gaza strip [22]:

No.	Year	Produce (Tone)	land area (Dunum)
1	1967-1968	1.5	1
2	1971-1972	12	12
3	1975-1976	50	25
4	1979-1980	900	300
5	1983-1984	1300	327
6	1987-1988	1400	358
7	1991-1992	1500	373
8	1995-1996	4098	1360
9	1999-2000	4800	1675
10	2003-2004	5757	1919
11	2004-2005	7500	2500
12	2005-2006	2500	2500
13	2006-2007	2200	2500
14	2007-2008	2350	2500

1.5.1 The Most Commonly Grown Strawberry Cultivars in Gaza Strip

At the end of 1960s and the beginning of 1970s following items are planted:

Fresno, Aliso and Tioga.

During 1980s following items are planted:

Douglas, Sharon, Ofera, Dorit, Rosalinda and Shandler.

During 1990s following items are planted:

Osso Grandi, Yael 329, Camarosa, Tamar 328 and Sweet Charlie.

'Sweet Charlie' strawberry (*Fragaria x ananassa*) offers strawberry growers in Florida, and other areas with relatively mild winter climates, a short-day cultivar that is early fruiting and produces fruit that has a distinctively sweet and flavorful due to a consistently low acid content and is resistant to anthracnose (caused by Colletotrichum acutatum) (Figure 1).

Figure 1: 'Sweet Charlie' strawberry plant and fruit.

1.5.2 Problems Faced Planting Strawberry in Palestine

a-   Inability to grow high quality strawberry mother plants in local greenhouse.
      It's totally relayed on Israeli companies to do that.

b-   Strawberry mother plants which are got from Israeli companies are expensive.

c-   Strawberry is planted in one governorate in Gaza Strip.

d- Strawberry is exported through Israeli companies since Palestinian companies are not able to do that.

e- Continuous closer of the border between Gaza Strip and Israel. As a result, exported crops stay on the border and it spoils as fungi spread in it.

1.5.3 Impact of the Closure on Gaza Strip Exports of Strawberries

Despite the relatively small area of farmland used for sector, it is considered that enjoy high economic and social value. Strawberries annual income is estimated at $US 10-12 million. In addition, it contributes to the creation of employment opportunities. Strawberry farmers are estimated at 450. Indirect employment opportunities related to the Strawberry sector (production, marketing, services, etc.) are estimated at 7,500 jobs.

The total losses of farmers in the sector of strawberry for last season amounted to $US 11.25 million. Approximately 450 strawberry farmers.

The strawberry sector losses were estimated by the researcher according to official statistics. The yield of 1 dunum is 2 tons and the dunums that export strawberries are 750 dunums. The price of 1 exported tons 30,000 NIS (an average of $US 7,500). The total loss is 750 x2 x7500 = 11,250,000 $US [23].

Therefore, building tissue cultures planting unit which is able to produce strawberry mother plants with high quality and resistance to common fungal and bacterial diseases. Hence, we, Palestinians will be independent on the Israeli side. Moreover, we will be able to produce by ourselves with low a price which suits the abilities of our farmers. We will also be able to choose the item which fit in with the requirements of international marketing web.

1.6 Significance

Our study is the first in Gaza, so the establishment of a model for plant tissue culture and determining the essential factors that influence the success of tissue culture technique present a very important point in the biotechnology field in our country, especially in the cell culture domain. The choice of strawberry was economically successful in the agriculture field. It is noticed that strawberry average production between 1983 and 2005 was 3 Tons per dunum. However, average production of strawberry was less than a Ton per dunum during 2006 – 2008.

Durind 1983- 2005, farmers used to depend on strawberry mothers plants imported from Israel. On the other hand, average production was less during 2006 – 2008 that farmers had to use plants they had due to the Israeli closure on Gaza strip.

Above stated facts show the importance of strawberry tissue culture in our country.

1.7 Aim of the Study

In vitro propagation of strawberry (*Fragaria × ananassa* Duch.) by using runner tips.

1.7.1 Objectives

The main objectives of the present study were:

- To test methods of avoiding problems in the beginning of the micropropagation.
- To prepare a particular and selective media for culturing *Fragaria × ananassa* Duch.
- To overcome the problem of oxidation and minimize phenolic exudation.
- To find out the effect of some regulators and supplements on the growth of shoots and roots of this plant.
- To refine the reorganization of the strawberry micropropagation stages.

CHAPTER 2

Literature Review

2.1 Palestine Climatic and Agriculture

Gaza Strip lies on the eastern coastal of the Mediterranean Sea. Gaza Strip lies in the moderate northern area of the horizontal lines 31.13 – 31.36 to the north of the equator which gives it good a climate along the seasons of the year. The total area of historical Palestine is 27009 Km^2, among which Gaza occupies only 1.35%. According to survey conducted in 2006 by Palestinian Center Bureau of Statistics (PCBS), 48% of Gaza Strip land is cultivated; out of which, 32% is cultivated with fruits, 32% is cultivated with vegetables and 36% is cultivated with field crops [24].

2.2 Strawberry

2.2.1 Taxonomy and Origin of Strawberry

Strawberries are members of the family Rosaceae, subfamily Rosoideae, and genus Fragaria. Closely related genera include Duchesnea, the mock strawberry, and Potentilla, the cinequefoils. Fragaria species can be grouped by ploidy: there are nine diploids, two tetraploids, one hexaploid, and four octoploids. Diploids (2n=14) include *F. vesca* Duch., *F. viridis* Duch., *F. nilgerrensis* Schlect., *F. daltoniana.* J. Gray, *F. nubicola* Lindl. ex Lacaita., *F. iinumae* Makino, *F. yezoensis* Hara, *F. nipponica* Makino, and *F. mandschurica* Staudt [25].

The alpine strawberry, *F. vesca*, is the most geographically widespread. Cytogenetic studies indicate that this species may be a diploid progenitor of the octoploid strawberries. The two tetraploids (2n=28) are *F. orientalis* Losinsk and *F. moupinensis* (Franch.) Card. The lone hexaploid (2n=42), *F. moschata* Duch. Or musky strawberry is found in northern and central Europe into eastern Russia. This species was domesticated in the early 1600's and fruit were commonly known as 'Hautbois' or 'Hautboy'. Cultivated plantings still exist in Europe.

Four octoploids (2n=56) are known: *F. iturupensis* Staudt, *F. chiloensis* (L.) Duch., *F. virginiana* Duch., and *F. ×ananassa* Duch. *F. iturupensis* is found in Iturup Island of the Kuril Islands (northeast of Japan) [26]. Taxonomic characteristics include obovate sub-glaucous leaves (similar to *F. iinumae*), hermaphroditic flowers, and almost spherical fruit. The beach or Chilean strawberry, *F. chiloensis*, is found along the Pacific coast from Alaska down through central California, along the beaches of Chile and inland to the

Andes Mountans, and on top of mountains in Hawaii. They were once extensively cultivated in Chile, Peru, and possibly Ecuador. Wild populations are primarily dioecious although hermaphrodites have been found in California [27, 28]. Plants are low-spreading, vigorous, and produce many runners. Leaves are thick, dark-green and very glossy. Fruit are dull to bright red, firm, white fleshed, pungent, and large. Four subspecies are recognized based on morphology and distribution [29]: ssp. chiloensis (South America), ssp. lucida (Washington to California), ssp. Pacifica (California to Aleutian Islands), and ssp. sandwicensis (Hawaii).

The scarlet or Virginia strawberry, *F. virginiana*, is found North America from the Southeastern U.S. north to Newfoundland and as far west as the Yukon Territory into Alaska, oftentimes in meadows. Plants are slender, tall, and have many runners. Only females and hermaphrodites are observed in the eastern U.S. while all three sexes are equally found in western populations [30, 31]. Fruit are soft, round, up to 1.5 cm diam, light red, aromatic, with deeply embedded seeds, and white flesh. Both plant and fruit characters are highly variable. Four subspecies are recognized by (Staudt (1989) [25] ssp. virginiana Duch. (Eastern U.S. to Newfoundland and west to Yukon Territory); ssp. glauca (Wats.) Staudt [southern Arizona through the Rocky Mountains into northwest Canada and central Alaska, probably equivalent to F. ovalis [28] due to lack of hybridization barriers and intermediate characters]; ssp. platypetala (Rydb.) Staudt (California to British Columbia and in the Rocky Mountains in Colorado and Wyoming); and ssp. grayana (E. Vilmorin ex Gay) Staudt (Texas through Louisiana, Alabama and north to New York).

However, this classification scheme by Staudt has undergone considerable debate. New evidence suggests that these four "subspecies" are too closely related to be considered infraspecific taxa. Welsh et al. [32] suggested that ssp. *glauca* and ssp. *platypetala* completely intergrade and should be referred to as a single taxa var. glauca. Hokanson et al. [33] suggested that strawberries in the Black Hills and eastern front ranges of the Rocky Mountains may be introgressive swarms between ssp. *glauca* and ssp. *grayana*. Finally, Harrison et al. [34] using multivariate analysis, found that these strawberries from the Black Hills were morphologically intermediate between collections of eastern ssp. *virginiana* and western ssp. *glauca*. Furthermore, when multivariate analysis was done using RAPD data, this population of strawberries from the Black Hills was part of a large cluster group that encompassed the eastern ssp. *virginiana* and western ssp. *glauca*.

The dessert or pineapple (Ananas) strawberry, *F. × ananassa* is the most important cultivated strawberry worldwide. It arose as a chance hybrid of *F. chiloensis* x *F. virginiana* within European gardens during the mid-1700's. Naturally occurring hybrids have been found in coastal areas of southwest British Columbia, Washington, Oregon, and northern California. Staudt (1962) [29] recognized these hybrids as *F. ×ananassa* nm. cuneifolia (Nutt. Ex Howell). Many of the dessert strawberry's traits are intermediate to its parents. Plants have large fruit, high yields, and vigor.

2.2.2 Description of Fragaria x ananassa Duch.

The *F. x ananassa* is a perennial which arises from a crown of meristematic tissue or compressed stem tissue. Leaves, stems, runners, axillary crowns, inflorescences, and roots all arise from the crown. The plant has trifoliate leaves which spiral around the crown, with buds in the leaf axils giving rise to the runners. Runners have two nodes with a plant produced at the distal node. Strawberry blossoms contain many pistils, each with its own style and stigma attached to the receptacle. When fertilization occurs the receptacle develops into a fleshy fruit [35].

The fruit is called an achene which contains the seeds. The edible part is an accessory type fruit. The seeds are arranged on the outside of the receptacle tissue. The growth of the receptacle is dependent on successful fertilization of the ovules with its size and shape dependent on the number of achenes formed. Strawberry plants are day length dependent with cultivars being long day, short day or day neutral [35].

Following further hybridizations, especially since 1850, *Fragaria x ananassa* has developed into the large, fragrant, tasty red fruit that is now cultivated worldwide. The high degree of genetic heterozygosity present in Fragaria spp. enabled the development of strawberry cultivars adapted to widely varying environment conditions and resistant to several diseases and pests. Not only the genetic variability, but also a high adaptability and plasticity of the strawberry plant itself give this crop such a remarkable range of adaptation [28].

That heterozygosity was explained by (Gaafar and Saker 2006) as there are more than 20 *Fragaria species* worldwide, there are seven basic types of chromosomes that they all have

in common. However, they exhibit different polyploidy. Some species are diploid, having two sets of the seven chromosomes (14 chromosomes total). Others are tetraploid (4x = 28), hexaploid (6x = 42), octoploid (8x = 56) or decaploid (10x = 70) [36].

2.2.3 Histological Characteristics of Strawberry

Microscopic observations (Figure 2. a, b) showed that the apex of stipular shoots presented more sites of higher cell activity in comparison to axillary shoots. In the former, one axillary meristem was developed at nearly each foliar premordium. Moreover, the number of primordia on the apical meristem is more important in stipular than in axillary shoots [37].

Figure 2: Longtudinal section of (a) axillary shoot apex and (b) stipular shoot apex of strawberry cv, Elsanta. A.m = apical meristem; a.s = axillary shoot; f.p = foliar primordium. G × 100 [37].
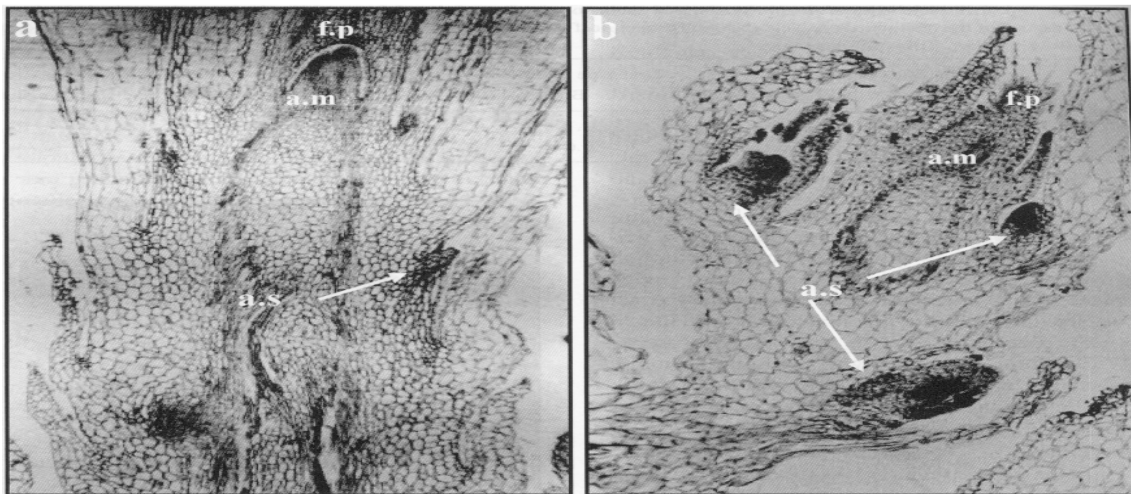
2.2.4 Strawberry Nutritional Value

For many centuries before, strawberries had been a favorite among the fruits of the temperate world. They were valued for delicious flavor and fragrance, for health-restoring qualities and as harbinger of spring [38].

Flavourful and nutritious, strawberries are enjoyed by millions of people in all climates, including and are predominantly used as fresh fruit. Their use in processed forms such as cooked and sweetened preserves, jams or jellies and frozen whole berries or sweetened juice extracts or flavorings, and their use in making a variety of other processed products

made them one of the most popular berry crops, more widely distributed than any other fruit [39].

Of its many positive characteristics, the nutritional value of strawberries is nearly perfect (Table 2). Eight medium strawberries contain more vitamin C than an orange, 20% of the recommended daily allowance for folic acid, no fat, no cholesterol and are considered high in fiber [40].

Strawberry is cultivated all around the world, not only for its digestive and tonic properties, but because of the nutritional value of its fruits, important source of folate, vitamin C, fiber, potassium, flavonoids, autocianidin, phytochemicals and antioxidants.

