IN VITRO PLANT REGENERATION OF STRAWBERRY (*Fragaria x ananassa* Duch.) FROM DIFFERENT EXPLANTS

A THESIS

BY

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STUDENT NO.:1105069
REGISTRATION NO.: 1105069
SEMESTER: January- June, 2013
SESSION: 2011-2012

MASTER OF SCIENCE (M.S.)
IN
BIOTECHNOLOGY

DEPARTMENT OF GENETICS AND PLANT BREEDING

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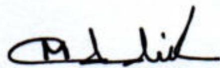

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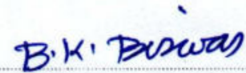

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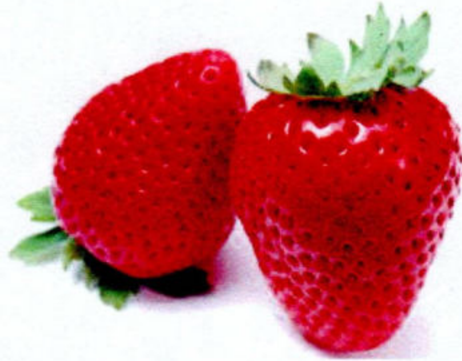

DEDICATED
TO MY
BELOVED PARENTS

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ABSTRACT

The present study was undertaken in the Tissue Culture Laboratory, Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur during the period from January to May, 2012, for callus induction and plantlet regeneration in Strawberry (*Fragaria x ananassa* Duch.). Leaf discs derived from two months old strawberry plants were cultured on MS media and supplemented with BAP. The cultures were incubated for 4 weeks in dark followed by another 4 weeks under 16/8 hr light regime. The effects of different concentrations of BAP (0, 1.5, 3.0 and 6.0 mg $L^{-1)}$ on callus induction were investigated. Among the concentrations 3.0 mg L^{-1} BAP showed the highest percentage (93.33%) of callus induction. To regenerate shoots, the calli derived from leaf discs were cultured on shoot induction media containing different combinations and concentrations of BAP (0, 1.5, 3.0 and 6.0 mg L^{-1}) and GA_3 (0.5, 1.0, 1.5 and 2.0 mg L^{-1}). The highest percentage of shoot regeneration (93.33%) and number of shoots (15.00) per leaf disc was found to be induced on the MS medium supplemented with 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3. The highest (83%) percentage of rooting in shoots was observed in MS medium in combination with 1.5 mg L^{-1} GA_3 and 1.0 mg L^{-1} IBA.

ABBREVIATIONS AND ACRONYMS

2,4-D	:	2,4-diclorophenoxyaceticacid
$AgNO_3$	:	Silver Nitrate
BA	:	Benzyl adenine
BAP	:	6-Benzylaminopurine
HSTU	:	Hajee Mohammad Danesh Science and Technology University
CRD	:	Completely Randomized Design
CV	:	Coefficient of variance
DMRT	:	Duncan's Multiple Range Test
GA_3	:	Gibberellins
HCL	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
IAA	:	Indoleaceticacid
IBA	:	Indolebutyricacid
KIN	:	Kinetin
LSD	:	Least Significant Difference
MS	:	Murashige and Skoog
NAA	:	α-naphthaleneaceticacid
NaOH	:	Sodium Hydroxide
pH	:	Negative logarithm of hydrogen ion (H^+) concentration
TDZ	:	Thidiazuron
O^0 C	:	Degree Celsius
ANOVA	:	Analysis of variance
BARI	:	Bangladesh Agricultural Research Institute
BAU	:	Bangladesh Agricultural University

BBS	:	Bangladesh Bureau of Statistics
cm	:	Centimeter
cv.	:	Cultivar
d.f.	:	Degrees of freedom
et al.	:	et alli (and other people)
etc.	:	Etiology centra (L.) and others
g	:	Gram
g/L	:	Gram per liter
ha	:	Hectare
hr	:	Hour
i.e.	:	That is
j.	:	Journal
Kg	:	Kilogram
mg L^{-1}	:	Milligram per Liter
mm	:	Millimeter
No.	:	Number
Sp.	:	Species
Univ.		University
UV	:	Ultra violet
Var.	:	Variety
Viz.	:	Namely

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CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

The strawberry (*Fragaria x ananassa* Duch.) is an important commercial fruit grown worldwide. *Fragaria* is a member of the Rosaceae family. Strawberry contains more vitamin C than orange .It is recommended for folic acid, no fat, no cholesterol and is considered high in fiber. The fruits are rich in bioactive phytochemicals, especially phenolic compounds with high antioxidant, and as a part of daily diet, it could be beneficial for human health (Hannum, 2004). Strawberry is a popular fruit eaten raw or used in making juice, desserts, jam, syrup and wine. It has been commercially cultivated in USA, Canada, Japan, Spain, Germany, Korea, Italy, Poland, Thailand and so many other countries in the world. Strawberries are now getting popularity in Bangladesh for its nutritional value and market demand. 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. 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.

One of the best loved fruits in many parts of the world are strawberries. Indeed, people from all walks of life truly enjoy the venerable strawberry. Strawberry belongs to the genus *Fragaria* in the Rosaceae family is one of the most important fruit plants for both fresh consumption and food processing in the temperate and subtropical areas. According to nutrient database for standard reference the strawberry fruits are rich in vitamin C, B1, B2, protein, calcium, potassium, copper and iron, most of the nutritious elements essential for human being (Chieng-Ying *et al.* 2009).

There are about 20 recognized species of strawberries in five chromosome groups ($x = 7$): ten diploids, four tetraploids, one pentaploid, one hexaploid and four octoploids (Staudt 1999; Jiajun *et al*. 2005). The cultivated strawberry is an octoploid ($2n = 8x = 56$). Flavourful and nutritious, strawberries are enjoyed by millions of people in all climates, including temperate, Mediterranean, sub-tropical and taiga zones (Hancock *et*

al. 1991) 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 (Childers 1980). The berry is valued for its low-calorie carbohydrate and high fiber contents.

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. 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.

Regeneration protocols of strawberry are species specific to their regeneration capacity (Passey *et al.,* 2003). No two plants share the same results when regeneration experiments are performed. Selection of the proper hormone combination, explants, and cultivar are the keys to successful regeneration of strawberry (Barcelo, 1998; Jimenez-Bermudez, 2002; and Passey *et al.,* 2003). The direct regeneration without intervening callus phase may provide useful alternative to the tedious, time consuming meristem culture. In this regard, direct shoot regeneration from strawberry leaf disks was described by several authors (Hassan, 1996 and Sutter *et al.;* 1999). Several reports indicated the possibility of *in vitro* regeneration of strawberry microplants via callus or cell suspension culture or another culture (Svensson and Johansson, 1994). Passey *et al.* (2003) studied

seven commercial cultivars of strawberry using leaf disks, petioles, roots, and stipules as explants material.

Leaf tissue of strawberry has been studied and shown to have the greatest regeneration capacity (Jelenkovic, 1991; Nehra *et al.*,1990; Passey *et al.*, 2003 and Popescu *et al.*, 1997).Callus production is also more prolific from the leaf tissue . Calluses induced from leaf disc explants of *in vitro* grown plants exhibited higher regeneration compared to those induced from greenhouse-grown plants (Khan and Spoor, 2004). The leaf discs from young greenhouse grown stock plants had a higher regeneration rate than older plants (Nehra *et al*, 1990). Explants collected during the months of May and June were the most suitable for *in vitro* propagation. Different hormonal combinations and leaf disc explants sources influence the number of regenerated plants (Adak *et al.*, 2001). A pretreatment in darkness is vital for callus induction and plantlet regeneration (Popescu *et al.,* 1997). Therefore, regeneration of strawberry is influenced by explants, hormonal combinations, light and season of the crop growth.

Plant biotechnology, a modern technique is mainly based on plant cell culture. Regeneration of plant from cell or tissue is an important and essential compound of biotechnology, which is required for the genetic manipulation of plants. Plant cell culture has become an excellent method for plant cell differentiation as well as a supplementary technique for plant breeding programs through the uses of new and expanded genetic variability (Nakamura and Maeda, 1989). To get disease free healthy plant materials it is very urgent to develop a protocol for *in vitro* propagation of strawberry.

In spite of plenty of information on tissue culture studies elsewhere in the world, no reports of studies are available in Bangladesh. That's why, to achieve this goal we have to look for suitable germplasms followed by developing varieties as well as rapid multiplication through tissue culture.

The objectives of present study was

i) To establish an efficient regenerative protocol from leaf discs of strawberry in the shortest possible period.

ii) To determine the optimum concentrations of growth regulators for *in vitro* regeneration in strawberry and

iii) To select desirable plantlets for commercial cultivation.

