Standardization of *in vitro* Protocol on Plant Regeneration of *Fragaria* × *ananassa* Duch. cv. Chandler and its Evaluation of Genetic Fidelity through Molecular Marker

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CERTIFICATE – I

This is to certify that the thesis entitled, **"Standardization of *in vitro* protocol on plant regeneration of *Fragaria* × *ananassa* Duch. cv. Chandler and its evaluation of genetic fidelity through molecular marker"** submitted by **SUVALAXMI PALEI** to the Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE IN AGRICULTURE (Fruit Science and Horticulture Technology)** is a faithful record of *bona fide* research work carried out under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma or published in any other form. The assistance and help received in the course of investigation have been duly acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled, **"Standardization of *in vitro* protocol on plant regeneration of *Fragaria* × *ananassa* Duch. cv. Chandler and its evaluation of genetic fidelity through molecular marker"** submitted by **SUVALAXMI PALEI** to the Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE IN AGRICULTURE (Fruit Science and Horticulture Technology)** has been approved by the student's Advisory Committee after oral examination on the same in collaboration with an External Examiner.

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ABBREVIATIONS

Ads	:	Adenine Sulphate
AFLP	:	Amplified Fragment Length Polymorphism
BA	:	Benzyl Adenine
BAP	:	Benzyl Amino Purine
Ca	:	Callus
cv.	:	Cultivar
DAI	:	Days After Inoculation
Fig	:	Figure
GA_3	:	Gibberellic Acid
Hcl	:	Hydrochloric Acid
HPLC	:	High Performance Liquid Chromatography
h	:	Hour
IAA	:	Indole-3- Acetic acid
IBA	:	Indole 3-bnutyric acid
ISSR	:	Inter Simple Sequence Repeat
Kn	:	Kinetin
LS	:	Linemaier –Skoog
ml	:	milliliter
mg	:	milligram

µM	:	Micro molar
Mm	:	Mill molar
MS	:	Murashige and Skoog's medium
NAA	:	Naphthalene Acetic Acid
NaOH	:	Sodium Hydroxide
NN	:	Nitsch and Nitsch medium
PCR	:	Polymerase Chain Reaction
PGR_S	:	Plant Growth Regulators
ppm	:	Parts Per Million
PVPP	:	Polyvinylpolypyrolidone
RAPD	:	Randomly Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
Sl. No.	:	Serial Number
SSR	:	Simple Sequence Repeat
SST	:	Sequence-tagged Sites
Tab	:	Table
TDZ	:	Thidiazuron
TE	:	Trolox Equivalent
2,4-D	:	2,4-dichloro phenoxy acetic acid

ABSTRACT

Delicious, rich-red, sweet, yet gently tangy strawberries are among the most popular berries. It is low in calories (32 cal/100 g) and fats but is a rich source of health promoting phyto-nutrients, minerals, vitamins and ellagic acid which are essential for optimum health. So, the present investigation was carried out on an alluring fruit crop, strawberry (*Fragaria* × *ananassa* Duch.) cv. Chandler belonging to family Rosaceae. The different vegetative parts i.e. runner tips, nodal explant, shoot apices, stem and leaves were used to standardize the regeneration protocol through *in vitro* culture. The explants excised from pot established matured plants and thereafter inoculated on Murashige and Skoog's medium fortified with various concentration of plant growth regulators for multiple shoot proliferation and callus induction. Maximum percentage of shoot induction was obtained from runner tips on the medium containing 1.5 mg/l BAP and 0.1 mg/l NAA. Upto 89.8% shoot multiplication was found having highest numbers of shoot (4.3) in the process of direct organogenesis on the same medium. This study also revealed that maximum percentage of multiple shoots per culture were achieved when inoculated under 3000 lux light intensity and 16 hour photoperiod at 25±2^{o}C incubation temperature in MS medium having pH 5.8 and agar concentration of 0.6%. Rooting of these daughter shoots were found to be best on ½ MS medium supplemented with 1.0 mg/l IBA. Callus induction from leaf and internodal cuttings were achieved using different concentrations of auxins and cytokinins. Maximum callus induction frequency (90%) from internodal explants was observed in the media containing MS basal salts supplemented with 1.0 mg/l 2,4-D and 0.1mg/l BAP. However, these greenish compact callus regenerates to healthy shoot when the MS medium fortified with different concentration of BAP and NAA . Best response was revealed from the medium in addition with 2.5 mg/l BAP and 0.5 mg/l NAA. These microshoots were rooted in most commonly used rooting hormones IBA recording 73.3% rooting at 0.25mg/l IBA on ½ MS medium. Finally all regenerates (direct and indirect) were hardened inside the greenhouse with 85% relative humidity for better field establishment and reducing the risk of mortality. The fidelity of *in vitro* derived regenerates were screened with 12 ISSR primers that have shown monomorphic among these plantlets which is a pre-requisite for mass multiplication and beneficial to identify any adulteration or clonal variation which may bear desired traits for the present economy oriented community.