(Table. 2) Nutritional values for 100 grams edible portion of strawberry (USDA, 1999) [41]:

Protein	0.61g	Sodium	1mg
Fat	0.37mg	Zinc	0.13mg
Fiber	2.3g	Copper	0.049mg
Carbohydrates	7.02g	Manganese	0.29mg
Calcium	14mg	Selenium	0.7 µg
Iron	0.38g	Vitamin B-6	0.059mg
Mangnesium	10mg	Folate	17.7µg
Phosphorus	19mg	Vitamin A, IU	3 µg
Potassium	166mg	Vitamin A, RE	0.14mg

2.2.5 Strawberry Phytochemicals and Human Health

Although strawberry is not an essential component of the diet, its delicious flavor and taste, attractive appearance and seasonal availability make this fruit an excellent crop. Even more, strawberries are rich in phytochemical compounds with potential antioxidant compounds, mainly ellagic acid and flavonoids, which can lower the risk of cardiovascular events and tumorogenesis [42].

These qualities have ensured that the economic importance of this crop has increased throughout the world and, nowadays, it remains as a crop of primary interest for both research and fruit production [43].

A growing body of data suggests that consumption of a phytochemical-rich diet reduces the risk of certain chronic human illnesses such as cancer, heart and neurodegenerative diseases. Strawberry (*Fragaria x ananassa* Duch.) fruits are a rich source of phytochemicals (plant chemicals) of which phenolic compounds predominate. Berry fruits are reported to contain a wide variety of phenolics including hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, flavanols, condensed tannins (proanthocyanidins) and hydrolyzable tannins [44].

Studies conducted *in vitro* indicate that berry phenolics have a wide range of biological properties such as anti-cancer, antioxidant, anti-inflammatory, and cell regulatory effects [45, 46].

2.2.6 Anticancer Effects

The anticancer effects of individual phytochemical constituents of strawberries, as well as whole strawberry extracts, have been demonstrated [45]. These anticancer effects are exerted through multi-mechanistic means of action including the antioxidant actions of the berry's phenolic constituents by protecting DNA from damage, and also through effects exerted beyond antioxidation [46]. The biological activities of strawberry phytochemicals include the regulation of phase-II enzymes and the modulation of gene expression and sub-cellular signaling pathways of cell proliferation, angiogenesis and apoptosis (programmed cell death). Although there have been many published reports on the anticancer effects of individual phenolics known to be present in the strawberry fruit [47].

Strawberry extracts have also been evaluated for their ability to inhibit mutation by the direct-acting mutagen methyl methanesulfonate, and the metabolically activated carcinogen, benzopyrene. Ethanol extracts from freeze-dried fruits of several strawberry cultivars were also evaluated and hydrolyzable tannin-containing fractions from strawberries were found to be most effective at inhibiting mutations [48].

2.2.7 Anthocyanins

Anthocyanins are natural pigments providing scarlet to blue colors in flowers, fruits, leaves and storage organs. The recent interest in the field of anthocyanin chemistry has been generated by restriction and limitation of the use of synthetic dyes as food ingredients.

Because of low toxicity of anthocyanins, they have a high potential as a food colorant as the substitute of synthetic red dyes. Recently, these anthocyanins have been thought to have pharmacological effects, such as lowering the atherogenic index [49] and decreasing triglyceride and free fatty acid levels [50]. Moreover, Kamei et al. [51] reported that anthocyanin was more effective to inhibit the growth of tumor cells than other flavonoids. Studies concerned with anthocyanin production using plant tissue cultures have therefore become very important.

Masayuki et al. [52] demonstrated that Fragaria ananassa (strawberry) callus, which produced high amounts of anthocyanin in the dark which accumulated more than 1000 μg of anthocyanin per g fresh cell. And Sato et al. [53] reported that in the suspension cultures of F. ananassa cells, anthocyanin content increased with the intensity of light irradiation from 2500 lx to 8000 lx. They also measured the ratio of pigmented cells in total cells at 8000 lx and found that the ratios were 26% in MS medium.

2.2.8 The Strawberry Economic in the World

Strawberry world production has shown a significant increase in the last 25 years. From 1980–2004, the land cultivated with strawberry increased by 25% and fruit production by 73%. In 2004, strawberry was cultivated on 214,118 ha, reaching a production of 3.1 million tones. Fifty seven countries contributed to this production, though only nine of them accounted for more than 72% of the total. During the period 2000–2004, average world production was 3.17 million tonnes, the major strawberry producer nations being the USA. The total value of USA production was 1.22 billion dollars in 2002, being the third fruit in value in the non citrus category behind grapes and apples. It is noteworthy that most strawberry production is localized in areas with mild winter climates [43].

Approximately 15% of world strawberry production is exported as fresh fruit and, at least in the USA, more than 25% is processed. The major fresh strawberry exporters are Spain (212,300 t in 2003, 81% of its strawberry production) and the USA (94,600 t, 11.2%). Spain is the main supplier for the European market during the winter period (February and March) and Germany, France and the UK are the major consumers of this production. In Spain, as well as other Mediterranean nations, the concentration of strawberry production over short periods often creates a product excess, reducing retail prices [54].

In the case of the USA, exported fruits are destined mainly for Canada and a small quantity for Japan and Mexico. California has more than 85% of the USA strawberry production. This region also supports the most active and successful strawberry breeding programs. In fact, the program of the University of California has released very popular varieties (e.g. Camarosa, Chandler, Seascape, Selva, Pajaro, Oso Grande), accounting for more than 50% of the world's strawberry production [43].

2.2.9 Strawberry Diseases

Several soil pathogens damage strawberry roots, resulting in vigor declines and ultimately death. Two very common problems across the world are red stele or red core caused by *Phytophthora fragariae* Hickman and Verticillium wilt caused by *Verticillium albo-atrum* Reinke & Berth. And *V. dahlia*. Black root rot is also widespread and is caused by a complex of organisms including *Pythium, Rhizoctonia* and the root lesion nematode (*Pratylenchus penetrans* Cobb) [55].

Fusarium wilt or Fusarium yellows (*Fusarium oxysporum* Schl. f. sp. fragariae Winks and Williams) is of major importance in Japan, Korea and Australia. Fumigation has been widely employed to control soil pathogens, but the impending ban on methyl bromide fumigation has stimulated increased interest in developing resistant cultivars. Without fumigation, cultivars yield 50% less fruit on average [55].

This chemical is, however, very hazardous to the environment and will be forbidden in 2005 at least in the EU countries. Biological and other solutions are therefore urgently needed to ensure the health and productivity of strawberry [56].

Among the foliar diseases, three are very widespread and can cause serious damage including, leaf blight [*Phomopsis obscurans* (Ell. and Ev.) Suton], Ramularia leaf spot, [*Mycosphaerella fragariae* (Tul.) Lindau] and leaf scorch [*Diplocarpon earliana* (Ell. & Everh.) Wolf]. Alternaria leaf spot or black leaf spot (*Alternaria alternata* (FR.) Keissler) causes serious damage in Europe, New Zealand and Korea [55].

Powdery mildew [*Sphaerotheca macularis* (Wallr. Ex Fr.) Jaez] is also found across most of the strawberry range, although it rarely does economic damage. Angular leaf spot,

Xanthomonas fragariae Kennedy and King, is a rapidly growing problem in strawberries all across the world [55].

Anthracnose is a common problem in strawberries, causing a wide array of symptoms including fruit rot, crown rot, and lesions of the stolons, petioles and leaves. Anthracnose diseases of strawberry are caused by *Colletotrichum fragariae* A. N. Brooks, *C. acutatum* J.H. Simmonds, and *C. gloeosporioides* (Penz.) Penz. & Sacc. In Penz. *Colletotrichum acutatum* is the primary pathogen causing crown rot in Europe, while *C. fragariae* is the most common cause of crown rot in the southeastern U.S.A. *C. acutatum* is the primary pathogen in Israel and California [55].

Phytophthora cactorum (Leb. & Cohn) Schroet also causes widespread incidences of severe crown (Cactorum crown rot) and fruit rots (leather rot), particularly in warm climates [55].

2.3 Common Problems and Prospect in Tissue Culture

2.3.1 Contamination

One of the main problems encountered with *in vitro* propagation was the massive bacterial contamination at the initiation and multiplication stages. After transfer of the bud on to solid sterile medium, a whitish exudate of bacteria was observed around the base of the explant after 2-3 d. The problem was complicated further by the latent nature of the contaminants. Turbidity of the liquid multiplication medium 1-2 d after splitting of the shoot clumps indicated the presence of bacteria. In some cases the contaminants appeared upon the sixth or seventh subculture. Contamination at the initiation stage caused rotting of the bud whereas at the multiplication stage, the rate of tillering bore an average three- fold decrease with subsequent death of the plantlets in about one month [57].

In most laboratories, losses due to contamination average between 3-15% of plants at every subculture [58], rendering commercial micropropagation less economical. It also leads to severe production losses, low progeny performance and rejections of entire shipments of plants due to quarantine regulations [59].

Bacterial contamination is one of the most crucial problems of plant tissue culture, both in research and commercial production. Often bacterial contamination is difficult to detect. Even healthy plants can contain several bacteria, and some plant exudates may look similar to bacterial growth. Contaminated plants may lack symptoms, have reduced multiplication rates, reduced rooting rates, or may die [60].

Contaminants in the xylem vessel which are protected from surface sterilization are endophytic bacteria detected even in meristem-tip explants .Endophytic bacteria have probably evolved a close relationship with their host plant through co-evolutionary processes and may influence plant physiology in ways that have not yet been elucidated .Inside the plant they have very little microbial competition and usually they do not cause visible symptoms to the plant. The bacteria may stay latent or symptomless up to several months after the initiation of culture and may not survive outside the plant tissue .Endophytic bacteria may even promote beneficial effects for field grown crops, but in stress conditions such as in vitro culture, latent endophytic bacteria may become pathogenic and detrimental to the growth and development of the plantlets [61].

Latent bacterial contamination during the proliferation phase adversely affects rooting due to changes in growth regulators and water potential of the media [62] and also kills young trees after weaning [63]. Many *Bacillus spp.* increase pH of the medium, inhibiting proton pumps involved in plant nutrient uptake and cause precipitation of many nutrients from the medium [62]. Cultures of apricot with latent bacterial contaminants showed high CO_2 and low O_2 concentration in the vessel headspace, thereby decreasing the proliferation and photosynthetic rates [64].

Organic soil amendments have been observed to increase the inoculum for the contamination of plants and thus organic material should be avoided in the preparation of plants for micropropagation. Also, explants taken from different locations have been observed to differ in their *in vitro* bacterial contamination. It has been demonstrated that many of these non fastidious bacterial contaminations are environment associated, and may be transmitted through water. Bacteria have been detected especially from tissues close to the soil [65].

The most serious problem for continuos use of antibiotics is the contaminant becoming resistant, although scientific reports of this problem are scare. Bacteria may be associated with plant species, which are also sensitive to antibiotics. Antibiotic at bactericidal concentrations may be toxic to the plant and reduce plant growth. On the other hand, many antibiotics have enhanced shoot regeneration, shoot development; delayed the loss of regeneration potential; stimulated callus growth and root formation; and enhanced plant differentiation from somatic embryos. Additionally, antibiotics may stimulate enzymes responsible for nitrogen metabolism; Hill reaction in chloroplasts, and increase chlorophyll, carotene, and xanthophyll content in leaves [61].

Piyarak et al. [60] detected the microbial contaminant in surface-sterilized strawberry runner explants partially submerged in half strength liquid MS Piyarak medium. 45 of 70 strawberry genotypes were found to be contaminated, there were more bacterial than fungal contaminants. Bacterial contaminants from 22 strawberry genotypes were isolated, purified, and identified to genus by standard biochemical tests such as Gram's stain, motility, oxidase, and gelatinase, and carbon source utilization (Biolog Microplates, Biolog, Inc., Hayward, CA). Among the 30 isolates identified with the Biolog system, the majority were fluorescent pseudomonads including *Pseudomonas fluorescens* types A, F, and G. *P. corrugata*, *P. tolaasii*, *P. paucimobilis*, *Xanthomonas campestris*, *Xanthomonas spp.*, and *Enterobacter cloacae* were also identified. Five Gram-negative and two Gram-positive contaminants could not be identified by the Biolog test. Biochemical tests were used to characterize the bacteria and to confirm Biolog test results. Bacteria identified in this study were soil, water, and plant related, indicating that efforts to reduce explant contaminant levels should be centered on the care of stock plants or the sterility of the watering system.

2.3.2 Phenolic Browning / Oxidation *in Vitro*

The presence of phenolic compounds causing death of explants has been another important problem of tissue cultures of woody perennials [66] in addition to various bacterial and fungal contaminants as discussed above. Some of these exudates appear as a reaction to injury and/or infection. In tissue culture they appear after tissue excision and are many times aggravated by growth media constituents [67]. Tissue blackening occurs due to action of copper-containing oxidase enzymes: polyphenoloxidases like tyrosinases, which

are released or synthesized in oxidative conditions after tissue wounding and they oxidize o-diphenols released due to cellular wounding to O-quinones [68, 69]. The onset of tissue browning has been found to be associated with changes in protein pattern, amino acid content, ethylene production and the occurrence of saccharose and accumulation of starch [70]. These changes eventually lead to growth inhibition or death of explants. Other types of phenolic exudates appear at the end of incubation period and are apparently products of dying cells [67]. The phenolic exudation is aided by light and is autocatalytic.

It is possible, that some external factors trigger stress symptoms such as browning in plant tissue. These factors may be pathogens or in some cases even agar. High concentrations of other medium components such as macrosalts, auxins, and sucrose have caused browning as well. In addition, there are some substances including 5, 6-Cl$_2$-IAA, yeast extracts, and phloridzin which directly enhance the production of phenolic compounds [61].

Several factors in the growth and culture conditions of the donor tissues have been tested, that may in later steps of the establishment of tissue cultures cause browning. *In vitro* formation of phenolic acids from explants diminished when donor tissues were preincubated on a medium with or without mineral salts treated with BA (200 mg\ L) or PVPP, ascorbic acid, cysteine, or citric acid [61].

Accumulation of phenolics is most obviously associated with the developmental stage of the plant and season, which was shown with woody species. Explants collected from November to February had produced low browning percentages *in vitro*, whereas the browning was at its maximum if explants were collected in April - August [71].

Differences in browning between varieties and in relation to the size of the explant were also observed. The authors concluded that a better way to overcome tissue browning was to optimize growth conditions of the source plants rather than by treating the explants afterwards. The most effective antibrowning treatment was growing the source plants in the dark, in the greenhouse, or use of explants from heat treated or cold-treated source plants [72, 73]. Simple, but effective inhibition was achieved by sealing the cut ends of explants with paraffin [74].

Many medium components have been observed to decrease or eliminate tissue browning such as inclusion of nitrate as a source of nitrogen; increased concentration of phytagel or using gelrite instead of agar. Many authors have also tested phenol traps such as activated charcoal; adsorbent resin; citric acid; cysteine; PVPP; and antioxidants such as ascorbic acid or glutathione (GSH). In addition, culturing *in vitro* plantlets under low light intensity has given good results [61].