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

The use of plant tissue culture technique has been proved as a useful tool for mass propagation of several crops. The technique is important especially for clonal multiplication of virus-free stock plant materials is strawberry. For this purpose, leaf discs culture is normally utilized for true-to-type propagation of strawberry plant. The literature relevant to the present study have been reviewed below-

2.1 Concept of Plant Tissue Culture

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including:

1. The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits. 2. To quickly produce mature plants. 3. The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds. 4. The regeneration of whole plants from plant cells that have been genetically modified. 5. The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens. 6. The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and Nepenthes. 7. To clean particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

2.2. 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.

The alpine strawberry, *F. vesca*, is the most geographically widespread. Cytogenetic studies indicate that this species may be a diploid progenitor of the octoploids 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). Taxonomic characteristics include obovate subglaucous 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 Mountains, 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. 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: 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. 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) 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 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. suggested that ssp. *glauca* and ssp. *Platypetala* completely intergrade and should be referred to as a single taxa var. glauca. Hokanson et al. 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. 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) recognized these hybrids as *F.* × *ananassa* nm. cuneifolia (Nutt. Ex Howell). Many of the dessert strawberry.s traits are intermediate to it parents. Plants have large fruit, high yields, and vigor.

2.3 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.

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.

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.

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).

2.4 In vitro regeneration of Strawberry

Otroshy Mahmoud *et al*. (2013) were investigate in the current work attempts made to culture of different explants derived from in vitro seedlings of Fragaria species. Effects of explants on regeneration response of strawberry were examined based on four

parameters, include number of shoot per explants, and shoot length per explants, number of root per explants and percentage of rooting. The best response for callus induction was observed for leaf explants was obtained on MS supplemented with BAP 2 mg/l and NAA 0.5 mg/l. After 4 weeks, the highest callus growth transferred in same medium callus induction for direct regeneration that was BAP 2 mg/l and NAA 0.5 mg/l. The highest efficiency and high frequency of shoot formation occurred after 30 days. Elongation of shoot buds was obtained on MS medium containing 2 mg/l BAP + 0.1 mg/l NAA. Regenerated shoots rooted best on the same medium of elongation. The regenerated plantlets have been successfully established in pit mass soil. Subsequently, they were shifted to the field conditions. Regenerated plantlets resemble the parent plants morphologically and cytological. Histological study of the cultures showed the presence of meristemoids, somatic embryos and embryoids. We concluded that the present protocol can be efficiently used for mass propagation of the strawberry.

Omar *et al.* (2013) were observed somatic embryo-like structures (SELS) were produced *in vitro* from leaf disk and petiole explants of two cultivars of strawberry (Fragaria × ananassa Duch) on Murashige and Skoog medium with different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and sucrose to check the embryonic nature of these structures histologically. A large number of SELS could be regenerated in both cultivars on media with 2 - 4 mg L-1 2, 4-D in combination with 0.5 - 1 mg L-1 BAP and 50 g L-1 sucrose. Histological examination of SELS revealed the absence of a root pole. Therefore these structures cannot be strictly classified as somatic embryos. The SELS formed under the tested culture conditions represent malformed shoot-like and leaf-like structures. The importance of these results for the propagation of strawberries via somatic embryogenesis is discussed.

Tanziman Ara *et al.* (2012) was achieved a simple and rapid protocol for micropropagation of strawberry (*Fragaria* x *ananassa* Duch.) using runner tips and nodal segments. The excised runner tips and nodal segments were cultured on MS medium containing 6-benzylaminopurine, 6-furfuryl amino purine, indole-3-butyric acid and gibberellic acid at various levels of concentration and combination for multiple shoot induction and proliferation. The highest percentage of shoot proliferation was found when both explants (92% from nodal segments and 83% from runner tips) were cultured

on MS medium supplemented with 1.5 mg/l 6-benzylaminopurine + 0.5 mg/l 6-furfuryl amino purine. Shoots were rooted most effectively in MS0 medium. Nodal segments were found more responsive explants than runner tips for rapid clonal propagation in strawberry.

Moradi K. *et al.* (2011) was carried out an experiment to examine the effects of different combinations of plant growth regulators *in vitro* micropropagation of strawberry. Strawberry of the Rosaceae family, is a perennial, stoloniferous herb and popular in genetic engineering. The present study showed the procedure for propagation of *Fragaria* using nodal segments from *in vitro* germinated plants. The best concentration of BAP for bud induction was 0.5 mg/l plus KIN 0.2 mg/l. Histochemical analysis showed that only a direct induction of more teratological protuberances that arise around the cut end of the explants. The maximum number of roots per explant was obtained in elongation medium in MS combined with BAP 0.1 mg/l with auxin IBA 0.2 mg/l. In this medium, all of them the seedlings had safe and excellent rooting. The rooted plants were transferred to the greenhouse condition where they normally grew, matured and flowered with a survival rate of 100%. It is concluded that the present protocol can be efficiently used for mass propagation of strawberry.

Valentina Isac *et al. (2010) was* described the use of tissue culture in the regeneration and commercial propagation of economically important plants is a comparative recent and radical development. Advances in biotechnology provided new methods for rapid production of high quality, disease-free and uniform planting material. Biotechnological tools like in vitro culture and micropropagation offer a valuable alternative in fruit trees propagation studies, virus control and management of genetic resources. At the Research Institute for Fruit Growing Pitesti the technique of in vitro micropropagation was employed since 1975. Its objectives were virus elimination from certain strawberry cultivars by meristem culture, rapid propagation under aseptic conditions and in vitro preservation of strawberry germplasm. Subsequently, the tissue culture research was extended too many other fruit species, such as blackberry, raspberry, currant, gooseberry, apple, pear, quince, plum, sweet cherry, and sour cherry. The applied research was focused on the improvement of in vitro technologies for fruit and ornamental species, oriented mainly towards optimization of the culture media and shortening the period for producing in vitro plants at low costs. Based on the results of tissue culture research and

advances in application of *in vitro* techniques, a range of micropropagation technologies were established, which enabled high quality and efficient plant production at commercial scale. Remarkable achievements have been made also in the medium and long-term in vitro maintenance of small fruit germplasm under cold storage conditions. In this review we will summarize advances in the application of plant tissue culture to fruit tree species, and highlight the achievements made in the last 25 years at the RIFG Pitesti in meristem culture, micropropagation, and germplasm preservation.

Chien-Ying Ko *et al.* (2009) was developed an effective method of disinfection protocol and micropropagation with an enhanced survival rate of explants and reduced phenol induced browning in strawberry. The survival rate of three genotypes was between 89.2 - 100%. Shoot tip were able to develop into plantlet on a hormone-free MS medium when cultured under dim light (500 lux). Two media, M1 and M2 were chosen to compare the effect on shoot multiplication of three genotypes. The shoot number per responding explants was between 5 - 8 and 11 - 14 after induction.

Mohamed *et al.* (2007) conducted an experiment where Leaf discs derived from either two-month-old greenhouse-grown (GHD) strawberry plants or *in vitro* plantlets were cultured on MS media amended with 2 mg L^{-1} TDZ, incubated for 4 weeks in the dark then for another 4 weeks under 16/8 h light regime. The regeneration capacity of leaf discs was compared to meristem-derived propagules in 6 strawberry cultivars, viz. Carlsbad, Camarosa, Chandler, Gaviota, Seascape, and sweet Charlie. Direct shoot regeneration occurred in all tested cultivars with different frequencies depending on explant source. From GHD leaf discs, the cultivars Camarosa, Gaviota and Seascape produced the highest number of shoots $explant^{-1}$ (38, 31 and 31 shoots, respectively). However, optimum number of shoots $explant^{-1}$ from *in vitro* leaf discs was achieved in the cultivars Carlsbad, Chandler and Sweet Charlie (13.3, 12.6 and 12.3 shoots, respectively). In general, regeneration capacity of GHD leaf discs was more than two-folds of that obtained from *in vitro* leaf discs.

Zhou *et al.* (2007) established an efficient and robust method for shoot regeneration of leaf discs of strawberry cv. Sachinoka. Five-week-old leaves, dark treatment for 5-6 weeks and addition of 1-4 mg L^{-1} $AgNO_3$ to the medium increased the shoot regeneration rate of the explants. The regeneration rate of strawberry cv. Sachinoka

reached the highest level of 98.9% with 3.8 shoots per leaf disc when cultured in MS medium supplemented with 4.0 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA after dark treatment for 6 weeks.

Sakila *et al.* (2007) used nodal segments of strawberry which gave multiple shoots when cultured on MS medium supplemented with different concentrations of BA with KIN or GA. The highest response of shoot multiplication was obtained in MS containing 1.5 mg 1G1 BA + 0.5-0.1 mg 1G1 KIN. The regenerated shoot lets 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 1G1 IBA. The planlets, thus developed were hardened successfully established in soil. The plants raised through tissue culture exhibited normal growth, flowering and fruit setting.