CHAPTER-1

INTRODUCTION

INTRODUCTION

"*Fragaria*" belongs to family Rosaceae, is a genus of the perennial, creeping herbs, found growing in the wild in different climatic zones of the world (CSIR, 1956). The cultivated strawberry (*Fragaria* × *ananassa* Duch.) is a monoecious octaploid hybrid of two largely dioecious octaploid species, *Fragaria chiloensis* Duch. and *Fragaria virginiana* Duch.(Darrow, 1996; Galletta and Bringhurst, 1990; Larson,1994). However, some strawberry cultivars are also derived from a subspecies of *Fragaria virginiana* Duch. and *Fragaria virginiana glauca* Standt. (Darrow, 1996; Scott and Lawrence,1975; Gallette and Bringhurst, 1990). There are 12 species of *Fragaria* and the genus is native to the north temperate regions and also extends into North Africa and down through tropical American mountains into temperate South America. The number of sets of chromosomes vary from species to species ranging from 2 sets in the Wild Strawberry *Fragaria vesca* to 8 sets in the modern garden strawberry (*Fragaria* × *ananassa* Duch.). There are a whole lot of individual female reproductive organs, termed carpels, in the flower of strawberry. Each carpel consists of a stigma, style and ovary and all the carpels are inserted on to a fleshy receptacle. In *Fragaria*, the receptacle swells into the red-coloured 'fruit' we know as a strawberry and over its surface are black dots, each dot being an individual true fruit (a true fruit being the ripened ovary). The individual true fruit are termed achenes; these being small, dry single seeded fruit that do not split open.

It is produced in 73 countries worldwide on 200,000 hectare area and produced 31 lakh metric tons strawberry (FAO, 2008). It has been commercially cultivated in Canada, USA, Japan, Spain, Germany, Korea, Italy, Poland, Thailand and so many countries in the world (Biswas *et al.*, 2007).

Strawberries have significantly high amounts of phenolic flavonoid phyto-chemicals called anthocyanins and ellagic acid. Scientific studies show that consumption of these berries may have potential health benefits against cancer, aging, inflammation and neurological diseases. Strawberry has an ORAC value (Oxygen radical absorbance capacity, a measure of anti-oxidant strength) of about 3577µmol TE per 100 grams. Fresh berries are excellent source of vitamin-C (58.8 mg/100 g), which is also very powerful natural antioxidant. Consumption of fruits rich in vitamin-C helps the body develop resistance against infectious agents, counter inflammation and scavenge harmful free radicals. The fruit is rich in B-complex group of vitamins. It contains very good amounts of vitamin B-6, niacin, riboflavin,

pantothenic acid and folic acid. These vitamins are acting as co-factors to metabolize carbohydrate, proteins and fats. Strawberries contain vitamin A, vitamin E and health promoting flavonoid polyphenolic antioxidants such as lutein, zea-xanthin, and beta-carotene in small amounts. These compounds act as protective scavengers against oxygen-derived free radicals and reactive oxygen species (ROS) that play a role in aging and various disease processes. Strawberries are known for their characteristics aroma, which is attributed to the presence of volatile ester. The most important aroma compound are ethyl hexanoate, methyl hexanoate, ethyl heptanoate, ethyl propionate, ethyl butanoate, methyl butanoate, furanone and linalool. The ripe fruits contain slightly more lipid than unripe one; with higher quantities of oleic acid and lesser linoleic acid (Mitra, 1991; Sharma and yamdagni, 1999). The red colour of the fruit is mainly due to the presence of anthocynin, pelargonidin 3- monoglucoside, and traces of cyanidin (Singh and Sharma, 1970; Pathak and Singh, 1971a; Mitra, 1991).