The initial browning develops later into intense tissue browning and deterioration which is reflected in reduced capacity for protein synthesis and changes in the free amino acid pools and protein patterns. Oxidized products, such as quinones, are known to be highly reactive and inhibit enzyme activity leading to the death of the explants. The toxicity of the oxidized compounds to larvae feeding on a brown callus caused death or reduced growth. As a consequence of browning, tissue senescence, recalcitrance in embryogenesis and regeneration have been observed [61].

2.3.3 The Difficulty in Using Explants from Mature Trees

There are numerous substantive phenotypic traits associated with juvenility, but they vary considerably among species. Commonly, the leaves on young plants are of a different shape than those on mature parts and may be simple rather than compound (or occasionally the reverse); juvenile leaves may also have a special type of cuticle and be arranged with a distinct phyllotaxy. Compared to their adult counterparts, young plants may have a modified resistance to pests and diseases. Juvenility in woody plants is often manifested by prolonged vigorous shoot growth [75].

Young healthy tissues that are rich in nutrients, and possibly endogenous hormone, are the best choices for the induction of cell division. While the woody plant material is generally a poor choice. Also, plant tissues that are high in oxidase activity pose a special problem since enzymatic browning retards cell division. The browning results from the activity of wound-induced copper oxidases (polyphenoloxidase). This may be suppressed, to some extent, by the use of an antioxidant mixture [15].

Juvenile plants have a different growth habit, leaf shape, and an enhanced ability to form adventitious roots. When petioles from the juvenile form were excised and treated with

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auxin *in vitro*, cortical parenchyma cells adjacent to the vascular bundles divided and formed root primordia. However, when petioles from adult leaves were treated in a similar manner, callus formed and some callus cells divided to form root primordia. The juvenile form had pre-existing competent cells that were able to respond to auxin and become determined to form roots. However, the adult form appeared to lack cells with pre-existing competence to form roots, but competence was acquired by some callus cells once they had been initiated [75].

Explants taken from mature shoots are frequently more liable than juvenile material to suffer necrosis, especially when surface disinfested and placed in culture, and juvenile explants are usually more readily established *in vitro* and grow and proliferate at a more rapid rate than adult material. This is particularly true with tree species where micropropagation of adult material is often difficult. Juvenile plants frequently grow more rapidly and have stronger apical dominance than adult forms. However, shoot explants from juvenile plants generally proliferate more axillary shoots than shoot explants from adult forms [19].

A safer and more promising alternative would be to use other plant parts such as apical buds and apical meristems for establishment of cultures. The apical bud is defined as the bud located immediately below the apical meristem and enclosed within the leaf sheath. Selecting these young tissues makes it possible to reduce infection since the apical zone displays better aseptic conditions because of the reduced size of the explant and the small area exposed to the external environment [57].

2.3.4 High – Temperature Degradation of Media Components

Several chemicals employed in plant tissue culture media degrade on exposure to steam sterilization. Gibberellins are rapidly degraded by high temperatures, and biological activity of a freshly prepared solution of GA_3 was reduced by more than 90% as a result of autoclaving. The auxins IAA, α-NAA, and 2,4-D are relatively thermostable depending on the inorganic basal medium and supplements. Aqueous solutions of kinetin, zeatin, and 2iP have been chromatographed on thin-layer silica-gel chromatograms before and after prolonged autoclaving with no breakdown products detected [15].

Vitamins have varying degree of thermolability. Most workers autoclave the vitamins with the remainder of the medium. Nicotinic acid, pyridoxine, and thiamine in a MS liquid medium (PH 5.5-5.6) showed no signs of degradation after autoclaving; nevertheless, thiamine is rapidly destroyed if the PH of the medium is much above 5.5. Calcium pantothenate can not be autoclaved without destruction. If the research study involves vitamin activity, then the vitamins should be sterilized by microfiltration. Since those employed in most our experiments are apparently thermostable, the vitamin supplement will be added to the medium prior to autoclaving [15].

One of the most frequently employed carbohydrates in media is sucrose. This disaccharide decomposes, to some extent, on autoclaving to release a mixture of D-glucose and D-fructose. A recent study indicated that 5 percent of the sucrose in an MS liquid medium was hydrolyzed during autoclaving. This degradation can be inhibitory to some cultured tissues. Presumably the toxicity is due to the degradation products of D-fructose. Steam sterilization may also catalyze reaction within the media between carbohydrates and amino acids [15].

Among various alternatives for reducing costs in so-called ''biofactories'', or commercial plant production laboratories, a potentially good alternative is to replace the autoclaving procedure described by some researchers such as using a microwave oven, and use of sodium hypochlorite or hydrogen peroxide with a low concentration in the media [76].

2.3.5 Hyperhydricity

Vitrification was first used as a term in conventional plant tissue culture to describe a morphological response of plant tissues to stresses [77]. The symptoms were water soaked, thick, elongated, wrinkled, curled, brittle and translucent leaves, shoots with shorter internodes, and the low number of shoots/explant. However, as a term vitrification is confusing, because in cryobiology vitrification (cryopreservation) describes the process where plant tissue is converted into ice during freezing preservation. Thus, later the same author suggested that the term vitrified should be substituted for hyperhydric [61].

Anatomically hyperhydric leaves have large vacuolated mesophyll cells showing a hypertrophy of the cells with large intercellular spaces filled with water instead of air.

Tissues have less lignin and the vascular system is abnormal. Leaves have less if any surface wax or the wax has an altered chrystalline structure, and thin cuticle with a lower level of cutin, pectin and cellulose. Several abnormalities have been observed also in hyperhydric cells. The chlorophyll content has been shown to be low and to contain abundant plastoglobuli. The guard cells contained high levels of K+ and were morphologically abnormal as well as the stomata them self. Also the photosynthetic rate was lower in hyperhydric cells compared to normal tissue [61].

Biochemically the activity of several enzymes is altered in hyperhydric leaves. The activity of superoxide dismutase was high in hyperhydric shoots resulting in hydrogen peroxide (H_2O_2) accumulation. Also, the blockage of the porphyrin pathway and lack of H_2O_2 detoxifying enzymes (catalase, ascorbate peroxidase, and glutathione reductase). And a deviation of the nitrogen metabolism induced by NH_3 towards polyamines caused the formation of toxic oxygen forms [77, 78].

 It is unlikely that nitrate level alone affects hyperhydricity, although plantlets showed changes in protein synthesis, which correlated both positively and negatively with nitrate content [61]. It was also observed that habituated (capable to induce callus without exogenous growth substances) and hyperhydric calli resembled each other biochemically [78].

Development of the morphological symptoms of hyperhydricity depends on many factors. Hyperhydricity has been observed to increase by zeatin, fructose, thiadiazuron, low sucrose concentration, BAP, and high relative humidity in the culture vessel. Visual symptoms occur only for a certain period of time and may vary between species. The visual symptoms can be reduced by controlling various factors. Medium components or growth conditions such as silver nitrate, yeast extract, potato juice, increased gelling agent, ABA, high KNO_3 : NH_4Cl_2 ratio, phloridzin , paclobutrazol, increasing gas exchange in the culture vessel, and cold or reduced temperature treatments and increased Ca concentration have decreased the abnormal hyperhydricity of tissues [61].

2.3.6 Shoot Tip Necrosis

Calcium deficiency in plants results in poor root growth and in the blackening and curling of the margins of apical leaves, often followed by a cessation of growth and death of the

shoot tip. The latter symptoms are similar to aluminum toxicity. Tip necrosis has been especially observed in shoot cultures, sometimes associated with hyperhydricity. It often occurs after several subcultures. After death of the tip, shoots often produce lateral branches, and in extreme cases the tips of these will also die and branch again [19].

Different approaches have been reported to overcome the loss of cultures by different means including more frequent subculture, using modified media by addition of silver nitrate, GA_3, fructose and calcium [79].

Calcium, as a component of cell walls, membranes and lignin, protects membranes from damage and leakiness, thus maintaining cell integrity and membrane permeability. Calcification strengthens plant cell walls and enables increased plant resistance to pathogen infection. Mitigation of calcium deficiency during shoot multiplication in potato was achieved by the use of calcium-rich media, and by periodically inverting of the culture tubes containing liquid medium supplemented with calcium gluconate for better interaction of the explant with the medium [79].

As calcium is not remobilized (nontranslocatable) within plant tissues, actively growing shoots need a constant fresh supply of ions in the transpiration stream. An inadequate supply of calcium can result from limited uptake of the ion, and inadequate transport, the latter being caused by the absence of transpiration due to the high humidity in the culture vessel (George et al, 2008). And can introduce undesirable anions. A temporary increase in Ca_2+ concentration to 1 or 10 M does not significantly alter the ionic environment within the cell, but is yet sufficient to trigger fundamental cell process such as polarized growth, response to gravity, plant growth substances, cytoplasmic streaming, and mitosis [79].

Addition of higher levels of calcium chloride in shoot multiplication as well as rooting medium did not result in complete alleviation of necrosis and this may be due to chloride toxicity caused by the supra-optimal level of calcium chloride in the medium. Excess calcium may also produce deficiencies in magnesium and potassium. Although calcium can be present in millimolar concentrations within the plant as a whole, calcium ions are pumped out of the cytoplasm of cells to maintain the concentration at around only 0.1 M.

This active removal of Ca_2+ from the protoplasm is necessary to prevent the precipitation of phosphate and interference with the function of Mg_2+ [2].

A remedy can sometimes be obtained by reducing the culture temperature so that the rate of shoot growth matches calcium supply, using vessels which promote better gas exchange (thereby increasing the transpiration and xylem transport), or by increasing the concentration of calcium in the medium. The last two remedies can have drawbacks: the medium will dry out if there is too free gas exchange [19].

2.3.7 Light Requirements

The illumination of plant cultures must be considered in terms of intensity, length of the daily exposure period, and quality. At the outset, it should be clearly noted that the light requirements of tissue cultures are not the same as those of autotrophically developing whole plants. In tissue culture, photosynthesisis not a necessary activity, except perhaps during the latter part of Stage III, since carbohydrate is adequately provided. Nevertheless, light is needed to regulate certain morphogenetic processes. It has been reported to be important for the formation of shoots, the initiation of roots, the differentiation of cladophylls, and in asexual embryogenesis. Instances of failure of tissue cultures are sometimes caused by the use of plant growth chambers of similar facilities where the light provisions have been intended for autotrophic plant development. The difficulty most often encountered with such facilities is the excessively high light intensity; sometimes the quality of illumination is also unsuited for tissue culture [10].

2.3.8 The Cost of Generating Plantlets

The cost of generating plantlets is very high. The most advanced micropropagation technology is still labour and cost intensive. Therefore, this process can only be profitable if species cannot be multiplied conventionally and a high return is expected from genetic gain, yield, disease-free stock, etc [11].

2.3.9 Somaclonal Variation

In addition to the variants/mutants (cell lines and plants) obtained as a result of the application of a selective agent in the presence or absence of a mutagen, many variants have been obtained through the tissue-culture cycle itself. These somaclonal variants,

which are dependent on the natural variation in a population of cells, may be genetic or epigenetic, and are usually observed in the regenerated plantlets. Somaclonal variation itself does not appear to be a simple phenomenon, and may reflect pre-existing cellular genetic differences or tissue culture- induced variability. The variation may be generated through several types of nuclear chromosomal re-arrangements and losses, gene amplification or de-amplification: non-reciprocal mitotic recombination events, transposable element activation, apparent point mutations, or re-activation of silent genes in multigene families, as well as alterations in maternally inherited characteristics [80].

Many of the changes observed in plants regenerated *in vitro* have potential agricultural and horticultural significance. These include alterations in plant pigmentation, seed yield, plant vigour and size, leaf and flower morphology, essential oils, fruit solids, and disease tolerance or resistance. The same types of variation obtained from somatic cells and protoplasts can also be obtained from gametic tissue [80].

One of the major potential benefits of somaclonal variation is the creation of additional genetic variability in coadapted, agronomically useful cultivars, without the need to resort to hybridization. This method could be valuable if selection is possible *in vitro* or if rapid plant-screening methods are available. It is believed that somaclonal variants can be enhanced for some characters during culture *in vitro,* including resistance to disease pathotoxins and herbicides and tolerance to environmental or chemical stress. However, at present few cultivars of any agronomically important crop have been produced through the exploitation of somaclonal variation [80].

 Although somaclonal variation can be used as a source for variation to obtain superior clones, it can be also a very serious problem in the plant tissue culture industry resulting in the production of undersirable plant off – types [80].

2.3.10 Acclimatization of Tissue-Cultured Plants

A substantial number of micropropagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environment. The greenhouse and field have substantially lower relative humidity, higher light level and septic environment that are stressful to micropropagated plants compared to *in vitro* conditions. The benefit of any

micropropagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro*. Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil [81].

The transfer of *in vitro* plantlets to *ex vitro* conditions is one of the most critical factors of the micropropagation process and a cause of higher production costs.

High mortality is often observed upon transfer to *ex vitro* conditions as the cultured plants have a poorly developed cuticle, nonfunctional stomata and a weak root system (Table 3). In order to increase growth rate and reduce mortality of plantlets at the stage of acclimatization, recent research has focused on control of the environmental conditions. One approach has been to modify the environmental conditions during acclimatization by increasing light intensity or both increasing the light levels and altering the CO2 concentration. Another approach has been to change the environment during the multiplication and rooting stages, including increasing light intensity and CO2 concentration in culture tubes, and decreasing the sugar concentration. All were found to be beneficial for plantlet growth in the later stages of micropropagation [82].

A biological approach to reducing the stress of acclimatization and providing faster growth of micropropagated plantlets is the establishment of vesicular-arbuscular mycorrhizae (VAM) on micropropagated plantlets during acclimatization. VAM colonization of horticultural plant roots can improve growth by increased uptake of phosphorus, zinc and other minerals and may reduce the incidence of disease. Moreover, colonization with VAM fungi may increase transplant uniformity and reduce both transplant mortality and injury. Recent work has also shown improvement in water relations of the host plant using VAM. These fungi may also improve drought tolerance by decreasing leaf water potential, by reducing stomatal and root hydraulic resistances, and by increasing transpiration rates. Several studies have focused on VA mycorizal formation during acclimatization of cultural plantlets [82].

Table 3: Anatomical and histological differences between roots, stems and leaves of plants in vitro and after transplanting ex vitro. George et al. [19].

Plant Organ and Tissue	*In vitro*	*Ex vitro*
Roots	Thin	Large root system
Epidermis	Uniseriate	Uniseriate and multiseriate
Cortex	Broad, irregular enlarged, hypertrophied individual cells, numerous intercellular spaces; loose arrangement of cortical arenchyma.	Uniform, compact cortical cell arrangement
Root hairs	Few or no, thick, short and straight, fine, delicate appearance, many fused together, usually abundant.	Long, thin, slender, fibrous, wiry and formed an interwoven mat.
Stems	Small diameter.	Large diameter.
Epidermis	Limited development.	Fully developed.
Cortex	Limited development, little collenchyma, few sclerenchyma fibers; starch grains in old stem.	Fully developed with continuous cylinder of collenchyma.
Leaves	Small, succulent, brittle and hyperhydrated.	Normal shape and size
Epidermis	Deformed thin cell walls irregularly shaped.	Normal cell walls.
Cuticle	Thin and discontinuous.	Thick and continuous.
Stomata	Irregular guard cells with thin cell walls, large stoma.	Normal guard cells.
Spongy Parenchyma	Highly vacuolated with large intercellular air-spaces.	Normal cells with regular air-spaces.
Chloroplasts	Low chlorophyll, abnormal non-functional chloroplasts, limited formation of grana, abundant stroma, and starch grains.	Normal chloroplasts with typical granal structure, increased chlorophyll content.