Debnath *et al.* (2007) studied 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, germplasm improvement and for gene conservation of this flavourful and nutritious berry crop. The *in vitro* propagation of *Fragaria* species using axillary bud proliferation, adventitious shoot regeneration and somatic embryogenesis has been investigated in a number of previous studies. The morphogenesis seems to be highly dependent on plant growth regulators and media used for culture, which is again genotype specific. In strawberry, genetic transformation has been developed using tissue culture systems with varying rates of success. This review presents the progress in-depth of various aspects of strawberry culture *in vitro*, on gelled and in liquid media using bioreactors, for its improvement and for commercial production. It also discusses the issues that still need to be addressed to utilize the full potential of plant tissue culture techniques in mass propagation, *in vitro* selection, somaclonal variation, haploid recovery, somatic hybridization, genetic transformation and in cryopreservation of strawberries. Application of molecular marker techniques should be useful to verify the clonal fidelity of micropropagated strawberries. Strawberry improvement using *in vitro* and molecular techniques will develop improved cultivars suited to the changing needs of growers and consumers.

Biswas *et al.* (2007) studied 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 and among them 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.

Qin *et al.* (2005) established an efficient and reliable method for shoot regeneration from leaf discs of strawberry (cv. Tayonoka). The effects of plant growth regulators (0.5, 1.0, 2.0 or 2.5 mg L^{-1} TDZ; 1.5 mg L^{-1} TDZ + 0.2, 0.4, 0.6, 0.8 or 1.0 mg L^{-1} IBA; and 1.5 mg L^{-1} TDZ + 0.4 mg IBA + 0.2, 0.4, 0.6, 0.8 or 1.0 mg L^{-1} 2, 4-D), coloured plastic films and darkness (for 1, 2, 3, 4 or 5 weeks) on shoot regeneration were studied. The optimum medium for shoot regeneration was the MS medium supplemented with 1.5 mg L^{-1} TDZ and 0.4 mg L^{-1} IBA. The highest percentage of shoot regeneration (100%) and number of buds per explant (9.05) were obtained through the exposure of explants to darkness for 4 weeks.

Debnath (2005) was developed an efficient system to *in vitro* regenerated shoots on excised sepals (calyx) of greenhouse-grown 'Bounty' strawberry (*Fragaria x ananassa* Duch.). Sepal cultures produced multiple buds and shoots without an intermediary callus phase on 2 to 4 μM TDZ containing shoot induction medium within 4-5 weeks of culture initiation. Young expanding sepals with the adaxial side touching the culture medium and maintained for 14 d in darkness produced the best results. In a second experiment, sepals proved more effective than the leaf discs and petiole segments for regenerating shoots. A third experiment compared the effects of six concentrations of two Cytokinins (TDZ at 0, 0.5, 2, and 4 μM and zeatin at 2 and 4 μM) for elongation of sepal-derived adventitious shoots. The media containing TDZ generally promoted more callus formation and suppressed shoot elongation. TDZ-initiated cultures transferred into the medium containing 2-4 μ M zeatin, produced usable shoots after one additional subculture. 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.

Wu *et al.* (2004) was established an ideal regeneration system of strawberry (*Fragaria ananassa*) *cv.* Toyonoka by leaf explants. The effects of plant growth regulators, dark periods and $AgNO_3$ concentrations on shoot regeneration from strawberry leaves were investigated. The highest regeneration rate of 72.33%, with 5.59 shoots per leaf disc, was

obtained on the MS medium supplemented with 2.0 mg L^{-1} TDZ and 0.8 mg L^{-1}IBA. TDZ was more effective than BA in inducing shoot regeneration from Toyonoka leaves. Two weeks of dark treatment could increase the shoot regeneration rate to 90.09%.

Khan and Spoor (2004) was evaluated the potential of an *in vitro* callus culture and regeneration system in strawberry cv. Tango. The effects of different hormonal combinations on leaf disc explant sources required to produce the highest number of plants in the shortest time were also determined. The effects of BA, IBA, NAA, and 2, 4-D on callus initiation and regeneration were also studied. BA at 2.25 mg L^{-1} in combination with the 0.18 mg L^{-1} NAA or 1.0 mg L^{-1} IBA increased shoots regeneration. Culture media containing 2, 4-D and BA (1:1) resulted in callus production while a higher 2, 4-D: BA ratio produced a substantial number of shoots in greenhouse-grown leaf disc explants (less than 0.5 per leaf disc).

Yuan *et al.* (2004) conducted an experiment where Strawberry (cultivars Darselect and Tongzi No. 1) leaf disc explants (3-5 mm^2) were cultured on MS medium containing different growth regulators at 25+or-2 degrees C and 16 h light (8 h dark) with a light intensity of 2000 Lx. Results showed that the induction rates for adventitious buds of Darselect and Tongzi No. 1 in media with 3.0 mg L^{-1} 6-BA +0.1 mg L^{-1} 2,4-D reached 94 and 60%,respectively; sole use of 2,4-D had no effect, so it must be combined with 6-BA. Amongst the 6-BA combinations with other growth regulators, the induction rate for adventitious buds cv. Darselect with 2,4-D was higher than with (IAA) and indolebutyric acid (IBA), whereas IAA was of benefit to cv. Tongzi No. 1.The induction rate for Tongzi No. 1 with 1.0 mgL^{-1} TDZ reached 80%, but the effect of TDZ on cv. Darselect was inferior to that of 6-BA. Kinetin used together with 6-BA for adventitious bud induction gave better results than kinetin alone.

Sukhjit and Chopra (2004) were used runner tips as explant on MS medium. The MS medium supplemented with 6-BA at 0.5 mg L^{-1} and IBA at 0.10 mg L^{-1} was used for shoot multiplication. Rooting was obtained on the same basal medium fortified with IBA at 1.00 mg L^{-1} alone. Micropropagated strawberry plants were hardened in polyethylene bags and plastic trays with 98 built-in small pots. The maximum survival during hardening was observed in polyethylene bags filled with soil and farmyard manure in a 1:1 ratio.

Berljak *et al.* (2003) conducted an experiment where somatic tissue excised from *in vitro* multiplied strawberry plants were tested on ability for plant regeneration. Leaves, petiole and stipules were inoculated on initial medium with BA and 2,4-D, or on medium with BA only after 1 hour pulse treatment with 2,4-D. Callus was induced on all sliced surfaces of explants inoculated on initial medium with growth regulators BA and 2,4-D during first 7 days of culture. Explants inoculated on initial medium with BA, after pulse treatment with 2, 4-D did not develop callus but abundantly produced fenolic compounds, and tured necrotic in the first 24 hours. Spontaneous plant regeneration was noticed on leaves explants with less developed callus tissue on initial medium with growth regulators during second week of culture. High percentages of explants with regenerated shoots were obtained after transfer on hormone-free medium. The highest plant regeneration ability was in leaf tissue, less in petiole and stipules. Callus induced in leaf tissue showed ability for constant plant regeneration during three months of culture and careful 4-week interval transfer on basal MS medium with 4.4 μ M BA and 40 g/l sucrose

Murari *et al.* (2003) described an efficient method for shoot regeneration, proliferation and rooting from runner tip explants of strawberry cultivars Chandler, Oso Grande and Ofra on MS and Knop's media was developed. Ofra recorded the maximum shoot regeneration (100%) after 7 weeks of incubation on recorded the maximum shoot regeneration (70%) after 7 weeks of incubation on Knop's medium supplemented with IBA at 4.0 mg L^{-1} +BAP at 0.4 mg L^{-1} +GA_3 at 0.4 mg L^{-1}. In all cultivars, the maximum number of shoots per explant was observed in MS medium supplemented with BAP at 4.0 mg L^{-1}. Ofra recorded the highest number of multiple shoots (13.8 + or -3.6) and range of shoots per explant (4-36) than the other cultivars. Maximum rooting of 100% was observed in both full-and half-strength MS media supplemented with IBA at 1.0 mg L^{-1}. On the other hand, maximum rooting of 60% was observed on Knop's medium supplemented with IBA at 4.0 mg L^{-1} +BAP at 0.4 mg L^{-1} +GA_3 at 0.4 mg L^{-1}.