Among various cultivars of strawberry, Chandler is a highly acceptable present day cultivar of north plains of India. It was developed in California by crossing "Douglas" and "Cal 72.361-105". Its fruits have exceptionally high dessert quality with outstanding colour, flavour and texture. Fruit size is large (15-20 g) and have low soluble solids (7-8%). But because these exceptionally large, they are damaged easily when transported to distant markets (Sharma, 1998a; Sharma,1998b; Sharma, 2003) . It is a high yielding, short-day variety, quite resistant to physical damage.

Propagation of strawberry, through seed is easier, but the plants are not true to type. Large numbers of seed per fruit as compared to other fruit crops but seeds are very small and required stratification, the effective chilling temperature ranges from -2 to 6.5°C. (Voth and Bringhurst, 1958; Takai, 1970; Korenberg and Durner *et al.*, 1986) and duration of chilling period is usually 2-4 weeks (Larson, 1994). Treatment of seeds with H_2SO_3 or GA_3 and Thiourea also break seed dormancy and facilitate germination. Strawberry plants also spread vegetatively using runners and this enables them to be easily transplanted and propagated as clones. Most strawberry cultivars produce hermaphrodite flowers and are self- fertile. However, some also produce male or staminate, imperfect and female or pistillate flowers. Hermaphrodite flowers are self fertile and pistillate flowers require cross pollination for fruit production.

Strawberries are affected by over 30 viruses and phytoplasmas, many of which can greatly reduce yield, rapidly spread in the field, and may not cause obvious

symptoms. Strawberry mottle virus (SMoV), Strawberry crinkle virus (SCV) and Strawberry mild yellow edge virus (SMYEV) are some of the most common viruses of strawberries. Some severe strains of SMoV can reduce yield as much as 30%. Often, modern cultivars are symptomless when infected with just one virus but in a mixed infection with more than one virus, a decline disorder can develop in which the leaves turn red, and plants weaken and may die (Martin and Tzanetakis, 2006).

Micropropagation is the first major and widely accepted practical application of plant biotechnology that has gained the status of a multibillion dollar industry throughout the world. Tissue culture is the *in-vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions often to produce the clones of plants. The resultant clones are true-to type of the selected genotype (Thorpe, 2007). The controlled conditions provide the culture an environment conducive for their growth and multiplication. These conditions include proper supply of nutrients, pH of medium, adequate temperature and proper gaseous and liquid environment. Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. Small pieces of tissue can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (Akin-Idowu *et al.*, 2009). In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants. The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities (Brown and Thorpe, 1995). Commercial production of plants through micropropagation techniques has several advantages over the traditional methods of propagation through seed, cutting, grafting and air-layering etc. It is rapid propagation processes that can lead to the production of virus free plants (Garcia-Gonzales *et al.*, 2010). *In vitro* regeneration technique in strawberry has been standardized (Sharma and Singh , 1999). Different explants like meristem tip, anthers, immature embryos and first axillary buds of stolon have been used to produce millions of plants in the year (Waithaka *et al.*, 1980; Wang *et al.*, 1998; Jones *et al.*, 1988; Boxus, 1989).

In vitro production of plants involves the application of plant growth regulator, such as auxin, for initiation. Nevertheless, these auxins are known to be associated with genetic instability in plants, a phenomenon called somaclonal variation (Karp, 1989; Cullis, 1992). Mostly somaclonal variation also occurs as responses to the stress imposed on the plant in culture conditions and are manifested in the form of DNA methylations, chromosome rearrangements and point mutations (Phillips *et al.*, 1994). Although somaclonal variations may be used as a source for variation to get superior clones, it could be a serious problem in plant tissue culture industry where the aim is to develop identical propagules of a desired variety resulting in the production of undesirable traits or plant off-types (Karp, 1993; Cassells *et al.*, 1999). Scaling up of any micropropagation protocol is severely hindered due to incidence of somaclonal variations, so a stringent quality check in terms of genetic similarity of progeny becomes mandatory. Any system, which can significantly reduce or eliminate variation generated during tissue culture, can be of much practical utility. Traditionally, morphological description, physiological supervision, karyological analysis, biochemical estimations and field assessment were used to detect any types of genetic variations, but presently molecular markers have complemented over traditional methods to detect and monitor the genetic fidelity of tissue culture derived plantlets and variety identification. This is apparent in studies conducted to screen somaclonal variations produced in tissue cultured derived plants such as in turmeric (Salvi *et al.*, 2001), *Lillium* (Varshney *et al.*, 2001), Strawberry (Gaafer and Saker, 2006) and *Swertia chirayita* (Jhosi and Dhawan, 2007). Out of the available techniques, ISSR markers have proven to be a reliable, reproducible, easy to generate, inexpensive and versatile set of markers that relies on repeatable amplification of DNA sequences using single primers.