2.4 Use of Charcoal in *in vitro* Culture

Effects of charcoal on tissue response in *in vitro* culture appear to be dependent not only on the kind of charcoal and their degree of activation, but also on the plant species cultured. The addition of activated charcoal to tissue culture media may have either a beneficial or an adverse effect on growth and development, depending upon the medium, tissue used, and/or objective(s) of the researcher [83].

Use of charcoal can make a major difference in the success or failure of a given culture attempt. In general, effects of charcoal on *in vitro* cultures could be dealt with under the following headings:

2.4.1 Providing a dark Environment

Charcoal provides a degree of darkness during *in vitro* culture. Light is a major factor of the culture environment and has been shown to have an effect on organized development *in vitro*. Light requirements for differentiation involve a combination of several components, including intensity, daily light period and quality. The properties of light affect tissue cultures, and influences their growth and development. Reduction of light at the base of a shoot can provide an environment conducive to the accumulation of photosensitive auxin or co-factors.

Addition of activated charcoal in the rooting medium improved the potential for adventitious rooting, not only in terms of rooting rates, but also an enhancement of the number and the length of the roots, as well as root score. This stimulating effect of activated charcoal on root ability was particularly associated with mature explants.

Charcoal may affect the activity and/or stability of plant growth regulators by reducing or excluding light in *in vitro* culture. Light promoted degradation of IAA and IBA in both liquid and solid agar media [83].

2.4.2 Adsorption of Undesirable or Inhibitory Substances in *in vitro* Cultures

It is widely accepted that some of the beneficial effects of activated charcoal can be attributed to the removal of inhibitory substances from the media, produced either on autoclaving the media [84] or released by the tissue itself [85]. Charcoal may be able to adsorb toxic brown/black pigments (phenol-like compounds and melanin) as well as other unknown colourless toxic compounds. Fridborg et al. [85] found that differentiation occurred in Daucus and Allium cultures in activated charcoal-containing media. The media without activated charcoal contained phenolic compounds and other metabolites which inhibited embryogenesis and morphogenesis.

A major breakdown product of autoclaved sugars, which has been shown to reduce growth of plant tissue *in vitro*, is HMF (5-hydroxymethyl-2-furaldehyde).

The inhibition of growth of tobacco anther cultures by 100 mg \ L HMF was eliminated by addition of activated charcoal, while the addition of 0.3% activated charcoal increased plantlet yield by 200–300% in all the anther cultures [84].

It is normal for the pH of the medium to drift. Typically, during tissue culture experiments, pH is adjusted prior to autoclaving to between 5.2 and 5.8. Tissue culture media are poorly buffered and in the presence of developing tissue the pH may go up or down. Johansson et al. [86] showed that the pH of the medium had increased by almost 0.5 pH units in one hour. After eight hours the pH appeared to be reasonably stable. As stated previously, however, AC may have acidic character depending on how it was manufactured.

2.4.3 Adsorption of Plant Growth Regulators in *In Vitro* Culture

The concentration and combination of auxins and cytokinins in the nutrient medium is usually a key factor which determines successful plant regeneration. The use of activated charcoal as a culture component for adsorption of toxic plant metabolites is known. Activated charcoal is able to adsorb high concentrations of the growth regulators BA, IAA, IBA, NAA and Kinetin, in both liquid and solid media [87].

Ethylene is a gaseous hormone produced by plant tissue and/or the medium. Activated charcoal was able to adsorb ethylene released by the medium and by *Nicotiana tabacum* nodes [88]. More ethylene was produced by *Anemone caronaria* seedlings than agar alone [89]. The authors also noted that the effect of activated charcoal on ethylene is dependent on culture conditions, such as container volume and shape, medium volume, and surface exposed to the inner atmosphere.

 Takayarna et al. [90] reported that root formation and growth were inhibited by higher concentration of BA, but this inhibition was completely reversed by the addition of activated charcoal.

Root formation and growth were better in BA-free medium containing activated charcoal than in the medium without activated charcoal. Similar results were observed in bulb

formation. This could be due to activated charcoal regulating internal physiological processes [83].

2.5 Strawberry Tissue Culture

One of the important goals of the agricultural policy in the world is to increase the acreage of strawberry to meet the demand of local fresh market, processing and export. Importing mother plants is expensive. Healthy stocks used for propagation through conventional methods are not available.

Micropropagation of strawberry plants were introduced in 1974 (Boxus) [91]. Immediately, the most important European nurseries producing several millions plants per year, were interested in this technique. The two basic reasons that gave the importance to this technique were: it gave a definitive answer to the problems of soil fungi, causing a lot of damage to the strawberry fields and by another way, tissue culture plants seemed to produce more runners per mother plant in a short time. However, strawberry tree is difficult to propagate by seed due to genetic variation and specific requirements of seed germination. In addition, rooting percentage of cuttings is relatively low [92].

In general, strawberry can be managed easily under *in vitro* conditions. Meristem tips, generally obtained from runners of virus-free plants, are commonly used to establish *in vitro* cultures, which are employed for mass propagation or as a source of plant material for regeneration and transformation experiments [43].

Micropropagation of strawberry by axillary buds has been studied intensively for a long time [93]. On a commercial scale, tissue culture-derived strawberry plants are estimated to cost four to five times more than plants produced by conventional propagation [6]. However, micropropagated strawberry has several advantages, such as its ability to multiply virus-free stock rapidly and, in particular, the improved capacity of these plants to produce runners for planting in the field [94, 6]. On contrast, when used directly in the field for fruit production, micropropagated material shows a decrease in fruit size and weight [94].

Several parameters used during the *in vitro* phase can affect the behavior of micropropagated strawberry in the nursery, e.g. plant genotype, mineral formulation, type and concentration of cytokinin in the medium and the number of subcultures. It is generally recommended not to exceed four to five subcultures to avoid loss of trueness-to-type of the propagated material [54].

This technique is useful in case of the introduction of new cultivars. Moreover, the storage of tissue cultured propagules requires less space than traditional runner plant and the *in vitro* storage can be initiated at any time during the production cycle [95].

Prior experiences with strawberry micropropagation indicate that *vitro* plants are more uniform, produce higher number of runners, have better survival in the field, and the fruit yield increases in 24% than plants propagated by the traditional method [96].

Another advantage of micro-propagation is the elimination of pest and pathogen stress during the production cycle, assuming that the initial stock plant is free of diseases. Therefore tissue culture technique was applied to evaluate its feasibility for a wider use. Several authors have dealt with this subject from several aspects. Boxus 1983, [97] reported that each m^2 of growing area of strawberry can produce 40000 plantlets year-[1], but Abramenko 1983, [98] has cultured apical meristem of strawberry and found that from one meristem 3000 shoot primordial were produced in 6 months and half of these developed into normal plants. These plants were vigorous and after transplanting in the soil some produced up to 500 new runner plants and found that new runner plants were 10 times more than that produced by conventional material.

The production program suggested by Vit et al. [99] for commercial planting material is based on the selection of economically important clones; freeing these of virus by a combination of heat therapy and meristem culture. Tissue culture aimed particularly at the rapid propagation of breeding material [100].

2.5.1 Strawberry Organogenesis

2.5.1.1 Strawberry Direct Organogenesis

Most commonly used explant for strawberry micropropagation is the meristem from the tip of runners. The explant is placed on a medium containing no or low levels of auxins and higher levels of cytokinins to promote axillary budding while preventing callus formation. The cytokinins are used to overcome apical dominance and enhance the branching of lateral buds from the leaf axis. Additional shoots are produced through further axillary bud growth [101].

Strawberry *Fragaria × ananassa* Duch. Was the first micropropagated *in vitro* on a commercial scale by Buxus in 1974. There after this technique has been widely used especially in Europe and USA.

In 1974, [91] Boxus experimented with BA to determine its influence on shoot and root production *in vitro*. He established that shoots would proliferate in the presence of the cytokinin but roots would not form until the explant was without cytokinin. He concluded with the statement that micro-propagated strawberry plants would replace traditional methods of propagation for the commercial trade.

Mullin et al. [102] grew strawberries with strawberry mild yellow edge (SMYE) viruses for 6 weeks in a 36°C growth chamber before excising 0.3 to 0.8 mm meristematic tips with leaf primordia. The result was that 33% to 75% of the resulting plants were SMYE-free according to leaf insert graft indexing to indicator strawberry plants.

Liu and Sanford [103] reported that the methods were developed for efficient shoot regeneration from leaf and runner tissues of the strawberry *Fragaria x ananassa* Duch. cultivars 'Allstar' and 'Heneoye'. They showed that optimal regeneration condition differed for 'Allstar' depending on whether leaf tissue was derived from plantlets grown *in vitro* or from plants grown in the greenhouse. 'Allstar' leaf tissues derived from *in vitro* culture regenerated shoots most efficiently in a Linsmaler- Skoog (LS) medium containing 2.5 mg BA and 0.5 mg IBA /liter, 'Allstar' leaf tissues derived from greenhouse plants regenerated shoots best in (LS) medium containing 3.0 mg BA and 0.1 mg IBA /Liter. Addition of casein hydrolysate (CH) at either 400 or 600 mg/l stimulated shoot production.

A supplement of KNO_3 at 2000 mg/l also enhanced regeneration efficiency of greenhouse - grown leaf tissues. 'Hsotoye' had lower regeneration potential in most treatments than 'Allstar' and only produced shoots in a (LS) medium containing 5.0 mg BA, 0.5 mg IBA, and 400mg CH /Liter. Shoots from runner tissues of both cultivars were best obtained using (LS) medium containing 10.0 mg BA, 2.0 mg IAA, and 500mg CH /Liter. Shoots proliferation from runners was dependent on the diameter of the runner, with diameters of more than 2.0 mm having poor regeneration potential.

Boxus 1989, [104] claimed that this clonal propagation process proceeded exclusively through axillary branching which normally leads to true-to-type progenies.

Jelenkovic et al. [105] studying different cultivars than Nehra or Finstad studied, tested hypocotyls, runners, petioles and lamina. Only young, fully expanded leaves were used in the lamina study. They determined in preliminary tests that BA and 2, 4-D were the most effective phytohormones to use. Regeneration was noticeably lower in inoculated explants than in controls possibly due to the explants dying from the toxic effects of the bacteria. The researchers expressed frustration over the inconsistency of response of the same explant material exposed to the same conditions.

Lopez - Aranda et al. [94] they studied *in vitro* culture of strawberry cvs. 'Douglas' and 'Chandler' for effects of mineral salts, BA levels and the number of times an explant was subcultured on the regeneration capacity. The field behavior of the regenerants was also tested in the comprehensive investigation.

The best quality regeneration was achieved with a formulation of $N_{30}K$ salts formulation for both cultivars. Best results on rooting were obtained with either the Knop or $N_{30}K$ salts formulation. Addition of 500 mg/l activated charcoal also enhanced rooting; in contrast, high BA levels during multiplication decreased the rooting capacity of shoots. They noted that micropropagated plants showed a higher runner production capacity than their control counterparts. This increase in quantity was not linked with decreased quality. They also concluded that salts formulation, BA levels (1.48 µM - 4,44 µM) and the number of the subcultures (1-8) seemed not to affect the behaviour of plants in the nursery. No adverse effects on total fruit production or fruit size could be associated with the mineral formulation used during the *in vitro* phase. High BA concentrations adversely affected total fruit production although differences between treatments were not significant.

The authors concluded that the field results of micropropagated plants are greatly influenced by their *in vitro* culture.

Jemmali 1994a, [106] suggested that Stipular buds (SB) formation occurred spontaneously on Gorella strawberry leaf stipules during proliferation phase on Boxus medium. These buds gave rise to normal shoots on the inner median zone between the stipule tips. Some stipular buds also have been observed on other parts of adaxial surface of the stipule. Stipular bud formation took place directly on the stipule without an intermediate callus. The first stage consisted of subspherical pretruions of small cells which progressively differentiated into shoots that were able to prolifrate and to root. Scanning microscopy was used to examine the developmental stages of this adventitious organogenesis and to describe morphologic abnormalities, e.g, multiapex formation and fasciation. *In vitro* cloning of plantlets was made from axillary and SBs produced by the same tufts. The greater flowering abundance of stipular plants resulted in a drastic reduction of the commercial production and the fruit caliber when compared to axillary plants. However, the general phenotype of the mother cultivar (Gorella) was not affected in either case.

Nehra et al. [107] They studied the Effect of *in vitro* propagation methods on field performance of two strawberry cultivars, 'Redcoat' and 'Veestar', propagated by meristem culture (MC), callus culture (CC) and direct shoot regeneration (DR) from leaf disks were compared for their vegetative and reproductive characters with standard runner (SR) propagated plants under field conditions. They observed that in the planting year, *in vitro* propagated plants of both cultivars had the same number of leaves as SR plants, but *in vitro* propagated 'Redcoat' produced fewer stolons per plant than SR plants. However, in the following year, *in vitro* propagated mother plants of both cultivars had more leaves and higher runner production than SR mother plants. Flowering and fruiting behavior of 'Veestar' was not appreciably influenced by *in vitro* propagation methods. However, *in vitro* propagated plants of 'Redcoat' flowered earlier and produced more flowers and fruits than SR plants, but still maintained normal berry weight. Among *in vitro* propagated plants, DR plants of 'Redcoat' were the earliest to flower, whereas MC plants produced more flowers and fruits. The field performance of the first daughter plants derived from the *in vitro* propagated plants was consistent with their respective mother plants. They also observed that leaf shape of both cultivars was not altered by *in vitro* propagation. Phenotypic abnormalities were mainly confined to occurrence of yellow leaf variants in

MC and CC plants and occasional appearance of plants with irregular flowering and growth habit among CC plants.

Jemmali et al, (2002)[37] demonstrated that adventitious stipular bud formation occurred *in vitro* in many strawberry cultivars during the proliferation phase on medium containing Knop macronutrients, MS micronutrients, vitamins, amino acids, 2.22μM BAP, 2.46μM IBA and 0.29 μM GA_3. As described previously for cultivar Gorella, cultivar Elsanta also showed adventitious stipular buds developing on the abaxial median zone between the stipule tips. To compare the shoots produced from both types of buds, clonal propagation was initiated from stipular buds and from axillary buds on the Knop macronutrients and MS micronutrients medium. Stipular buds were separated from the meristem-tip initiated plantlet and cultivated in the presence of a lower BAP concentration (1.33 μM) to prevent further stipular bud formation. During proliferation cycles, stipular originated propagules were very easily distinguished by their specific leaf phenotype and light green colour in comparison to plantlets cloned for an axillary bud. Their multiplication rate and cytokinine content were also higher than for axillary buds. No significant diference was observed in auxin content.