Yin *et al.* (2003) showed that the adventitious shoots of strawberry (*Fragaria ananassa* cultivars Darselect, Xinxing No. 2, Honeoye and Tudla) were induced by different combinations of TDZ and IAA. The results showed that the shoot inducing rates of cultivars Xinxing No.2 and Tudla were 94.2 and 87.9% respectively. The numbers of shoots per leaf disc were 1.9 and 1.7 in MS + TDZ 2.0 mg L^{-1} + IAA 0.1 mg L^{-1}medium,

respectively. The regeneration frequency from Honeoye was highest (89.1) in medium containing MS + TDZ at 2.0 mg L^{-1}+ 2,4-D 0.1 mg L^{-1}, while Darselect obtained the highest level of 79.6% in medium containing MS + TDZ at 8.0 mg L^{-1}+ IAA at 0.1 mg L^{-1}. The regeneration from the disc explant was better than that from the petiole of the same genotype.

Flores et al. (2003) were determined the effects of 2, 4-D and picloram (0, 5, 10, 15, and 20 μM), and BA (5, 10 and 15 μM) on the callogenesis and morphogenesis of *in vitro* cultured strawberry cv. Vila Nova. Leaf disks were cultivated in MS salts and vitamins, myo-inositol, sucrose and agar. The culture was maintained in the dark for 3 weeks and then transferred to the growth chamber. Callus formation was observed for all auxin treatments. Increasing BA concentrations favoured the formation of compact callus. 2, 4-D was more effective for the formation of compact callus, whereas picloram favoured the formation of friable callus. Callus growth was highest with 5 μ M BA + 14.3 μ M picloram treatment. In the presence of different 2,4-D concentrations, higher intensity of callus formation was observed with 10 μ M BA + 13.6 μ M 2,4-D treatment, whereas in the absence of auxins, 10 μ M BA was superior in terms of shoot regeneration is concerned. No shoot formation on callus was observed with 2, 4-D or picloram treatment alone.

Mereti *et al*. (2003) was studied the *in vitro* rooting of strawberry in glass jars containing 70 ml of WPM media supplemented with either IBA (0, 2.5, 5, 10 micro M) or IAA (0, 10, 20 μM). The highest rooting percentages were achieved in 10 μM IBA (92%) and 10 μM IAA (82%). Equal volume (70 ml) of peat: perlite (1:4, v/v) mixture was added during *in vitro* rooting. This novel medium resulted in high survival rates and well-branched roots, in all auxin concentrations, reaching up to 90% of the acclimatization rate for 10 μM IAA.

Zebrowoska and Hortynski (2002) was examined plant regeneration from leaf explants (petioles and leaf blades) at various concentrations of BAP (0; 1.6; 3.2; 6.4 mg L^{-1}) in MS medium in clone B-302 and cultivar Kama. Leaf explants regenerated only at concentrations of 3.2 mg $L^{-1}$and 6.4 mg L^{-1} BAP in the medium. Shoot formation was better in clone B-302 than in cultivar Kama. Regeneration from petioles of clone B-302 was the best and reached 30% and 17% from leaf blades with an average of 6 and 8 shoots per explants (respectively) at 3.2 mg L^{-1} of BAP. At the same concentration of

BAP in MS medium no shoot formation was observed in cultivar Kama which regenerated only at concentration of 6.4 mg BAP. Generally, in both genotypes, regeneration from petioles was better than from leaf blades although number of plantlets per leaf blade was higher when compared with that obtained from petioles.

Karhu and Hakala (2002) observed that micropropagated strawberry plants comparatively better in different characters (crown size, number of runners, flowering time and yield of berries) than conventionally propagated runner plants. Although production of propagules through runner has been reported to contribute 90% of total Dutch strawberry production, the product in Elsanta cultivars was found to be susceptible to several fungal diseases.

Taji *et al*. (2002) described that *in vitro* techniques are important tools for modern plant improvement programs to introduce new traits into selected plants, to multiply elite selections and to develop suitable cultivars in the minimum time.

Hemant *et al.* (2001) compared between *in vitro* and *ex vitro* rooting of micropropagated shoots of strawberry. For *in vitro* rooting, micropropagated shoots of strawberry cv. Chandler were transferred to MS medium of different nutrient strengths supplemented with IBA (1 mg L^{-1}) and charcoal (200 mg L^{-1}). Highest root induction frequency obtained was 95.23% on 1/4 MS medium with shoots were prepared overnight with IBA (2 mg L^{-1}) from one week-old *in vitro* shoots. For *ex vitro* rooting, only 4 weeks time required for root production whereas, 8 weeks needed for *in vitro* rooting.

Adak *et al.* (2001) conducted an experiment on the clonal propagation of strawberry cultivars Camarosa, Chandler and Oso Grande using meristem culture techniques. The most suitable explant collection times were also determined. BA (0.5, 1.0, 1.5 or 2.0 mg L^{-1}) combined with 1 mg L^{-1} IAA and 1 mg L^{-1} GA_3 were tested during the propagation stage. IBA and NAA (0.1, 0.4, 0.8 or 1.0 mg L^{-1}) and activated charcoal (1.0, 2.5 or 5.0 g L^{-1}) were tested during rooting stage. May and June collected explants were the most suitable for *in vitro* propagation. During the propagation stage, the use of 1 mg L^{-1} BA combined with 1mg L^{-1} IAA had the highest number of shoots and quality compared to the other treatments. During the rooting stage, the use of activated charcoal was more successful than IBA and NAA for all the cultivars. In addition, 5 g activated charcoal L^{-1} had more rooting success than 1 and 2.5 g activated charcoal L^{-1}.

Wei *et al.* (2001) revealed that the most suitable medium for inducing adventitious buds in shoot tip culture of cv. Saiwa was MS medium supplemented with 0.5-1.0 mg L^{-1} BA + 0.05-0.1 mg L^{-1} NAA. The most suitable regeneration medium was MS +0.5-1.0 mg L^{-1} BA +0.05-0.1 mg L^{-1} IBA, and the most suitable medium for rooting was modified MS + 0.1-0.5 mg L^{-1} BA, which gave a rooting percentage of up to 99%.

Mesa et al. (2000) performed particle bombardment of strawberry tissue. They used *Agrobacterium*-coated gold particles and a gene gun to bombard 'Chandler' leaf tissue. After observing the explants for twenty-five weeks; they reported a 20% transformation rate.

Bhatt and Dhar (2000) described micropropagated wild strawberry (*F. indica* Andr.) using node culture. Nodal segments (2.5-3 cm), treated with Salon (an antiseptic containing 3% antimicrobial agent centrimide + detergent) for 15 min, were immersed in 80% ethanol for 30 sec prior to surface sterilization in HgCl2 (0.05%) containing few drops of Tween 20 for 5 min and then cultured on MS medium supplemented with 4 μ M BA and 0.1 μ M α-naphthalene acetic acid (NAA). The same medium was used for shoot multiplication

Sajjad Mahmood *et al.* (1994) has been reported that a micropropagation system in *Fragaria x ananassa* Dutch cv. toro by using apical meristem. A number of media combinations were tested, and it has been observed that fairly low levels of BAP enhanced growth rate. Maximum number of leaves (8) with three stolons was noted after eight weeks of culture. Root formation was observed on the same cultures with six weeks of incubation.

CHAPTER III

MATERIALS AND METHODS

The experiment was carried out during the period from January to May, 2012 at the Tissue Culture Laboratory, Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

3.1 Experimental materials

3.1.1 Plant materials

The planting material (leaf) of *Fragaria x ananassa* Duch. were collected from BARI, Rangpur, for the establishment of culture. Leaves were used as explants to fulfill the objectives of the present study.

3.1.2 Culture media

The degree of success in any technology employing cell, tissue and organ culture is related to relatively few major factors. A significant factor is the choice of nutritional components and regulators. MS (Murashige and Skoog, 1962) medium supplemented with different phytohormones as per treatments was used as culture medium for shoot induction, shoot multiplication and maintenance and regeneration of roots from multiplied shoot. The composition of MS medium has been presented in Appendix-1. Hormones were added separately to different media according to their requirements. For the preparation of media, stock solutions were prepared at the beginning and stored at $4\pm1°$ C. The respective media were prepared from the stock solutions.

3.2 Preparation of stock solutions

The first step in the preparation of the medium was the preparation of stock solutions. The various constituents of the medium were prepared into stock solution for ready use to expedite the preparation of the medium. Separate stock solutions for macronutrients, micronutrients, irons, vitamins and amino acids, growth regulators etc. were prepared and stored appropriately for use.

Table 1. The composition of MS (Murashige and Skoog, 1962) medium

Constituents	Concentration (mg/L)
a) Macronutrients (10X)	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
b) Micronutrients (100X)	
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.20
$ZnSO_4.7H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
c) Iron sources (10X)	
$FeSO_4.7H_2O$	27.80
$Na_2EDTA.2H_2O$	37.30
d) Vitamins/ Organic Nutrients(100X)	
Glycine	2.00
Nicotinic acid	0.50
Pyrodoxine HCl	0.50
Thiamine HCl	0.10
e) Myoinositol	100
f) Sugar	30000
g) Agar	9000

3.2.1 Stock solution of Macronutrients (A)

For the preparation of this stock solution, 10 times (10X) of the concerned salt required per liter of the medium was weighed accurately and dissolved in 750 ml of distilled water. The final volume was made up to 1000 ml by further addition of distilled water. The stock solution was filtered and poured into a sterilized bottle and stored in a refrigerator at 4° C temperatures for later use.