On the above fact, the present investigation with following objectives;

Objectives:

- To standardise the protocol for *in vitro* plant regeneration of strawberry by using plant growth regulators, growth culture condition and photoperiods.
- Induction of rooting and acclimatization of *in vitro* raised plantlets.
- To evaluate the genetic fidelity of the micropropagated plant through DNA marker.

CHAPTER-2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Strawberry (*Fragaria* x *ananassa* Duch.) is a major berry crop around the world. Production of strawberries has been attempted to meet the demands for improved yields, fruit size and quality traits. However, the narrow genetic base of the cultivated strawberry, combined with the polyploid nature of the crop hinder the traditional breeding methods. *In vitro* approaches are the alternative efficient strategy to implement strawberry improvement. Strawberries are members of the family Rosaceae, subfamily Rosoideae, and genus *Fragaria*. *Fragaria* species are grouped according to polyploidy. There are nine diploids (2n=2x=14), two tetraploids (2n=4x=28), one hexaploid (2n=6x=42), and four octoploids (2n=2x=56). Cultivated pineapple Strawberry *Fragaria*× *ananassa* Duch., is a cross between *Fragaria chiloensis* × *Fragaria virginiani*, is an octapliod one (2n=8x=56) . 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 (Darrow, 1966). The blossoms of *Fragaria* 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 achiness formed. Strawberry plants are day length dependent with cultivars being long day, short day or day neutral. Micropropagation has also been widely used in the USA in commercial propagation of strawberries and in breeding programs to produce many plants rapidly (Zimmerman, 1981). Most of the European nurseries producing several millions plants per year through *in vitro* technique as it gave a definitive answer to the problems of soil fungal flora, 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 period (Mohan *et al.*, 2005). The present review highlighted the *in vitro* studies as well as genetic identity assessment of strawberry.

2.1 Status of nutritional value

Strawberries were valued for delicious flavour and fragrance, for health-restoring qualities and as harbinger of spring (Wilhelm *et al.*, 1974). According to Nutrient Database for Standard Reference (USDA, 2003) the strawberry fruits are resources of the nutritious elements essential for human being. Strawberries had been a favourite among the fruits of the temperate world. Flavourful and nutritious, strawberries are enjoyed by millions of people in all climates 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 flavourings, 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 fruits (Debnath *et al.*, 2005; 2006). Strawberry is cultivated all around the world, not only for its digestive and tonic properties, but also because of the nutritional value of its fruits, important source of foliate, vitamin-C, fiber, potassium, flavonoids, autocianidin, phytochemicals and antioxidants (Appendix-I).

2.2 Phytochemicals enrichment

Although strawberry is not an essential component of the diet, it's delicious flavour and taste, attractive appearance and seasonal availability makes this fruit an excellent crop. Strawberry (*Fragaria x ananassa* Duch.) fruits are rich sources of phytochemicals of which phenolic compounds are predominating. 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 (Machiex *et al.*, 1990). Anthocyanins are natural pigments providing scarlet to blue colours 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 and decreasing triglyceride and free fatty acid levels (Igarashi *et al.*, 1990). Moreover, Kamei *et al.* (1995) 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. Sato *et al.* (1996) reported that in the suspension cultures of *F. ananassa* cells, anthocyanin content increased with the intensity of light irradiation from 2500 to 8000. Masayuki *et al.* (1999) and Igarashi *et al.* (1991) demonstrated that *Fragaria* × *ananassa* (strawberry) callus, which produced high amounts of anthocyanin in the dark , get accumulated more than 1000 μg of anthocyanin per gram fresh cell . 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 (Hannum , 2004). Strawberry extracts have also been evaluated for their ability to inhibit mutation by the direct-acting mutagen methyl methane sulfonate, 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 (Hope *et al.*, 2004). *In vitro* studies indicate that berry phenolics have a wide range of biological properties such as anti-cancer, antioxidant, anti-inflammatory, and cell regulatory effects (Seeram, 2006 a,b; Seeram and Heber,2006). These anticancer effects are exerted through multi-mechanistic means of action including the antioxidant actions of the berries phenolic constituents by protecting DNA from damage, and also through effects exerted beyond antioxidation (Seeram *et al.*, 2006). The biological activities of strawberry phytochemicals include the regulation of phase-II enzymes and the modulation of gene expression and subcellular signalling 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 (Seeram 2006 a,b). These qualities have ensured that the economic importance of this crop has increased throughout the world and, now-a-days, it remains as a crop of primary interest for both research and fruit production (Mercado *et al.*, 2007).