Meria et al. [108] demonstrated that actively growing shoots of potted greenhouse - grown strawberry tree(*Arbutus unedo* L.) were initially sterilized and established in basal woody plant medium containing 11.1 μM BA. Optimum shoot proliferation was achieved on a basal WPM containing MS vitamins, sucrose, agar and 22.2 μM BA. They observed in preliminary testes with MS basal salts instead of WPM basal salts resulted in extensive oxidative browning and failure of explants after culturing for 3 weeks, This browning was due to a high concentration of salts or, in particular, due to high NH_4NO_3 concentration in MS medium. Microshoots rooted successfully in basal *in vitro* medium containing 10 μM IBA or IAA, but their survival rate during acclimatization was low. Addition of a mixture 1 part peat: 4 parts perlite in the basal *in vitro* rooting medium (1:1 v/v) containing 10 μM IAA resulted in high rooting percentage and plantlets with branched roots. These plantlets were successfully acclimatized. This novel rooting medium can be exploited further due to its potential in commercial applications.

Zhou et al. [109] found that rooting strawberry plantlets under photoautotrophy conditions significantly increased plant height and weight after acclimatization. This effect was related to a better photosynthetic performance of *in vitro* plants.

Biswas et al, [110] studied about the effects of colour illumination on multiple shoot regeneration from runner tip explants of strawberry. Six colour (mixed, white, red, yellow, blue and green) illuminations were used in this study. They observed that the mixed colour illumination showed the high percentage of shoot proliferation. Fresh and dry weights were also significantly higher under the mixed colour condition. Proliferated shoots showed 100% rooting in half strength of MS media. Plantlets were established successfully in soil. Madany et al. [111] suggested that an efficient method of micropropagation based on an increased percentage survival of explants and reduced phenol-induced browning in strawberry (*Fragaria x ananassa* Duch.) cv. Camorosa and Selva has been developed. The effect of hormone concentration growth regulator balance and kind of antioxidant in medium on the direct shooting of meristem culture was studied. They showed that the best results were obtained on MS basal medium with B-5 vitamins supplemented with 1 mg/l IAA and 2 mg/l BAP for Camorosa and 2 mg/l IAA and 2 mg/l BAP for Selva. Use of 0.2 % activated charcoal was better than 2% PVPP to eliminate inhibitory substances from *in vitro* cultures. Excised shoots rooted on MS medium with 2 mg/l IAA and 0.3 mg/l BAP.

Sakila et al. [112] showed that nodal segments of strawberry gave rise to multiple shoots when cultured on MS medium supplemented with different concentration of BA with KIN or GA_3. The highest response of shoot multiplication was obtained in MS containing 1.5 mg/l BA + 0.5-0. mg/l KIN. The regenerated shootlets were rooted on MS basal medium with different concentrations IBA and IAA. The maximum frequency of rooting and highest number of roots was produced on medium containing 1.0 mg/l IBA. The plantlets, thus developed were hardened and successfully established in soil. The plants raised through tissue culture exhibited normal growth, flowering and fruit setting.

Emarah 2008, [36] demonstrated that an efficient method for shoot regeneration, root formation from runner tips and acclimatization of strawberry plantlets was developed. Runner tips of 1-2 cm long were used as source of explnts. After surface sterilization apical meristems of 3-5mm long were isolated and used as explnts. At multiplication stage, results indicated that the highest vegetative parameters (shoot number, shoot length and

leaf number) were observed when MS medium supplemented with 1 mg/l BA followed by the medium contained 1 mg/l BA and 0.1 mg/l IBA. However control treatment showed a significant similar result in shoot length only. Results of this study indicated that, BA was more effective in enhancement the growth of strawberry *in vitro* compared to Kin and thidiazuron. He also demonstrated that at rooting stage, it was clear that MS medium at full strength containing 30 g/l sucrose significantly surpassed all other combinations of MS strengths and sucrose concentrations in increasing root number and length per plantlet and fresh weight/plantlet. The same treatment enhanced the shoot length but without significant difference compared to some other combinations. The treatment contained 3 g/l agar with 6 g/l perlite significantly enhanced root formation (number and length) as well as shoot length, fresh weight/plantlet, and leaf number/plantlets. Finally, plantlets were successfully acclimatized and the soil mixture contained peatmoss: perlite (2: 1, V/V) observed high percentage of survival of plants (80%) with enhancing both root number and length/plantlet, plantlet height and leaf number/plantlets.

2.5.1.2 Adventitious Shoot Regeneration

Adventitious shoot regeneration has been achieved in several cultivars using a broad range of explants. These include leaf explants, petioles, stipules, stem tissue, runners, mesophyll protoplasts, anthers, cotyledons, roots and immature embryos [43].

Jemmali et al. [113] focused that adventitious buds of strawberry 'Gorella' arise between the two leaf stipule points and having different numbers of subculture when these adventitious shoots are into contact with Boxus medium, they proliferate fastly by axillary branching. They concluded that the performances of the plants regenerated from stipular shoots are similar to the control ones concerning DNA ratio and preliminary analysis by flow cytometry gave no modification of their ploidy level, epigenetic or genetic variations are not excluded, the plants used as control are indeed a mixture of different shoot origin and that can explain why they did not observe real difference between the treatments. And they noted also that all roots of stipular plantlets were exclusively white, whereas the control ones were more or less colored with anthocyanin. This observation was confirmed in another experiment and could be used as a marker if it remains stable under variable culture condition.

Rugini and Orlando [114] studies about adventitious shoot regeneration by used stipules explants, they found great differences in shoot regeneration ability from calluses among cultivars when leaf, petiole and root tissues were used as initial explants. However, these differences disappeared when whole leaves, including stipules, were used as explants.

Passey et al. [115] studied seven commercial cultivars of strawberry using leaf disks, petioles, roots, and stipules as explant material. They started out by establishing and growing runner tips *in vitro* and subculturing them every three weeks until enough material was produced to begin the experiments. The leaf disks had the highest regeneration rates for all cultivars with greater than 90% of explants producing shoots.

Samir 2005, [116] reported an efficient system to regenerate shoots on excised sepals (calyx) of greenhouse-grown 'Bounty' strawberry (*Fragaria - ananassa* Duch.) was developed *in vitro*. He show that Sepal cultures produced multiple buds and shoots without an intermediary callus phase on 2–4mM 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron, TDZ)-containing shoot induction medium within 4–5 wk of culture initiation. And he also shows that young expanding sepals with the adaxial side touching the culture medium and maintained for 14 days in darkness produced the best results. In a second experiment, he observed that sepals proved more effective than the leaf discs and petiole segments for regenerating shoots. A third experiment he compared the effects of six concentrations of two cytokinins (TDZ at 0, 0.5, 2, and 4mM and zeatin at 2 and 4 mM) for elongation of sepal-derived adventitious shoots. The results indicated that the media containing TDZ generally promoted more callus formation and suppressed shoot elongation. TDZ-initiated cultures transferred into the medium containing 2–4mM zeatin, produced usable shoots after one additional subculture. And shoots were rooted in vitro in the same medium used for shoot regeneration, but without any growth regulators. When transferred to potting medium,   85–90% of *in vitro* plantlets survived.

Qin et al. [117] reported that 'Toyonoka' strawberry leaf explants cultured for 10 days in shoot regeneration medium in the presence of $AgNO_3$ not only enhanced shoot regeneration efficiency but also expedited the inhibition of adventitious buds. Being an ethylene inhibitor, $AgNO_3$ can markedly promote organogenesis in strawberries.

Samir 2006, [118] worked about the effects of 0, 2, 4 or 8 μM thidiazuron (TDZ) and explant type on adventitious shoots regenerated on excised leaf disks, sepals (calyx) and petiole halves of greenhouse-grown 'Bounty' strawberry (*Fragaria - ananassa* Duch.). He saw that a moderate concentration (2 – 4 μM TDZ) supported bud and shoot regeneration without an intermediary callus phase on young expanding sepals, or on leaf disks, or on petiole, all maintained for 14 d in the dark. Sepals proved more effective than leaf discs and petiole segments for regenerating shoots. Another experiment, he was compared the effects of 0, 0.5, 1, 2 and 4 μM TDZ or zeatin on shoot proliferation and rooting of sepal-derived adventitious shoots. The results indicated that the media containing TDZ generally promoted more callus formation and suppressed elongation and rooting of shoots. And Shoots proliferated and roots developed best when explants were cultured in medium supplemented with 1 or 2 μM zeatin.

2.5.2 Strawberry Indirect Organogenesis

Kartha et al. [119] successfully regenerated 'Redcoat' using a combination of BA, IBA and GA3 as a precursor to a cryopreservation study of the cultivar. Kartha et al. (1980) studied the effects of light intensity and media on greenhouse-grown and *in vitro* grown cultures of 'Redcoat'. Calli from *in vitro* grown cultures had very poor regeneration capacity. Both greenhouse-grown and *in vitro* cultures formed callus and shoots when BA and 2, 4-D were used at different concentrations. Severe browning occurred when fully expanded leaves of greenhouse grown 'Redcoat' were used. Browning was caused by BA and NAA and that it occurred even when young leaves were used. They suggested that the browning would not have occurred in the presence of activated charcoal added to the medium. However, activated charcoal will inhibit callus formation. They also saw a relationship between hormone concentrations and explant polarity when tissue from the upper part of the leaf regenerated at a higher frequency with higher concentrations of hormones than those on the basal end exposed to lower concentrations of hormones.

Narender et al. [120] reported that Plant regeneration using immature leaf explants taken from in vitro shoots and greenhouse grown plants of the strawberry cv. Redcoat. Both types of explants formed callus and multiple shoots at various frequencies in the presence of benzyladenine (BA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) at different concentrations. Highest shoot regeneration (31%) occurred with the calli of greenhouse

leaf explants at 5 µM each of BA and 2,4-D in 24 weeks. Improved regeneration (50%) with vigorous shoot proliferation was achieved in 10 weeks by transferring the 5 week old green compact calli derived from greenhouse leaf explants to MS basal medium supplemented with 10 µM BA and 1 µM 1-naphthaleneacetic acid (NAA) and incubating the cultures at 16 h light (62.5 µE. m -2 s-l). The calli from *in vitro* leaf explants had very poor shoot regeneration capacity. Seven other cultivars of strawberry also responded to this regeneration protocol albeit at different frequencies. The protocol may be useful for improvement of strawberry through somacional variation and selection of desirable traits.

Saifullah et al. [121] demonstrated that the potential of callus culture and regeneration was evaluated in strawberry (*Fragaria ananassa*). The effect of different hormonal combinations and explant sources was studied in- order -to produce maximum number of plant in shortest possible time. Young leaves developing from glasshouse and *in vitro* grown strawberry plants of cultivar Tango cultured on MS mineral salt. They observed that the organic potential of explants from *in vitro* culture shoots compared with those taken from glasshouse grown plants was significantly different for the sane media. The calli induced from *in vitro* grown plant exhibited high regeneration as compared to those induced from glasshouse grown plants. They also saw that maximum shoot regeneration from *in vitro* was found on regeneration media 29 containing BAP/IBA 2.25/1.0 mg\l whereas, glasshouse grown explants gave maximum shoots on medium 13 containing BAP/NAA 2.25\1.0 mg\l. The poor shoot regeneration on leaf explant taken from *in vitro* shoots may be due to a hormonal effect between NAA and IBA interaction caused by accumulation of IBA in the leaf tissue.

Wojciech et al. [122] demonstrated that the growth *in vitro* cultures of strawberry (*Fragaria × ananassa* Duch.) depending on different photoperiods. The investigated photoperiods influenced growth of strawberry clones *in vitro* on Boxus media supplemented with glucose. The application of 22/2 (d/n) photoperiod was not advantageous as it did not improve shoot proliferation of both 'Elsanta' and 'Senga Sengana' in vitro cultures, whereas stimulated the growth of callus at the explant base and caused chlorosis of leaves when compared to control [16/4 (d/n)] .Photoperiod 4/2 (d/n) significantly enhanced shoot proliferation of 'Senga Sengana' cultures. However, in principle such reaction of 'Elsanta' cultures was not distinct as the significantly better proliferation of shoots under 4/2 (d/n) photoperiod was only recorded in the third passage.

The leaf blades of both clones grown under 4/2 (d/n) cycle were significantly reduce. On the other hand, the leaf petioles of 'Elsanta' were visibly elongated under mentioned photoperiod.

2.5.3 Strawberry Anther Culture and Haploid Recovery

Anther culture involves the aseptic culture of immature anthers to generate fertile haploid plants from microspores. The production of haploid plants through anther culture is widely used for breeding purposes, as an alternative to the numerous cycles of inbreeding or backcrossing usually needed to obtain pure lines in conventional breeding. Chromosome doubling of haploids could result in immediate establishment of homozygosity for all loci. The success achieved with anther culture has led to the development of microspore culture to regenerate homozygous plants. Furthermore, isolated microspores are very attractive for protoplast isolation and applications aiming at transformation as they are unicellular and transgenic homozygous plants could be provided in a comparatively short time [39].
Haploid recovery in strawberry through aseptic anther culture was unsuccessful in some early reports [123, 124].

Owen and Miller [125] obtained haploid plants from cultured anthers of 'Chandler', 'Honeoye' and 'Redchief' strawberries. The highest shoot regeneration across cultivars (8%) was obtained when a semi-solid MS medium contained 2 mg/l IAA, 1 mg/l BA and 0.2 M glucose. Chromosome counts of root tip cells from ex vitro-grown plants confirmed that haploid plants were obtained from all three cultivars.

Hamdouni et al. [126] demonstrated that the achenes of two strawberry cultivars 'Chandler' and 'Tudla' when scarified and sterilized by H_2SO_4 (36N), showed a maximum capacity of germination after a short time on MS inorganic salts ('Chandler' 94% and 'Tudla' 63%). On the contrary, the hydrogen peroxide treatments did appear to affect neither the time nor the rate of germination. The culture on the same medium supplemented with 3% sucrose of achenes cut and treated by H_2SO_4 1N, as well as isolated embryos give very good results (97% and 100% for 'Chandler' and 74% and 85% for 'Tudla', respectively for cut achenes and embryos), and shown the role played by teguments in the dormancy of achenes and reduced the time to 50% of final germination to only 3 days.

2.5.4 Strawberry Somatic Embryogenesis

Somatic embryogenesis research with strawberries is still in a preliminary stage and some more efforts would be required to develop the technology [127].

Wang et al. [128] reported somatic embryogenesis from strawberry cotyledons on MS medium supplemented with 22.6 μM 2, 4-D, 2.2 μM BA and 500 mg/l casein hydrolysate where few of the embryogenic tissues developed into somatic embryos. Morphologically normal plants were obtained from somatic embryos that were transferred to MS medium containing 2.89 μM GA3 or 2.22 μM BA + 0.54 μM NAA. Maintenance of the embryogenic cultures was, however, unsuccessful.

Donnoli et al. [129] reported somatic embryogenesis in 8% of the embryogenic calli in strawberry cultivar 'Clea' on MS medium supplemented with 4.88 μM BA and 4.90 μM IBA.