3.2.2 Stock solution of Micronutrients (B)

In preparing of micronutrients stock solution, 100 folds (100X) of the particular salt required per liter of the medium was weighed accurately and dissolved in 750 ml distilled water. The final volume was made up to 1000 ml by further addition of distilled water. The stock solution was filtered and poured into a sterilized bottle and stored in a refrigerator at 4° C temperatures for use later whenever necessary.

3.2.3 Stock solution Iron (C)

This solution was prepared at 10 folds (10X) the final strength of $FeSO_4$ and Na_2-EDTA in 100 ml of distilled water and chelated by heating on a heater cum magnetic stirrer. Then the volume was made up to 100 ml by further addition of distilled water. Finally, the stock solution was filtered and stored in a refrigerator at 4° C for use.

3.2.4 Stock solution Vitamins and amino acids (D)

Each of the desired ingredients except myo-inositol was taken at 100 times (100X) of their final strength in a measuring cylinder and dissolved in 400 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots stored at 20° C temperatures. Myo-inositol was used directly at the time of media preparation.

3.2.5 Stock solution of Growth regulators (E)

Stock solution of growth regulators were prepared separately at 100 ppm by dissolving the 100 mg quantity of ingredients in appropriate solvent and made the final volume of 1 liter with distilled de-ionized water.

The following growth regulators were used in the present investigation.

Auxins
Indolebutaric acid (IBA)
Cytokinins
6- Benzylamino purine (BAP)
Gibberellins (GA_3)

The above-mentioned hormonal supplements were dissolved in proper solvent as shown below.

Hormones	**Solvents**
IBA	70% Ethyl alcohol
BAP	0.1 N NaOH
GA_3	0.1 N HCL

To prepare a stock solution of any of these hormones, 10 mg of the hormone was taken on a clean watch glass and then dissolved in 1 ml of particular solvent. The mixture was then washed off with distilled water and collected in a 100 ml measuring cylinder and was made up to 100 ml with distilled water. The solution was then poured into a clean round bottom flask and stored at 4° C and used for maximum period of four weeks.

3.3 Preparation of culture media

After the preparation of the stock solution the next step was to prepare culture media. To prepare one liter of MS medium 100 ml of macronutrients (A), 10 ml of micronutrients (B), 100 ml of irons (C) and 10 ml of vitamins were taken into a two liter Erlenmeyer flask on a heater cum magnetic stirrer. 500 ml distilled water was added to dissolve all these ingredients. 100 mg of Myo-inositol was added directly to the solution and dissolved well. 30 g pure sucrose was added to this solution and agitated gently to dissolve completely. Different concentrations of hormonal supplements were added to the solution either in single or in combination as required and mixed well. Since 100 ml of each hormonal stock solution contained 10 mg of hormonal salts. The addition of 10 ml of any hormonal stock solution to prepare one liter of medium resulted in 10 mg/L concentration of those hormonal supplements. Similarly, for 0.5, 1.0, 1.5, 2.0, 3.0 and 6.0

ml/L concentration of the hormonal supplemented 5, 10, 15, 20, 30, and 60 ml of stock solution were added respectively. The whole mixture was then made up to 1000 ml with further addition of distilled water. pH of the medium was adjusted to 5.8 with a digital pH meter by adding of 0.1 N NaOH or 0.1 N HCL. After adjusting the pH, 9.0 g agar was added to solidify the medium. The mixture was then gently heated without boiling with continuous stirring till complete dissolution of agar. Required volume of hot medium was dispensed into culture vessels or conical flasks. After dispensing the medium the culture vessels were plugged with cork and marked with different codes with the help of glass marker to indicate specific hormonal combination.

3.4 Sterilization

To ensure aseptic condition under *in vitro*, all instruments, glass wares and culture media were sterilized by autoclaving with 15 Ibs/sq. inch (1.16 kg/cm^2) pressure at 121° C for 30 minutes.

3.4.1 Sterilization of culture medium

The glass vials containing the medium were autoclaved with 15 Ibs/sq. inch (1.16 kg/cm^2) pressure at 121° C for 30 minutes. After autoclaving, the culture vessels were allowed to cool under normal condition. All vials were marked with permanent marker to indicate specific treatment.

3.4.2 Sterilization of glassware's and instruments

Beakers, distilled water in conical flaks, Petri dishes, metallic instruments like forceps, scalpels, needles etc. were wrapped with aluminum foil; vials were capped with plastic cap and then were sterilized in an autoclave at a temperate of 121° C for 30 min at 1.16 kg/cm^2 pressure.

3.4.3 Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all floors and walls with a detergents followed by wiping with 70% ethyl alcohol and salvon; the process of sterilization was repeated at regular intervals. Generally laminar air flow cabinet was

usually sterilized by switching on the cabinet and wiping the working surface with absolute ethyl alcohol for 30 minutes before starting the transfer work.

3.4.4 Precaution to ensure aseptic conditions

All inoculation and aseptic manipulations were carried out under Laminar Air flow cabinet. The cabinet was switched on for at least half an hour before use and cleaned with absolute ethyl alcohol to overcome the surface contaminants. The instruments like scalpels, needles etc. were sterilized by alcoholic dipping and flaming method inside the Laminar Air Flow Cabinet. Other requirements like Petri-dishes, distilled water and glass wares were sterilized by autoclaving. While not in use, the instruments were kept inside the laminar air flow cabinet. Hands were also sterilized by wiping with 70% ethyl alcohol. Aseptic conditions were maintained during each operation to avoid the chances of contamination.

3.5 Culture Techniques

The following culture techniques were employed in the present investigation

3.5.1 Preparation of Explants

Two months old leaves of strawberry were first washed with tap water followed by a wash with distilled water. For surface sterilization, leaves were first sterilized with 70% (v/v) ethanol for 30 seconds. The leaves were than rinsed with sterile distilled water. Afterwards the leaves were surface sterilized by immersing in 0.1% $HgCl_2$ solution containing one drop of Tween twenty for 6-8 minutes and then washed 3-4 times with double water inside the Laminar Air Flow chamber.

3.5.2 Explants culture

Sterilized leaves were cut into strips (0.5 × 0.5 mm) avoiding the midrib and placed adaxial side down onto the media with concentration of growth regulator BAP (0.0, 1.5, 3.0 and 6.0 mg L^{-1}) for callus induction.

3.5.3 Incubation period

The culture vials containing explants were placed under dark condition in a room with controlled temperature (25±2° C) for the first four weeks followed by four weeks of 16hr light/day by white florescent tubes. The vials were checked at a regular interval to note the respond and development of contamination.

3.5.4 Subculture

Cultures were transferred to fresh media regularly, at an interval of four weeks after dark period for callus induction.

3.5.5 Shoot initiation and root formation

The leaf derived calli were cultured on MS medium incorporated with combination of various concentrations of BAP (1.0, 1.5, 3.0 and 6.0 mg L^{-1}) and GA_3 (0.5, 1.0, 1.5 and 2.0 mg L^{-1}) for shoot initiation and the primary shoots from calli were cultured on MS medium with combination of GA_3 (0.5, 1.0, 1.5 and 2.0 mg L^{-1}) and BAP (0.5, 1.0 and 1.5 mg L^{-1}) for root formation.

3.6 Treatments

Three experiments were conducted to assess the effect of different concentrations and combinations of BAP, GA_3 and IBA.

Experiment 1. Effect of different concentrations of BAP on callus induction from leaf discs of *Fragaria* x *ananassa* Duch.

In this experiment, leaf discs were used as explant to investigate the effect of different concentrations of BAP on callus induction. Four levels of BAP (0, 1.5, 3.0, 6.0 mgL^{-1}) and MS medium were used as treatments.

Experiment 2. Effect of different combined concentrations of BAP and GA_3 on shoot iniciation from leaf derived calli of *Fragaria* x *ananassa* Duch.

In this experiment, leaf derived calli were used as explant to investigate the effect of different combined concentrations of BAP (1.0, 1.5, 3.0, 6.0 mgL^{-1}), GA_3 (0.5, 1.0, 1.5, and 2.0 mgL^{-1}) and MS medium on shoot initiation.

Experiment 3. Effect of different concentrations of GA_3 and IBA on rooting of regenerated shoots of *Fragaria* x *ananassa* Duch.