2.3 Disease in strawberries

Several soil pathogens damage strawberry roots, resulting in vigour 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 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*) (Hancock *et al.*, 2008). *Fusarium* wilt or *Fusarium* yellows (*Fusarium oxysporum*) 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 (Hancock *et al.*, 2008). Among the foliar diseases, three are very widespread and can cause serious damage including, leaf blight (*Phomopsis obscurans*), Ramularia leaf spot, (*Mycosphaerella fragariae*) and leaf scorch (*Diplocarpon earliana*). Alternaria leaf spot or black leaf spot (*Alternaria alternata* (FR.) Keissler) causes serious damage in Europe, New Zealand and Korea (Hancock *et al.*, 2008). Powdery mildew (*Sphaerotheca macularis)* is also found across most of the strawberry range, although it rarely does economic damage. Angular leaf spot, (*Xanthomonas fragariae*) is a rapidly growing problem in strawberries all across the world (Hancock *et al.*, 2008). 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, C. acutatum.*

2.4 *In vitro* studies of Strawberry

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. Healthy stocks used for propagation through conventional methods are not available. Micropropagation of strawberry was established in 1974 by Boxus because of two important factors i.e. soil fungi causing a lot of damage to the strawberry fields and *in vitro* raised plants seemed to produce more runners per plant in a short time. This technique is also useful for 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 (Swartz *et al.*, 1981). Boxus (1983) reported that each m^2 of growing area of strawberry can produce 40000 plantlets per year. These plants were vigorous and after transplanting in the

soil, some produced up to 500 new runner plants and it also indicated that new runner plants were 10 times more than that produced by conventional material.

Micropropagation of strawberry through axillary buds has been studied intensively for a long time (Boxus, 1989; 1992). On a commercial scale, tissue culture-derived strawberry plants are estimated to cost more than plants produced by conventional propagation. But, micropropagated strawberry has several advantages, such as its ability to multiply virus-free stock rapidly and, the improved capacity of these plants to produce runners for planting in the field (Lopez-Aranda *et al.*, 1994).

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, whitish exudates of bacteria was observed around the base of the explant after 2-3 days. 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 bored an average three- fold decrease with subsequent death of the plantlets in about one month (Moutia and Dookum, 1999). 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.

Several parameters used during the *in vitro* study which affect the behaviour of micropropagated plant 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 (Faedi *et al.*, 2002). 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 (Mohan *et al.*, 2005). Prior experiences with strawberry micropropagation indicate that *in 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 (Kikas *et al.*, 2006). 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. 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 (Mercado *et al.*, 2007). The details *in vitro* study of strawberry (*Fragaria x ananassa* Dutch.) is presented in Table 1.

2.5 Role of phenolic during *in vitro* growth

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 (Scalbert *et al.*, 1988; Marks and Simpson,1990). 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 sucrose and accumulation of starch (Lindfor *et al.*, 1990).

Accumulation of phenolics is most obviously associated with the developmental stage of the plant and season, which was shown in woody species. Explants collected from the month of November to February had produced low browning percentages *in vitro*, whereas the browning was at its maximum if explants were collected in month of April to August (Wang *et al.*, 1994). Differences in browning between varieties and in relation to the size of the explants were also observed. 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 (Seneviratne and Wijesekara, 1996). 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 macrosalts, auxins, and sucrose concentration in the culture medium have caused browning as well. In addition, there are some substances including 5,6-Cl_2-IAA, yeast extracts, and floridzin which directly enhance the production of phenolic compounds (Marjo, 1999). Many medium components have been used to 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 significant results (Marjo,1999). Oxidized products, such as quinones, are known to be highly reactive and inhibit enzyme activity leading to the death of the explants. As a consequence of browning, tissue senescence, recalcitrance in embryogenesis and regeneration has been observed (Marjo, 1999; Bhat and Chandel, 1991).