2.5.5 Strawberry Somaclonal Variation

The variation in the regeneration capacity amongst different cultivars has also been observed in other studies:

Strawberry plants spread vegetatively using runners and this enables them to be easily transplanted and propagated as clones. Commercial production of strawberry using micropropagation processes bears several risks. Plant off-types, i.e. non true-to-type and genetically not identical to the mother plant, may be among the resulting plants. These plants can simply the result of hardening errors and not arise from a change in the genetic make up of the plant [130].

In vitro production of plants involves the application of plant growth regulator, such as auxins, for process initiation. Nevertheless, these auxins are known to be associated with genetic instability in plants, a phenomenon called somaclonal variation. Although, somaclonal variation may be used as a source for variation to get superior clones, it could be also a very serious problem in the plant tissue culture industry resulting in the production of undesirable plant off - type [130].

However, through micro-propagation of strawberries (*Fragaria x ananassa*, Duch.), several morphological abnormalities were detected as somaclonal variation. Changes include leaf color variants and dwarf plants, among others [131]. Callus-derived "Redcoat" strawberry plants differ significantly from standard runner plant for several vegetative characteristics [132]. These variants pose a problem for production of uniform, true-to-type plants.

Nehra et al. [133] found that two cultivars of strawberry responded differently to various forms of *in vitro* propagation and in both cases variants were found in callus-derived plantlets, but not those derived from meristems or via direct leaf regeneration. Some studies have shown that modified characteristics are epigenetic and disappear over time [134]. Numerous authors have reported that genetic changes including insertions, deletions, point mutations and rearrangements occur during tissue culture [135], but few of the phenotypic symptoms found are heritable [136, 137]. Most somaclonal variations occur in plants regenerated from cultures that have undergone a differentiation phase.

Some studies indicating that a strong genetic component determines the success of adventitious regeneration. Recently, a role has been suggested for endogenous H_2O_2 in the process of bud primordia formation from strawberry calli [138]. Along this line, Yonghua et al. [139] found that shoot regeneration was enhanced when explants were cultured under red or green plastic films, and this was correlated with an increase in activity of antioxidant enzymes and endogenous hormone concentration.

Presently, there are various methods available which can be used to detect and monitor tissue culture-derived plants and cultivar identification. The most reliable methods are the molecular marker techniques that identify the variance depending on the plant proteins, which are expressed from defined regions of DNA, or DNA polymorphisms. RAPD (random amplified polymorphic DNAs) is a powerful technique for identification of genetic variation. It has the distinct advantage of being technically simple and quick to perform, requiring only small amounts of DNA compared to restriction fragment length polymorphism (RFLP) analysis. Strawberries (*Fragaria × ananassa* Duch.) have been extensively analyzed using randomly generated markers for clone identification and diversity studies [130].

Damiano et al. [140] studies about somaclonal variation and in vitro regeneration of strawberry cultivar 'Teodora' and 'Clea', micropropagated on MS medium, they proved that the variability induced by the in vitro culture has been shown at callus, cell level and after regeneration from well organized tissues. They shown that petioles and laminas, produced poor callus only, but the stipules are highly competent for organogenesis, and the BAP alone is sufficient to induce regeneration, while 2, 4-D is strongly inducing callus formation. Cell suspention and calli can be selected for deferent characterization of growth carves and polymorphisms of the isoenzymes.

Polymorphisms also appear in acid-phosphatase, glutamate-dehydrogenase and peroxidase of regenerated plants. The *in vitro* naturally occurring variability has been used for selecting cell lines to be resistant *Colletotrichum acutatum* crude culture filtrates; the selection effectiveness is shown to be dependent on the age of the fungus cultures and on the dosage of filtration liquid. The regenerated plants show polymorphism at RAPDs analysis. The sensitivity to the treatment with culture filtrate of *C. acutatum* was measured with test of viability (fluorescein diacetate). The viability is varying according to the dosage, the length of the incubation of fungus and the genotypes.

Adel 2007, [131] studied the somaclonal variation in micro-propagated strawberry detected at the molecular level. Meristem tips of three Strawberry cultivars, namely Chandler, Sweet Charlie and Gaviota were excised and cultured for 5 weeks on the starting medium. Subculture was carried out for five weeks on shooting medium, and finally shoots transferred to the rooting medium. The obtained plantlets were acclimated under greenhouse condition in medium consisting of peat moss and sand (2:1). DNA extracted from *in vitro*-derived plantlets and standard propagated plants were analyzed by RAPD-PCR to detect possible drift in genetic stability of micro-propagated plants. He showed that most of the obtained bands from *in vitro*-derived plantlets in all primers used with the three cultivars were found to be present in the fingerprints of standard propagated plants, demonstrating no variation in the pattern obtained with DNAs from the two sources of strawberry plants. It is concluded that mass propagation via meristem tip culture is reliable in producing genetically similar plants to the mother ones.

CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Source of Explants

Mother plants of strawberry (*Fragaria x ananassa* Duch.) cv. Sweet Charlie grown in the farm land of Bith Lahia city, Gaza Strip, Palestine, were used as source of explants during this study. Runner tips of 1-2 cm long were taken from mother plants as that long was suitable for sterilization procedures.

3.1.2 Chemicals

The chemicals that were used are listed in table

Table 4. A list of the chemicals used in this work.

Chemicals	Manufactures
Macro elements • **NH_4NO_3** • **KNO_3** • **$CaCl_2.2H_2O$** • **$MgSO_4.7H_2O$** • **KH_2PO_4** • **Na_2EDTA** • **$FeSO_4.7H_2O$.**	**Sigma Company**
Micro Elements • **H_3BO_3** • **$MnSO_4.4H_2O$** • **$ZnSO_4.7H_2O$** • **KI** • **$Na_2MoO_4.2H_2O$** • **$CuSO_4.5H_2O$,** • **$CoCl_2.6H_2O$**	**Sigma Company**
Organic Constituents • **Glycine - Myo-inositol - Nicotinic Acid Pyridoxine HCl - Thiamine HCl** • **Sucrose** • **Agar**	**Hi Media Company**
Hormones • **Benzylaminopurine (BA)** • **Indolbutyric acid (IBA)**	**Hi Media Company**
Detergents NaOCl - Tween 20 - $HgCl_2$ - Ethyl alcohol 70%	**Hi Media Company**
Suppluments • **Polyvenylpyridazone (PVPP)** • **Activated charcoal (AC)**	**Hi Media Company**
Another • **NaOH – HCl - KOH**	**Sigma Company**

3.1.3 Equipments

The main equipments that were used are listed in table.

Table, 5. A list of the main equipments used in this work.

Instruments	Manufactures
• **Growth chamber**	**Islamic Universty Lab.**
• **PH meter**	**Made in Germany.**
• **Autoclave**	**Henna (Made in Germany).**
• **Analytical balance**	**a-e ADAM PW 254**
• **Stirrer**	**Heidolph. MR 3001K**
• **Micropipette**	**Tomos Biotools Co., Ltd.**
• **Lux Meter**	**Made in Japan.**
• **Water Bath**	**Gemmy Industrial Corp YCW – 01**
• **Microfilter**	**Hi media Company**

3.2 Methods

3.2.1 Surface Sterilization of Explants

Explants were rinsed under running tap water for 30 minutes. For surface sterilization, explants were immersed in different concentrations (0.5, 1, 1.5, 2.0 and 2.5%) of sodium hypochlorite solution containing two to three drops of Tween 20 per 100ml for 20 minutes. The plant materials were then, rinsed three times with distilled water and were treated with 0.1% mercuric chloride solution for 10 minutes. The explants were then thoroughly washed (4 - 5 washings) with sterilized distilled water to remove the traces of $HgCl_2$. After sterilization, explants were shortened to remove the surfaces of explants and meristems of 3-5mm long were isolated as final explants. These explants were cultured with good contact on the surface of the medium.

3.2.2 Culture Medium

The media was formulated in the lab according to the composition of Murashige and Skoog [4] referred as MS medium was selected as the optimal culture medium including (0.2%) PVPP.

Stock solutions of generally 4x macroelements, 1000x for microelements, 100x for the organic constituents were prepared. These stock solutions were stored in a freeze chest at 4^oC and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days.

Table 6: Murashige and Skoog's Medium (1962) [4] Constituents Amount (mg/l).

No.	Macro Elements	Conc. mg/L
1	NH_4NO_3	1650
2	KNO_3	1900
3	$CaCl_2.2H_2O$	440
4	$MgSO_4.7H_20$	370
5	KH_2PO_4	170
6	Na_2EDTA	37.31
7	$FeSO_4.7H_2O$	27.81
No.	Micro Elements	Conc. mg/L
1	H_3BO_3	6.2
2	$MnSO_4.4H_2O$	22.3
3	$ZnSO_4.7H_2O$	8.6
4	KI	0.33
5	$Na_2MoO_4.2H_2O$	0.25
6	$CuSO_4.5H_20$	0.025
7	$CoCl_2.6H_2O$	0.025
No.	Organic Constituents	Conc. mg/L
1	Glycine	2.0
2	Myo-inositol	100
3	Nicotinic Acid	0.5
4	Pyridoxine HCl	0.5
5	Thiamine HCl	0.1
6	Sucrose	30.000
7	Agar	0.6%

The reagents used were of Analytical Reagent Grade. Each salt was dissolved separately one after one to avoid precipitation.

3.2.3 Sterilization of the Nutrient Media

Following preparation of the medium and prior to addition of agar, the pH was adjusted to 5.8. The medium was poured into culture jars of size 350ml where each jar contained 30 ml of the medium and capped with plastic caps. The culture jars were autoclaved at 121 ^{o}C and 1.2 kg/cm2 air pressure for 20 minutes.

3.3 Strawberry Micropropagation

3.3.1 Effect of Pretreatments on Explant Browning

To study the effect of various pretreatments on explant browning and survival percentage, different sets of sterilized explants were dipped in three different concentrations of antioxidant solution (1, 1.5 and 2% PVPP) and another set dipped in sterilized distilled water without antioxidant solution as a control.

Meristem tips were cultured on hormone-free MS solid medium to establish clean and healthy materials for next experiments.

Cultures were kept for chilling treatment at 4 ^{o}C for 10 days and then incubated for 4 weeks in the growth room.

All cultures of this study were incubated at 25 ^{o}C $\pm$ 2 day and night. Light was provided by fluorescent tubes giving an intensity of 1500 lux at the level of culture vessels for 16 hours per day.

3.3.2 Multiplication Stage

Meristem - derived plantlets were multiplied by sequential subculturing into fresh medium in 300 mL jars each with 30 mL of culture medium supplemented with 1.0 mg/L BA hormone and closed with plastic caps. At this stage of culture, the shoots proliferated rapidly within one month. Two subcultures were then conducted.

3.3.3 Rooting Stage

3.3.3.1 Effect of Different Strengths of MS Medium

Shoots of strawberry obtained from multiplication stage were cultured on different strengths of MS, 1/8, 1/4, 1/2, and full strength of MS medium. All treatments of this set of experiment contained hormone free media. After 4 weeks, data were recorded as, root number/plant, root length, shoot length and fresh / plant.

3.3.3.2 Effect of Different Concentrations of IBA

Shoots of strawberry obtained from multiplication stage were cultured on different concentration of IBA, 0.5, 1.0, and 1.5 mg/L. After 4 weeks, data were recorded as, root number/plant, root length and shoot length and fresh /plant.

3.3.3.3 Effect of Different Concentrations of Activated Charcoal

Shoots of strawberry obtained from multiplication stage were cultured on different concentration of activated charcoal, 0.2, 0.5 and 1.0 mg/L. All treatments of this experiment contained hormone free media. After 4 weeks, data were recorded as, root number/plant, root length and shoot length and fresh /plant.

3.4 Statistical Analysis

All experiments were repeated twice and the represented data were averages. Results of these experiments were analyzed by analysis of variance (ANOVA).

CHAPTER 4

Results and Discussions

4.1 Source of Explant

The strawberry plant used in this investigation were chosen on the basis of being the most commercially successful varieties currently grown in the Gaza strip, In addition cv. Sweet Charlie was selected for two reasons: it is the most economically important cultivar currently grown in Israel and Gaza strip and it has many characteristics that could be improved in a genetic modification programme. It is generally agreed that plants regenerated from shoot tips or buds are genetically stable and free from somaclonal variations associated with plants differentiated from callus [141].

In vitro culture of strawberry greatly affects the quality of material obtained and its field performance [94]. Generally, cultures are most easily established from explants harvested from the active growth of the mother plant. The best time for collecting explants is usually when stock plants are producing new vigorous shoots (i.e. in temperate regions, in spring to early summer), but many exceptions have been observed. The optimum time for collecting explants may depend on the kind of culture, on the particular genotype to be used, on the relative amounts of contamination, and browning of the explant occurring at each season [19]. However, in our experiment it was observed that establishment stage *in vitro* culture of strawberry depends on several factors such as: the quality of material obtained, shoot collecting season of explants, contamination treatment without any effect on the explants and reduction browning due to reaction between the phenol exudation secretion from the explants and some chemical reagent such as sodium hypochlorite.

4.2 Surface Sterilization of Explants

One of the main problems encountered with *in vitro* propagation was the massive bacterial contamination at the initiation and multiplication stages. After transfer of the bud to solid sterile medium, a whitish exudate of bacteria was observed around the base of the explant after 2-3 d. The problem was complicated further by the latent nature of the contaminants [142].

In general, it is accepted that contamination of plant tissue cultures can be caused mainly by insufficient aseptic techniques during manipulations, incomplete surface sterilization of the explant and endogenous microflora present in the explant.

We think that the most difficult stage in tissue culture is a contamination and browning. These two problems are combined with each other. To decrease the browning, we should be careful during the surface sterilization process of the explant. However, none of the reports mentioned the percentage of clean cultures obtained nor the occurrence of phytotoxic effects. This result agrees with Moutia et al. [57] he reported that disinfection with 2.7% sodium hypochlorite (pH 6) proved to be most phytotoxic, resulting in 100% necrosis and death of buds.

Sodium hypochlorite and mercuric chloride have been used by many authors for surface sterilization of strawberry explants, Passey et al. 2003 used (0.525% sodium hypochlorite) Bhatt and Dhar [143] used (0.05% $HgCL_2$) none of them have dealt with the problems of bacterial contaminants and browning occurring during culture.

In the experiments reported here, both chemicals gave good reduction of bacterial contaminants and gave the maximum aseptic cultures and maximum explant browning in cv. Sweet Charlie except the treatment with 1.5% Sodium hypochlorite which gave the best results, especially, when the explant treatment with 1 and 2% PVPP for 1-2 h before culture, the browning percentage were 80 and 40% respectively, and survival percentage were 20 and 60% respectively in August season (Table 7).

Moutio et al. [57] explain that none of the surface-sterilizing agents used achieved total elimination of bacterial contaminants when using sodium hypochlorite alone and mercuric chloride alone, and they demonstrated that the disinfection with 1% mercuric chloride gave 56.3% clean cultures as opposed to 46.8% with 2.7% sodium hypochlorite. However, in our experiment we think that the combination with two treatments increase the reduction of contamination.

According to these notes in our experiment is acceptable that we could reach the suitable concentrations in sterilization and browning methods. By this way we have got survival explants.