In this experiment, the effects of GA_3 and IBA on root formation of the micropropagated shoots were investigated. GA_3 (0.5, 1.0, 1.5, and 2.0 mgL^{-1}), IBA (0.5, 1.0, and 1.5 mgL^{-1}) and MS medium were used as treatment.

3.7 Parameters under study

Data were collected on the effects of different treatments on callus induction, shoot initiation and rooting. The following parameters were recorded.

3.7.1 Callus induction

3.7.1.1 Days required for callus induction

The number of callus initiated over a number of days was recorded.

3.7.1.2 Percentage of callus induction

Number of leaf discs formed callus was recorded at 50 days after culture and the percentage of callus induction was calculated as:

$$\text{Percent callus induction} = \frac{\text{Number of explants showing callus}}{\text{Number of explants incubated}} \times 100$$

3.7.2 Shoot initiation from callus

3.7.2.1 Days required for shoot initiation

This was calculated from days of callus induction to days when new shoot emerged.

3.7.2.2 Percentage of explant induced shoot

Leaf derived calli was used for shoot initiation. Percentage of explant induced shoots was calculated by using the following formula.

$$\text{Percent of explant induced shoot} = \frac{\text{Number of explant induced shoots}}{\text{Total number of explant inoculated}} \times 100$$

3.7.2.3 Number of shoots explant^{-1}

Number of shoots explant^{-1} was recorded from the calli.

3.7.2.4 Length of shoot

The shoot length was recorded by using a scale.

3.7.3 Root initiation

3.7.3.1 Days required for root initiation

This was calculated from days of inoculation to days when new root emerged.

3.7.3.2 Percentage of shoots showing roots

Excised shoots were used for root initiation. The percentage of shoots induced roots were calculated using the following formula.

$$\text{\% of shoots showing roots} = \frac{\text{Number of shoot induced roots}}{\text{Total number of shoots inoculated}} \times 100$$

3.8 Statistical analysis of data

The data for the parameters recorded in the present study were statistically analyzed by the program MSTATC and Microsoft Excel wherever applicable. The experiment was arranged in Completely Randomized Design (CRD). The analysis of variances for different parameters was performed and the means were compared by Ducan's Multiple Range Test (DMRT).

CHAPTER IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

In vitro callus induction and plant regeneration capability of strawberry *(Fragaria* x *ananassa* Duch.) were studied. Leaf explants were used for regeneration purpose. Three basic experiments were conducted to fulfill the objectives of this study. The results of the present investigation have been presented and discussed in this chapter. Analyze of variance in respect of all parameters have been presented in Table (3, 6, 8).

4.1 Experiment 1. Effect of different concentrations of BAP on callus induction from leaf discs explant of *Fragaria* x *ananassa* Duch.

In this experiment, the effects of different concentrations of BAP on callus induction of strawberry were studied. The results obtained from this experiment have been presented and discussed under the following headings.

4.1.1 Days required for callus induction

The cultured explants showed significant variation on days required for callus induction by the influence of different concentrations of BAP (Plate 1, 2, 3) where the least number of days required for callus induction was 45.67 at 3.0 mg L^{-1} BAP. The maximum numbers of days (49.33) was required for 6.0 mg L^{-1} BAP (Table 2).

4.1.2 Percentage of callus induction

Explants had shown significant variation in callus induction as influenced by BAP. The concentration of BAP exhibited significant influence on the percentage of callus induction. The highest percentage (93.33%) of callus induction was observed in 3.0 mg L^{-1} BAP and lowest percentage (80.67%) was found with 6.0 mg L^{-1} BAP. MS medium without BAP had no callus formation (Table 2).

Table 2. Effect of BAP on callus induction from leaf explants of strawberry

Treatment ($mg\ L^{-1}$)	Days required for callus induction	Percentage of callus induction
BAP		
0.0	-	0.0d
1.5	46.33b	88.33b
3.0	45.67b	93.33a
6.0	49.33a	80.67c
CV (%)	1.56	3.57
$LSD_{(0.05)}$	1.11	4.67

Means having common letter(s) are statistically identical at 5% level.

Table 3. Mean square of callus induction

Source of variation	Degrees of freedom	Mean square	
		Days required for callus induction	Percentage of callus induction
Replication	2	1.58	0.08
Treatment	3	5816.31**	1672.22**
Error	6	5.47	0.31

**= indicates significant at 1%level of probability

*= indicates significant at 5%level of probability

NS= indicates not significant

Table 4: Anatomical and histological differences between roots, stems and leaves of plants *in vitro* and after transplanting ex vitro. George et al.

Plant Organ and Tissue	*In vitro*	*Ex vitro*
Roots	Thin root	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 nonfunctional chloroplasts, limited formation of grana, abundant stroma, and starch grains	Normal chloroplasts with typical granal structure, increased chlorophyll content.

4.1.3 Anatomical and histological Description of Strawberry:

Straw berry plants generated both from in vitro and *ex vitro* were made comparison on anatomical and histological characteristics. Three plant organs like, roots, stems and leaves were considered for this study.

Root system

The roots of the *ex vitro* generated plants produced larger and stronger as compared to *in vitro* generated plants (Table 4). The cortical tissue of the *in vitro* plants showed loosed arrangement along with numerous multicellular spaces .On the other hand, the cortex of the *ex vitro* plants produced uniform and compact cell arrangement. The root hairs of the *in vitro* plants were not developed; looked delicate in appearance and several root hairs were fused together.

Stems

The diameter of the *in vitro* plants was narrower than that of ex vitro generated plans. In addition, the epidermal cells of the *in vitro* plants were not well developed, depicted weakness of the plants. While the plants produced in *ex vitro* exhibited profound development in epidermis. The cortex of the *in vitro* plants was apparently weak as determined by the little presence of sclerenchymatous cells. The cortex of the *ex vitro* had fully developed with continuous cylinder of collenchyma.

Leaves

The cuticle was thin and discontinuous of the *in vitro* generated plants; contrary it was thick and continuous of the *ex vitro* plants. The epidermal cells appeared irregular in shape as compared to normal observed in *ex vitro* plants. The leaves looked light green, while the leaves of the *ex vitro* plants were normal green in appearance. The anatomical observation revealed highly vacuolated cells with large intercellular spaces. On the other hand, the cells of the *ex vitro* plants had normal with regular air-spaces.

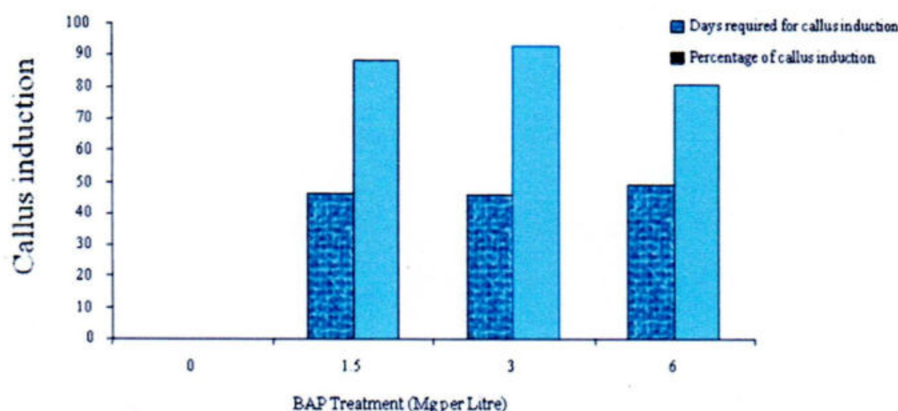

Fig 1: Effect of BAP on callus induction from leaf explants of strawberry

4.2 Experiment 2. Effect of different combined concentrations of BAP and GA_3 on shoot initiation from leaf derived calli of *Fragaria* x *ananassa* Duch.

For shoot regeneration, leaf derived calli were cultured on shoot induction media containing different combined concentrations of BAP and GA_3. The results on the effect of different concentrations of BAP and GA_3 for shoot initiation have been discussed below.

4.2.1 Days required for shoot initiation

Cultured explants were carefully observed in regular basis for data collection on days required for shoot regeneration. The growth regulator concentrations significantly affected the days required for shoot initiation. Table 5 shows that a combination of 3.0 BAP and 0.5 mg L^{-1} GA_3 performed better and needed least number of days (8.67) for shooting. Maximum number of days (18.0) was required in the combination of 1.0 mg L^{-1} BAP and 2.0 mg L^{-1} GA_3.

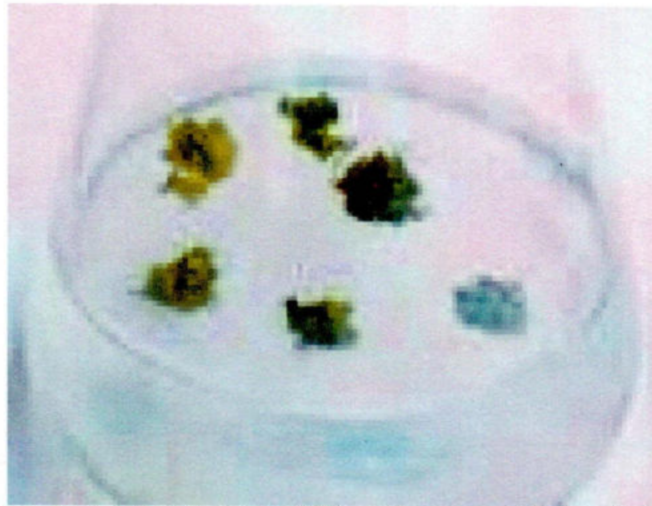

Plate 1. Callus from the leaf explant in MS medium supplemented with 3.0 mg L^{-1} BAP after 45 days of incubation.

Plate 2. Callus from the leaf explant in MS medium supplemented with 1.5 mg L^{-1} BAP after 45 days of incubation

Plate 3. Callus from the leaf explant in MS medium supplemented with 6.0 mg L^{-1} BAP after 45 days of incubation.

4.2.2. 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.

In most laboratories, losses due to contamination average between 3-15% of plants at every subculture, 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.

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.

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. Latent bacterial contamination during the proliferation phase adversely affects rooting due to changes in growth regulators and water potential of the media and also kills young trees

after weaning. 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. Cultures of apricot with latent bacterial contaminants showed high CO2 and low O2 concentration in the vessel headspace, thereby decreasing the proliferation and photosynthetic rates.

Organic soil amendments have been observed to increase the inoculum for them 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.

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.

Piyarak *et al.* 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 Grampositive 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 explants contaminant levels should be centered on the care of stock plants or the sterility of the watering system.

4.2.3 Percentage of explant induced shoot

The percentage of shoot initiation was significantly influenced by the concentration of the growth regulators used in the experiment. The highest shoot initiation (93.33%) was observed with the supplementation with 3.0 BAP and 0.5 mg L^{-1} GA_3 to the medium (Table 5).

4.2.4 Number of shoots explant^{-1}

The number of shoots produced per explant varied with different combined concentrations of hormone BAP and GA_3 (Plate 4, 5, 6). The combination of 3.0 BAP and 0.5 mg L^{-1} GA_3 showed the highest (15.00) number of shoots per explant and the lowest number (3.33) of shoots per explant was observed in the combined concentration of 1.0 BAP and 0.5 mg L^{-1} GA_3 which is statistically similar to the combined application of 1.0 mg L^{-1} BAP and 1.5 mg L^{-1} GA_3 (Table 5). Similar shoot regeneration frequency was also reported by Mohamed *et al.* (2007).

4.2.5 Length of shoot

The length of shoots was also significantly influenced by growth regulators used in the experiment. The combined concentration of 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3 resulted in the longest shoot (2.0 cm) and the shortest shoot (0.60 cm) was observed of 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3 (Table 5).

After considering the above parameters, it was clear that the different combined concentrations of BAP and GA_3 responded spontaneously for shoot induction, among them the combination of 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3 performed better than any other treatments.

Table 5.Main effect of hormone concentrations on shoot initiation

Hormone concentrations ($mg L^{-1}$)		Days required for shoot initiation	Percentage of explant induced shoot	Number of shoots $explant^{-1}$	Length of shoot (cm)
BAP	GA_3				
1.0	0.5	12.33b-e	**41.67g**	**3.33i**	**0.60b**
	1.0	16.00a-c	56.00fg	4.67hi	1.30b
	1.5	16.67ab	50.00ef	**3.33i**	0.83b
	2.0	**18.00a**	61.60d-f	4.67hi	0.63b
1.5	0.5	13.33a-e	78.33a-d	9.33de	1.50b
	1.0	17.00ab	73.33b-f	12.00bc	1.43b
	1.5	10.67c-e	88.33ab	10.33cd	0.97b
	2.0	14.33a-d	63.33c-f	7.33fg	1.17b
3.0	0.5	**8.67e**	**93.33a**	**15.00a**	**2.00a**
	1.0	9.33de	86.67ab	13.67ab	1.56b
	1.5	12.67a-e	78.33a-d	9.00d-f	1.13b
	2.0	11.67b-e	73.33b-f	8.33ef	0.87b
6.0	0.5	15.00a-c	80.00a-c	10.00de	1.47b
	1.0	9.33de	88.33ab	9.33de	1.43b
	1.5	12.33b-e	76.67a-e	6.33gh	0.83b
	2.0	14.00a-e	73.33b-f	7.33fg	1.43b
CV (%)		21.02	12.89	14.13	23.45
$LSD_{(0.05)}$		4.63	15.75	1.70	1.37

Means having common letter(s) are statistically identical at 5% level.

Table 6. Mean squares of different hormone concentrations on shoot initiation

Source of variation	Degrees of freedom	Mean square			
		Days required for shoot initiation	Percentage of explant induced shoot	Number of shoots explant^{-1}	Length of shoot (cm)
Factor A (Treatment)	3	56.14**	1917.58**	123.25**	0.71**
Factor B (Explants)	3	10.14NS	172.58NS	27.42**	0.75**
Factor (A×B)	9	19.08*	253.42*	9.25**	0.26**
Error	30	7.71	89.23	1.40	0.08

**= indicates significant at 1%level of probability

*= indicates significant at 5%level of probability

NS= indicates not significant

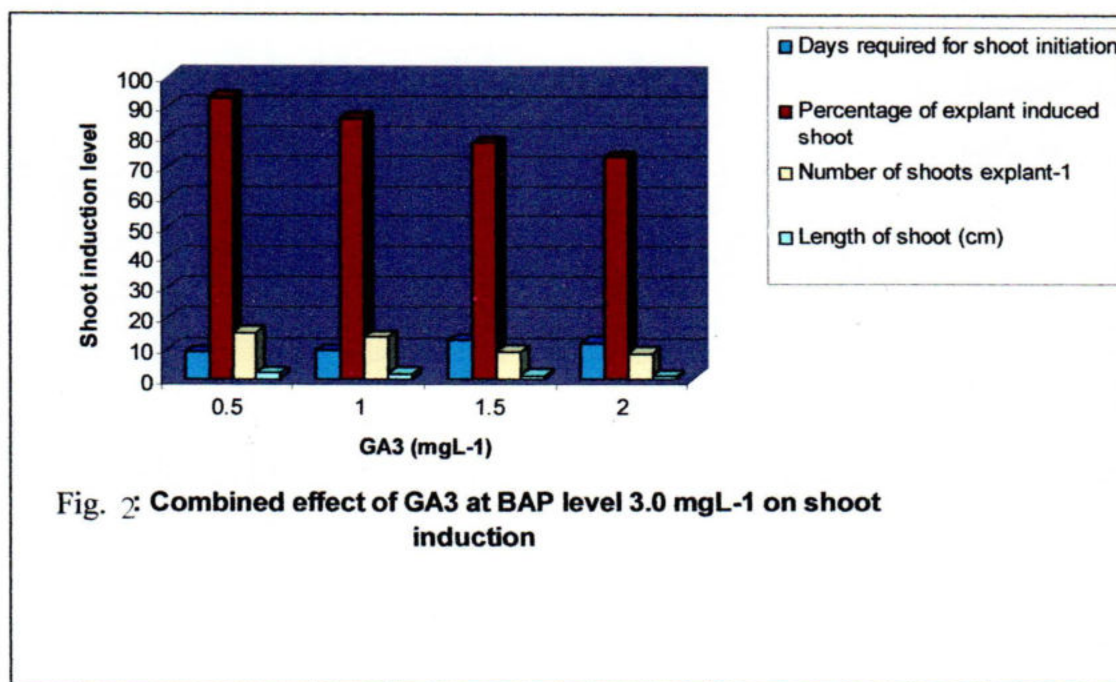

Fig. 2: **Combined effect of GA3 at BAP level 3.0 mgL-1 on shoot induction**

Plate 4. Multiple shoots from leaf derived calli in MS medium supplemented with 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3

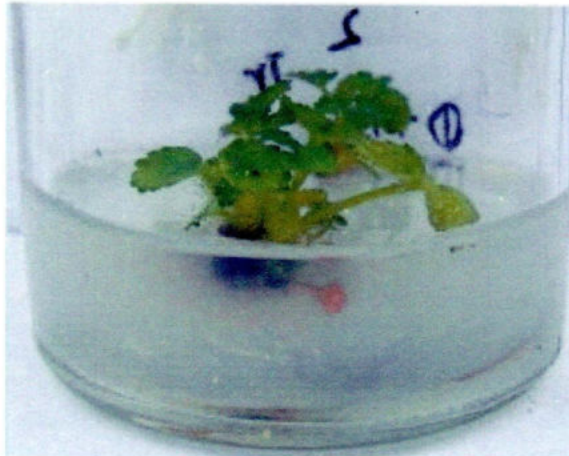

Plate 5. Multiple shoots from leaf derived calli in MS medium supplemented with1.5 mg L^{-1} BAP and 1.0 mg L^{-1} GA_3