While the same experiment was applied on the explants but was collected in October season and from other field, we observed that all the samples were failed to reduce explants contamination. We believed that these results depend on the selection of the

material in the field and choice a good farm land which used better bacterial and fungal treatment before and after culture, or that the shoot collection season proved to play important role in reducing contamination. Boxus (1999) [144] reported some ways to select the best mother plants in the field. The very young runner tips are the best plant material, and must be collected in a fruit production field in June, before the runners have formed leaves and roots. This very young material has fewer hairs surrounding the meristematic zone and results in little contamination. Swartz et al. [95] noted that the contamination problems occurred with cultures initiated during late Summer and Autumn, or some laboratories keep the mother plants in screen houses for several years, and always use the same plants for the meristem excision.

Persistence of contamination after strong disinfection and the sudden appearance of visible bacterial growth during later *in vitro* stages led to the hypothesis of endogenous, latent bacteria in the explants. Such observations have also been suggested by many authors working with *in vitro* culture of ornamental, fruit and crop species. Many bacteria found in old plant tissue cultures are known not to produce symptoms on the plant or visible growth on the propagation medium *in vitro* and these are usually described as being latent, endophytic or endogenous. The growth of these bacteria is often limited and multiplication occurs as a result of changes induced by the plant. Such bacteria are not detected by visual assessment of plant cultures and are propagated together with the plant material. Multiplication rates of plant cultures contaminated with such bacteria were, however, shown to decrease and in some cases, plants were killed by the phytotoxic metabolites produced by the bacteria [145].

This high percentage of endogenous saprophytes could be explained by the age of the buds used. It is generally accepted that ageing of tissue is accompanied by an increase in microbial infection. The West Indies Central Sugar Cane Breeding Station in Barbados reported that older and mature buds of sugarcane were more susceptible to infection than younger ones [146].

Table 7. Surface sterilization treatments for the elimination of bacterial contaminants in in vitro bud culture of strawberry cv. Sweet Charlie (The treatment condition as mentioned in section 3.3.1)

Sterilizing agent	Percentage of explant contamination	Percentage of explant browning (1% PVPP)	Percentage of explant browning (2% PVPP)
Sodium hypochlorite 1.0%	0	100	100
Sodium hypochlorite 1.5%	0	80	40
Sodium hypochlorite 2.0%	0	100	100
Sodium hypochlorite 2.5%	0	100	100

Another investigation, in our study the shoot collection season plays an important role in reduction of browning and bacterial contaminants. Indra et al. [147] reported that shoot collection season proved to play important role in reducing percent browning and inducing bud break. Explants collected during winter (November - December) gave the maximum response as measured by percentage of explants with axillary bud break *in vitro*, and they observed that seasonal changes greatly influence explant establishment and cultures could not establish in rainy season due to heavy fungal and bacterial contamination.

The effect of tissue culture on leaf formation was studied by Zebrowska et al. [148] who revealed that number of leaves per microplant in the Autumn after planting and in the next year of cultivation was significantly higher in comparison to the standard propagate seedlings.

4.3 Effect of Pretreatment on Explant Browning:

In the experiments reported here, to study the effect of various pretreatments on explant browning and survival percentage. The results of the present study demonstrated that the concentration of PVPP was beneficial to reduce exudates and inhibit the browning in the October season, the highest percentage were observed in the control and 2% PVPP compared with 1.5% and 1% PVPP respectively (Table 8) and low survival percentage for all treatments, these results are considered abnormal.

Table 8. The effect of PVPP in the browning inhibition.

Time	1% PVPP	1.5% PVPP	2% PVPP	Control
6 days	3 clear 7 moderate 0 high	5 clear 3 moderate 2 high	7 clear 2 moderate 1 high	10 clear 0 moderate 0 high
12 days	2 clear 7 moderate 1 high	4 clear 4 moderate 2 high	7 clear 2 moderate 1 high	10 clear 0 moderate 0 high

Clear = (without browning),   moderate = (moderate browning), high = (high browning)

However, the results can be explained on the basis that PVPP 0.2g/100ml when added into medium and subjecting cultures for ten days to cold treatment before transfer the explants to growth chamber was found most effective resulting in 100% browning inhibition in control. This finding is consistent with the fact that addition of PVPP into the media alone able to eliminate and prevent the browning. It is known that cell death is correlated with elevated levels of peroxides during tissue browning; in addition there are many factors able to induce browning after the explants treatment such as temperature and light intensity through the experiment.

In some plants higher peroxidase activity is induced by mechanical injury, environmental stress, H_2O_2, and polyphenols [149]. We speculate that wounding derived from these stress responses may be associated with the ability of peroxidase to detoxify oxidative damage and cause cell death, as well as subsequent tissue browning. When antioxidants were added to the plant tissue-culture medium, they reacted with polyphenols and decreased the concentration of harmful compounds. Antioxidants reduced the accumulation of peroxidase in strawberry tissue and protected cells from oxidative damage. Antioxidants also reduced the inhibition of polyphenols to cell growth and shoot formation; therefore, the frequencies of runner tips induction, shoot differentiation, and rooting were increased. The results from this study indicate the browning phenomenon in strawberry runner tissue can be greatly reduced by addition of 0.2g/100ml PVPP into the medium culture. Also, incubation in the cold at 4°C for the first 10 days may arrest the rate of tissue browning by slowing the enzymatic activity responsible for tissue oxidation (Table 7). Frequent subculturing to fresh medium may also assist so that toxic phenolic compounds do not hinder the activity of plant growth regulators on tissues. Madany et al. [111] show that addition of 0.2 % activated charcoal was better than 2% PVPP to eliminate inhibitory

substances from *in vitro* cultures of (*Fragaria x ananassa* Duch.) cv. Camorosa and Selva. PVPP added in the medium was also found effective in explant browning prevention from earlier reports on guava and *Cleistanthus collinus* [147].

Nehra et al. [150] Observed that the explants taken from fully expanded, mature greenhouse leaves of strawberry cv. Redcoat exhibit severe browning. The combination of BA and NAA caused browning even in the explants obtained from young leaves. Explants browning caused by this treatment could be effectively controlled by the addition of activated charcoal to the medium.

Indra et al. [147] explained that in the spring season maximum explants died due to phenolics. This is perhaps due to higher phenolic content in growing shoots.

Media containing only nitrogen as nitrate are used for the shoot culture of some plants, for example strawberry (Boxus, 1974) [91], which can be cultured with 10.9 mM NO_3 alone; supplementing the medium with 6 mM NH_4^+ causes phytotoxicity. However, shoot cultures of strawberry grown without NH_4^+ can become chlorotic: adding a small amount of NH_4NO_3 (or another source of reduced nitrogen) to the medium at the last proliferation stage, or to the rooting medium, may then give more fully developed plants with green leaves. On some occasions it is necessary to eliminate or reduce NH_4^+ from the medium for shoot cultures to prevent hyperhydricity [19].

Meria et al. [151] observed in preliminary testes that using MS basal salts instead of WPM basal salts in strawberry culture (*Arbutus unedo* L.) resulted in extensive oxidative browning and failure of explants after culturing for 3 weeks, this browning was due to a high concentration of salts or, in particular, due to high NH_4NO_3 concentration in MS medium.

We believe that incubation the culture in the cold at 4^oC for the first 10 days preventing high nitrogen content in the MS medium and alleviated the problem of browning and toxicity. This finding further supports the importance of this treatment in promoting organogenesis in strawberry. In that concern, Passy et al. [115] focused that pre-chilling of stock material at 4^oC enhanced subsequent shoot regeneration.

Some laboratories prefer MS medium to Knop medium as macronutrients during the rooting phase. Indeed, on this medium, the plantlets will grow faster. However, these MS plantlets are softer and also more susceptible during the weaning phase [146].

After transfer the samples to the growth room the runner tips starts swilling and about two weeks later, the excised meristem tips have developed into a rosette of leaflets, and has developed to a size of a few millimeters (Figure 3. A, B).

Within 3 - 4 weeks after this excision, the shoots reach to 2-3 centimeters (Figure 4. A, B), transfer to MS propagation media supplemented with 1mg/l BA hormones.

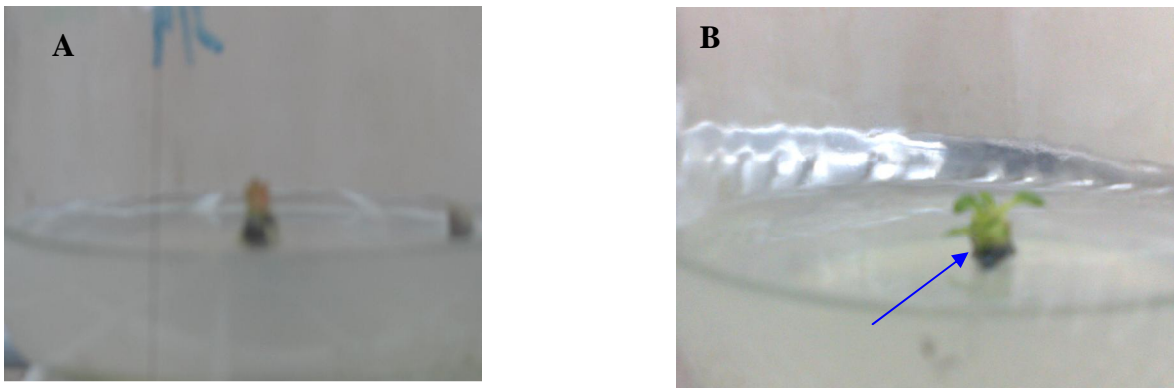*Figure3 :(A) Swilling of runner tips explants of cv. Sweet Charlie on MS medium free hormone after 8 days of culture.(B)   Development of bud from runner tips explants (arrow) after 15 days of culture.*
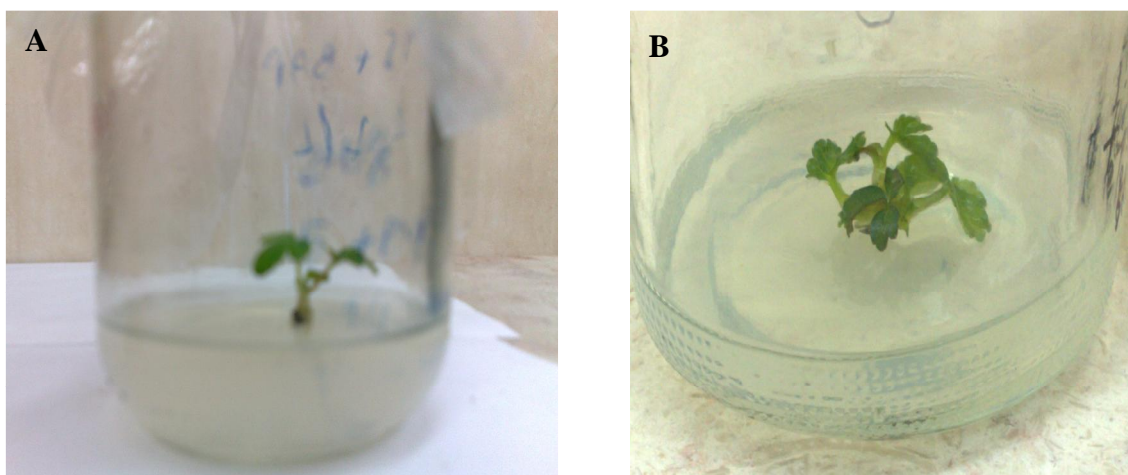

Figure 4. (A).   Runner tips explants of strawberry in MS media free hormone after 4 weeks culture the leaf reach to 2-3 cm.   (B). Explant after 6 weeks in MS media supplemented with 1.0mg/l BA.

Within 3 - 4 weeks after this transfer, two or three new buds appeared at the base of the petioles of the oldest leaves. These young axillary buds grow very quickly and, in turn, produce new axillary buds. After 6 – 8 weeks the axillary buds invade the base of the petioles of the oldest leaves (Figure 5). The initial plantlet is transformed into a cluster of buds, these clusters may contain more than 15 – 25 small buds. No roots are present on these buds.
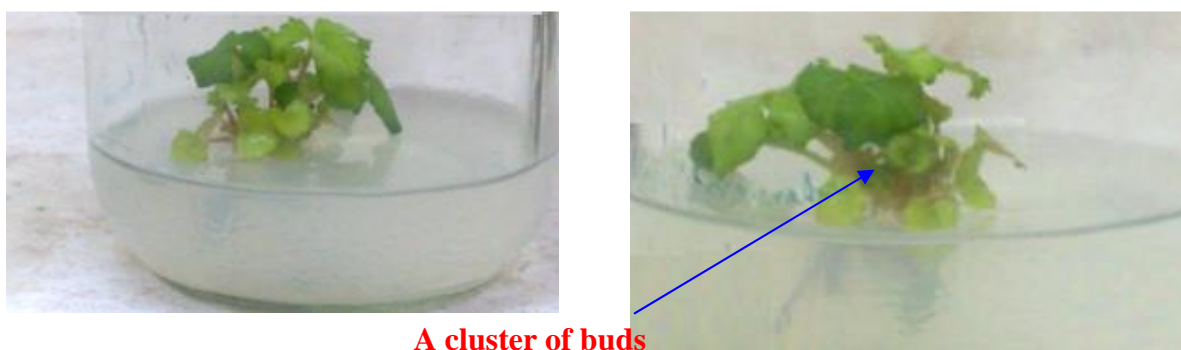

A cluster of buds

Figure 5: Explant in multiplication media after 8 weeks, the axillary buds invades the base of the petioles of the oldest leaves (first division).

4.4. Multiplication Stage

At this stage, the clusters0of buds may be aseptically separated and transferred onto a fresh medium to continue the clonal propagation. Every 6 – 8 weeks, small portion of the isolated buds produced high number of new axillary buds (Figure 6).

During the second division of the clusters, only a small fraction of the isolated buds is transferred to proliferation medium to continue the propagation.
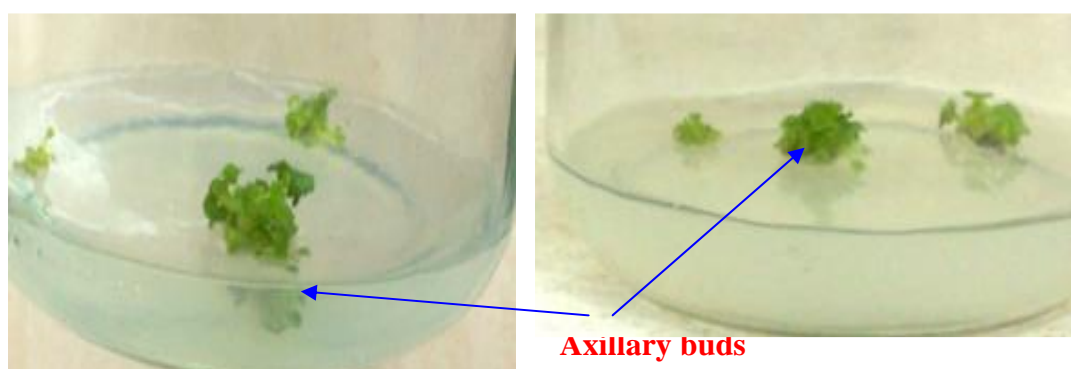

Axillary buds

Figure 6: Induction of multiple shoots from Runner explants on MS medium supplemented with 1.0 mg/L BAP, after 12 weeks of culture.