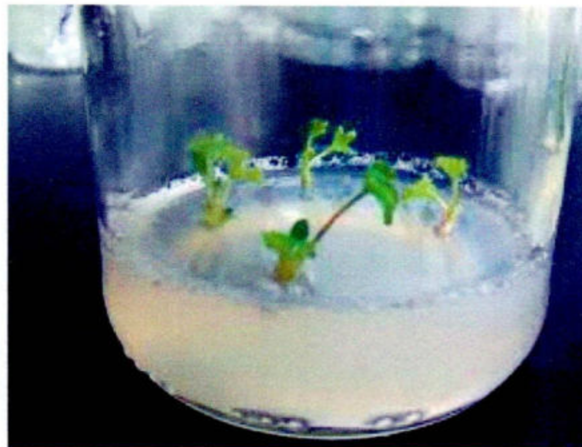

Plate 6. Multiple shoots from leaf derived calli in MS medium supplemented with 6.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3

4.3 Experiment 3. Effect of different concentrations of GA_3 and IBA on rooting of regenerated shoots of *Fragaria* x *ananassa* Duch.

The regenerated shoots were collected from *in vitro* grown plants of experiment 2. Those were subcultured on MS medium supplemented with combination of GA_3 (0.5, 1.0, 1.5 and 2.0 mg L^{-1}) and IBA (0.5, 1.0 and 2.0 mg L^{-1}) in order to allow root formation (plate 7). The results on rooting of regenerated shoots have been discussed below.

4.3.1 Days required for root initiation

The rooting of shoots was non-significant in terms of days required for root initiation. The combination of 1.5 mg L^{-1} GA_3 and 1.0 mg L^{-1} IBA performed best and required least number of days (52.00) for rooting. Maximum number of days (59.00) was required for the combination of 1.0 mg L^{-1} GA_3 and 0.5 mg L^{-1} IBA (Table 7). Similar rooting frequency was also reported by Mereti *et al.* (2003).

4.3.2 Percentage of shoots showing roots

The percentage of root initiation was significantly influenced by the concentration of the growth regulators used in the experiment. The highest root induction (83.00%) was observed with the combination of 1.5 mg L^{-1} GA_3 and 1.0 mg L^{-1} IBA and the lowest root induction (53.33%) was observed in the addition of 0.5 mg L^{-1} GA_3 and 0.5 mg L^{-1} IBA to the medium (Table 7).

After considering the above parameters, it was clear that the combined application of 1.5 mg L^{-1} GA_3 and 1.0 mg L^{-1} IBA performed better than any other treatments.

Table 7. Main effect of hormone concentrations on root initiation

Hormone concentrations (mg L^{-1}) GA$_3$	IBA	Days required for root initiation	Percentage of shoots showing roots
0.5	0.5	58.33ab	55.33g
	1.0	55.00d	72.33c
	1.5	57.67a-c	69.00d
1.0	0.5	59.00a	63.33e
	1.0	55.33cd	80.00b
	1.5	56.00b-d	65.00e
1.5	0.5	57.00a-d	59.00f
	1.0	52.00e	83.00a
	1.5	55.00d	70.67cd
2.0	0.5	57.67a-c	64.33e
	1.0	55.33cd	79.33b
	1.5	54.67d	73.00c
CV (%)		2.43	2.25
$LSD_{(0.05)}$		2.307	2.64

Means having common letter(s) are statistically identical at 5% level.

Table 8. Mean squares of different hormone concentrations on root initiation

Source of variation	Degrees of freedom	Mean square	
		Days required for root initiation	Percentage of explant induced root
Factor A (Treatment)	3	10.102**	74.69**
Factor B (Explants)	2	39.08**	990.19**
Factor (A×B)	6	2.71NS	36.08**
Error	22	1.86	2.44

**= indicates significant at 1%level of probability
*= indicates significant at 5%level of probability
NS= indicates not significant

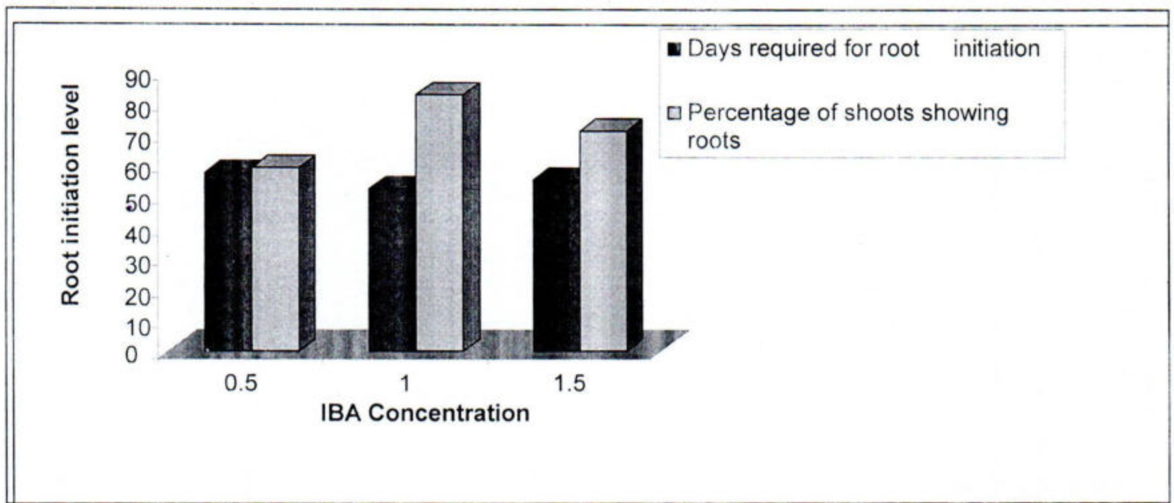

Fig 3: Combined effect of IBA at GA_3 level 1.5 on root initiation

Plate 7. Root formation from the regenerated shoots in MS medium supplemented with the combination of 1.5 mg L^{-1} GA_3 and 1.0 mg L^{-1} IBA

CHAPTER V

SUMMARY AND CONCLUSION

CHAPTER V

SUMMARY AND CONCULATION

The experiments were conducted at the Tissue Culture Laboratory, Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur during the period January to May, 2012. A detailed investigation was carried out to find out the best hormonal concentration for callus induction and plantlet regeneration by using leaf explant.

Leaf explant of strawberry (*Fragaria x ananassa* Duch.) was cultured on MS medium supplemented with different concentrations of BAP (0, 1.5, 3.0 and 6.0 mg L^{-1}) and placed under dark condition for four week to induce callus. The effect of BAP was highly significant on callus induction. For callus induction, minimum number (45.67) of days was required in 3.0 mg L^{-1} BAP. The highest callusing (93.33%) was found in MS medium supplemented with 3.0 mg L^{-1} BAP. No callus was observed in MS medium without growth regulator.

The different combination of GA_3 and BAP significantly influenced the shoot induction from calli. For shoot induction, minimum number (8.67) of days was required in the combination of 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3. The highest shooting (93.33%) was found in MS medium supplemented with combination of 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3. Number of shoots showed significant variation with BAP and GA_3. The maximum number (15.00) of shoots was obtained from combination of 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3. A combination of 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3 produced the highest (2.0 cm) shoot length.

The combination of GA_3 and IBA influenced the duration of root initiation. A combination of 1.5 mg L^{-1} GA_3 and 1.0mg L^{-1} IBA showed the minimum number (52.00) of days for root initiation. The highest rooting (83.00%) was observed in combination of 1.5 mg L^{-1} GA_3 and 1.0mg L^{-1} IBA.

From the above results, it may be concluded that 3.0 mg L^{-1} BAP showed the better performance in callus induction and induction percentage than any other concentrations. Considering all parameters for shoot induction, it was clear that the combination of 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3 performed superior than any other treatments. It was observed that the combination of 1.5 mg L^{-1} GA_3 and 1.0mg L^{-1} IBA performed better than the other combined concentrations of GA_3 and IBA for root formation.

In this experiment, an efficient and reproducible protocol for the plantlet regeneration from leaf disc explants in strawberry (*Fragaria x ananassa* Duch.) was developed. This protocol has the potential for the *in vitro* multiplication and genetic improvement of strawberry.

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