In our experiments the multiplication stage play an important role in micropropagation process, our results indicated that medium contained 1.0 mg/l BA alone proved to be the best for shoot formation, shoot number and number of buds. The results are in agreement with those of Emara (2008). [36] who reported that BA alone at 1.0 mg/l significantly showed the highest record of shoot number followed by the treatment contained 1.0 mg/l BA and 0.1 IBA that a significant similar response. Boxus (1999). [146] revealed that when shoot tips of strawberry were cultured on a medium supplemented with 0.5 mg dm-3 BA, 0.1 mg dm^{-3} GA$_3$ and 6.4 g dm^{-3} agar, shoots were multiplied, while Marcotrigiano et al. [152] found that BA at 0.3 mg/l was as effective for shoot proliferation as 1 and 3 mg/l. The importance of BA, in strawberry shoot formation, was also reported by Lal et al. [153] who found that the maximum number of shoots per explant was observed in MS medium supplemented with BAP at 4.0 mg/l.

Benzyladinine seems to be important in controlling proliferation of strawberry shoots. Marcotrigiano et al. [152] observed on a large number of strawberry cultivars that BA concentrations in the range 1.3-13.3 mM, did not produce significant differences in multiplication rate; in contrast, Simpson et al. [154] found clear differences among genotypes in their BA requirements for optimum shoot proliferation. In our case, optimum BA level for cv. Sweet charlie was 1.0 mg/l gave better results, Lopes et al. [94] indicated that the use of low BA concentrations on strawberry has been recommended by several authors, since it decreases the risk of phenotypic abnormalities after the field establishment of micropropagated plants [154].

In that concern, the use of 0.5 mg/l BA and 0.5 mg/l IBA was reported for shoot proliferation of strawberry. Singh and Pandey [155] reported that half strength MS medium supplemented with 1 mg / l BA and 1 mg/l IBA produced the highest number of shoots (13 per explant). Kaushal et al. [156] found that callus of strawberry differentiated into shoots after transferring to MS medium supplemented with 0.5 Kin + 2 mg/l BA + 0.25 mg/l NAA. Well developed shoots were transferred to multiplication medium containing 0.5 mg/l Kin + 0.5 mg/l BA and 1.0 mg/l GA$_3$. Weifeng et al. [157] revealed that Kin used together with BA for adventitious bud induction gave better results than Kin alone, but, Waithaka et al. [158] revealed that axillary dormancy was released by including a large level (50 μM) of Kin in the culture medium.

The results can be explained on the basis that different plants and even different organs of the same plant are characterized not only by their unique intrinsic biochemical make-up but also by the sensitivity of the endogenously supplied chemical stimuli.

Economically, the division within isolated buds is not very easy; it takes a lot of time. Therefore, after one or two transfers on proliferation medium, the cluster are now divided more roughly into four to five small tufts of buds. To maintain a high proliferation rate, the transfers must be done immediately after the appearance of the first roots. In this way, the proliferation rate is about four- to fivefold per month, i.e., several million from one meristem in only one year [146].

Lopes et al. [94] explained that the number of subcultures (3-8) did not affect productivity; in accordance with another observation they pointed out that problems arise when using material with over 10 subcultures.

4.5. Rooting Stage

4.5.1. Effect of Different Strengths of MS Medium

The results on the effect of MS strengths on root number and root length indicated that, highest response was obtained with full MS followed by 1/2   MS, but 1/4 and 1/8 MS came in the second order (Figure 7). In case of full MS the root number, root length and shoot elongate was high response, but 1/2 MS the root number was high but root length and shoot elongate was less than full MS.

Lower responses were obtained with the 1/4 and 1/8 MS with respect to root number and root length, but the shoots remained too short for rooting.

Figure 7: The effect of different strengths of MS, 1/8, 1/4, 1/2, and full strength of MS medium on rooting response after 4 weeks, highest response was obtained with full MS followed by 1/2 MS, but 1/4 and 1/8 MS came in the second order.

4.5.2. Effect of Different Concentrations of Activated Charcoal

The effect of different concentration of AC on root number and root length revealed that the highest response was obtained with 0.5 mg/L, similar to full MS, all other concentration showed lower effect in elongation of both the roots and shoots.

4.5.3. Effect of Different Concentrations of IBA

Regarding the effect of IBA on the root response, the results indicated that the IBA with (0.5, 1.0, and 1.5 mg/L) showed the lowest roots response compared to all other treatments. These findings are somewhat similar to those previously reported by Emara (2008). [36] who studied the effect of MS strengths on root length, who reveal that the highest significant record was obtained with full MS, all other strengths showed lower effect.

Induction of roots at the base of *in vitro* grown shoots is essential and indispensable step to establish tissue culture derived plantlets to the soil. The most effective auxins for rooting are IBA and NAA [18].

In that concern, some investigations did not recommend full MS for strawberry rooting, Kaushal et al. [156] found that rooting of strawberry was done in MS half strength with 1.0 mg/l IBA and 0.2 mg/l activated charcoal. The same strength was recommended for strawberry rooting by Yonghua et al. [139] who reported that half strength MS containing 1.0 mg/l $AgNO_3$ was optimum medium for rooting. Kikas et al. [96] found that high salt concentration of MS promoted active proliferation but the shoots remained too short for rooting, and with low salt concentration of MS, the proliferation rate was lower but shoots elongated enough. Gautam et al. [159] indicated that the highest root induction frequency obtained was 95.23% on 1/4 MS medium with IBA at 1.0 mg/l and charcoal (200 mg/l).

However, Lal et al. [153] found that maximum rooting was obtained in both full and half strength MS medium supplemented with IBA at 1.0 mg/l. Mereti et al. [160] found that the highest percentages of rooting were achieved in MS medium contained 10 µM IBA (92%) and 10 µM IAA (82%).

Passey et al. [115] indicated that the high levels of regeneration obtained from leaf discs (100% with Calypso and Tango), they placed the shoots on rooting media R13 containing 3.0 mg/L IBA for 3 days under the growth conditions and the shoots were then transferred to root elongation media R37 contains half-strength MS salts.

Boxus (1999), [146] revealed that when shoots tips of strawberry were cultured on a medium supplemented with 0.5 mg \L BA, 0.1mg \L and 6.4g \L agar, shoots were multiplied and subsequently rooted *in vitro* on the same medium without BA and GA$_3$.

In that concern, Kikas et al. [96] found that high salt concentration of MS promoted active proliferation, but the shoots remained too short during rooting period, but with low salt concentration of MS, the proliferation rate was lower but shoots elongated enough. Damiano et al. [140] found that the addition of 1 or 2 g of activated charcoal per liter to MS medium promotes elongation of both the shoots and roots of strawberry.

Nissen & Sutter [87] found that increasing the concentration of auxin promoted rooting without callusing, due to an increasing concentration of activated charcoal, which had an effective role in absorption of some of the medium components.

Root formation and growth were better in BA-free medium containing activated charcoal than in the medium without activated charcoal. Similar results were observed in bulb formation. This could be due to activated charcoal regulating internal physiological processes. It is also possible that a substance(s), which antagonizes cytokinin activity, was adsorbed by activated charcoal [83]. Other research has shown that activated charcoal had an effective role in absorption of unknown components, which were produced through chemical processes within the media. Sometimes these unknown components played the role of growth inhibitor at morphogenesis stages. In addition, the activated charcoal, which eliminated light and provided a reasonable physical environment for the rhizosphere, prevents undesirable callusing and helps rooting [87].

The poor root stimulation in our experiment may possibly be due to a hormonal imbalance caused by accumulation of auxin in leaf tissues. Contrary to our observation, in other genotypes of strawberry (Borkowska, 2001) [161] who studied the micropropagation of strawberry cultivars Senga sengana, Kent and Kama were rooted by a medium supplemented with 1.0 mg/L IBA. These possibility are somewhat similar to those previously reported by Nehra et al. [134] whereby it was noted that the calli from *in vitro* strawberry cv. Redcoat leaf explants had very poor shoot regeneration because the hormonal imbalance due to maintained the explant on a high cytokinin medium prior to explant preparation.

In our results, we think that different concentrations of IBA were necessary to optimize roots regeneration; these results are similar to those obtained in various other studies ([103, 162] for strawberry). Those researchers found that different concentrations of growth regulators were necessary to optimize regeneration from both types of explants.

Sometimes the organic potential of explants from *in vitro* culture shoots compared with those taken from glasshouse grown plants was significantly different for the same media. Saifullah et al. [121] observed that maximum shoots regeneration from *in vitro* was found on regeneration media containing BAP/IBA 2.25/1.0 mg/l whereas, glasshouse grown explants gave maximum shoots on medium containing BAP/NAA 2.25/0.1 mg/l. They explained that the poor shoots regeneration on leaf explant taken from *in vitro* shoots may possibly be due to a hormonal effect between NAA and IBA interaction caused by accumulation of IBA in the leaf tissue.

It is notable that using a full strength MS media free hormone on establishment and rooting stage of strawberry give us optimal results. We think that the hormones which present in the strawberry plant play an important role in shoot and roots stimulation. These possibilities are somewhat similar to those previously reported by Jammaly et al. [37] whereby it was noted that the six cytokinines and two auxins were isolated and identified in axillary shoots in strawberry. And they observed that Benzyladenine and riboside were the major cytokinines whereas the free IAA and its conjugate IAA aspartate showed higher concentrations in axillary shoots that means the amount of hormones inside the strawberry tissues gave optimal growth conditions in shooting and rooting response.

Actually, we had hoped to achieve this research as completely, but unfortunately the Israeli air force bombed the lab building at the Islamic university in 27-12-2008. Thus, this is caused demolishing the building completely as well as the special lab of tissue culture. As a result, there is a problem to repeat the experiment or to complete it. Even though, this action happened, but we would reach to rooting stage which is considered as a progress stage in this research. Finally, in this stage we wrote down data visually, but we couldn't take data and measurements which prove experiments procedures which lead us to the statistic analysis.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

This study illustrates a successful micropropagation system for strawberry cv. Sweet Charly plants and examined the application of micropropagation protocols to assist the production of strawberry mother plants with high quality and resistance to common fungal and bacterial diseases. For *in vitro* propagation of strawberry cv. Sweet Charlie, Key factors were investigated in this study include sterilization technique to establish contamination free culture, control of phenolic exudates in explant preparation. The selection of appropriate growth regulator levels to achieve successful *in vitro* regeneration and the selection of appropriate growth regulator levels and some supplemented levels to achieve the optimal rooting.

One of the most commonly encountered problems in *in vitro* culture establishment is the contamination, the maximum aseptic cultures was obtained when the runner tips were immersed in sodium hypochlorite solution (1.5%) containing two to three drops of 'Tween 20' per 100ml for 20 minutes and then in 0.1 % $HgCl_2$ for 10 min gave the maximum aseptic cultures and maximum explant browning in except the treatment with 1.5% Sodium hypochlorite which gave the better results, especially, when the explant treatment with 1% and 2% PVPP for 1-2 h before culture, the browning percentage were 80 and 40% respectively, and survival percentage were 20 and 60% respectively in August season. However, in October season all the samples were affected with contamination.

In spite of the low number of clean cultures obtained, micropropagation still remains the best alternative for the rapid bulking of promising varieties whilst maintaining genetic stability of the clone.

Further reduction in bacterial contamination can be achieved by growing mother plants, ideally originating from tissue culture or from nurseries and in a dry, clean environment, preferably a glasshouse. Overhead watering should be avoided and routine pesticide treatment and fertilization should be practiced, and can be obtained in specific season.

The apical bud is defined as the bud located immediately below the apical meristem and enclosed within the leaf sheath. Selecting these young tissues makes it possible to reduce infection since the apical zone displays better aseptic conditions because of the reduced

size of the explant and the small area exposed to the external environment. Taylor and Dukic [163] reported that when apical buds of sugarcane were used, contamination with saprophytic micro-organisms was less (20% to 40%) than in axillary buds (>90%). Since these buds are morphologically less developed than axillary buds, it is less likely that the vascular tissue or bud scales are colonized by saprophytic fungi, yeast and bacteria.

This study found that tissues of experimental plant were high in phenolic compounds. The oxidation of tissues was severe and proved deleterious to all tissues in the initial stages of explant preparation. So, for reduction of phenolic compounds explants were pre-soaked in antioxidant solution of 1% or 2% PVPP for 1-2 h prior to inoculation with addition of (0.2g/100ml) PVPP in the medium and subjecting cultures cv. Sweet charly during the initial ten days in cold treatment before transfer the explants to growth chamber. The effect of the treatments outlined above proved beneficial to explant survival *in vitro* of strawberry cv. Sweet Charlie runner tips.

To study the effect of various pretreatments on explant browning and survival percentage, the highest percentage were observed in the control and 2% PVPP compared with 1.5% and 1% PVPP respectively, survival percentage were low.

For successful explant establishment, the runner tips apices were cultured for 4 weeks on MS basal medium free hormone, the runner tips starts swilling and about two weeks later, the excised meristem tips have developed into a rosette of leaflets.

For successful explant multiplication, within 3 - 4 weeks after this excision, transfer to MS propagation media supplemented with 1mg/l BA hormone, two or three new buds will appear at the base of the petioles of the oldest leaves (within 3 - 4 weeks after this transfer). After 6 – 8 weeks the axillary buds invade the base of the petioles of the oldest leaves.

For successful rooted plantlets, the highest response was obtained when transfer the buds to the MS medium full strength and to MS medium supplemented with activated charcoal (0.5 g/l), all two media without hormone.

Present investigations have thus shown that the economically valuable strawberry cv. sweet charly could be regenerated *in vitro* via organogenesis. The protocol developed will be useful for rapid *in vitro* propagation of the species and also for the subsequent genetic manipulation studies.

5.2 Recommendations

Although, the tissue culture is new tool for plant propagation in our country, it is used in the developed countries many years ago for its economical importance. To establish this method on true basics must follow the following:

1. Establishing a special lab concerning with tissue culture equipments which comprise of success of experiments coming true.

2. Establishing a special greenhouse concerning with tissue culture that is treated by the true ways to facilitate sterilization methods and avoiding contamination problems.

3. The use of tissue culture technique in a comprehensive way including the importance plants in our country, such as potatoes and tomatoes etc…

4. The use of various methods with tissue culture through out the continuing experiments, we need a help from people those, who are specialized in this method, for example, the culture by use variety organogenesis, callus induction and embryogenesis.

5. The tissue culture methods must support different techniques for the genetic analysis unit (Genotyping) by using molecular marker to recognized and sure that seedling are true to type and without somaclonal variation.

For strawberry tissue culture planting in this research we recommend the following:

1. Continuing experiments procedure to reach to the final stage which we couldn't do it and that is seedling production able to acclimatization without side environment and without any troubles.

2. Concentrating on writing down notes culture procedures step by step to reach for optimum ways in doing experiment and saving a lot of efforts, costs and avoide the negative results.

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