Madany *et al.* (2007) 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.

2.6 Effect of explants source on micropropagation

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. 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 compound (John *et al.*, 1995). The juvenile explants had pre-existing competent cells that were able to

respond to auxin and became determined to form roots. However, the mature explants appeared to lack cells with pre-existing competence to form roots, but competence was acquired by some callus cells once they had been initiated. 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. 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 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 explants and the small area exposed to the external environment (Moutia and Dookum, 1999).

2.7 Strawberry Organogenesis

2.7.1 Direct Organogenesis

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

In 1974, 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 cytokinin but roots would not form until the explant was without auxin. He concluded with the statement that micro-propagated strawberry plants would replace traditional methods of propagation for the commercial trade.

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 (Debnath , 2003)

2.7.2 Adventitious Shoot Regeneration

Rugini and Orlando (1992) studies about adventitious shoot regeneration by used stipules explants, they found that there was great differences in shoot regeneration ability from calli 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.

Jemmali *et al.* (2002) 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.

Passey *et al.* (2003) 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.

Debnath (2005) reported an efficient system to regenerate shoots on excised sepals (calyx) of greenhouse-grown Bounty strawberry (*Fragaria - ananassa* Duch.) was developed *in vitro*. He found 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 abaxial 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. Shoots were rooted *in vitro* in the same basal medium used for shoot regeneration, but without any growth regulators.

Qin *et al.* (2005) reported that Toyonoka cv. Of 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.

Debnath (2006) worked 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 strawberry plant (*Fragaria* × *ananassa* Duch.). He observed that a moderate concentration (2 ,4 µM TDZ) supported shoot regeneration without an intermediary callus phase from leaf disks, or petiole, all maintained for 14 days 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. Shoots proliferated and roots developed best when explants were cultured in medium supplemented with 1 or 2 μM zeatin.

Biswas *et al.* (2007) 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.

Sakila *et al.* (2007) 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.

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 (Mercado *et al.*, 2007).

Emara (2008) 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 Kn 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 per litre 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.

2.7.3 Strawberry Indirect Organogenesis

Kartha *et al.* (1980) successfully regenerated cv. Redcoat using a combination of BAP, IBA and GA_3 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 BAP and 2, 4-D both were used at different concentrations. Severe browning occurred when fully expanded leaves of greenhouse grown Redcoat were used. Browning was caused by BAP 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.* (1990) reported that plant regeneration using immature leaf explants taken from *in vitro* grown 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 benzyl aminopurine (BAP) 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 BAP 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 BAP and 1.0 µM 1-naphthaleneacetic acid (NAA) and incubating the cultures at 16 h light (62.5 µE. $m^{-2}s^{-1}$). 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 somaclonal variation and selection of desirable traits.

Saifullah *et al.* (2000) 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 same 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.* (2005) demonstrated that the growth of *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 (day/night) 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 (day/night)] .Photoperiod 4/2 (day /night) 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 (day/night) photoperiod was only recorded in the third passage. The leaf blades of both clones grown under 4/2 (day/night) cycle were significantly reduce. On the other hand, the leaf petioles of Elsanta were visibly elongated under mentioned photoperiod.

2.8 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.

Owen and Miller (1996) 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.

Haploid recovery in strawberry through aseptic anther culture was unsuccessful in some early reports (Rosati *et al.,* 1975; Niemirowicz-Szczytt *et al.*, 1983). 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 (Debnath *et al.*, 2007).

2.9 Strawberry Somatic Embryogenesis

Wang *et al.* (1984) 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 GA_3 or 2.22 µM BA + 0.54 µM NAA. Maintenance of the embryogenic cultures was, however, unsuccessful. Further Donnoli *et al.*, (2001) reported that 8% of the embryogenic calli in strawberry cultivar Clea was developed on MS medium supplemented with 4.88 µM BAP and 4.90 µM IBA. Somatic embryogenesis research with strawberries is still in a preliminary stage and some more efforts would be required to develop the technology (Graham , 2005).

2.10 Transplantation of *in vitro* grown 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. The transfer of *in vitro* plantlets to *ex vitro* conditions is one of the most critical factors of the micro-propagation 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, non-functional stomata and a weak root system. 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 CO_2 concentration. Another approach has been to change the environment during the multiplication and rooting stages, including increasing light intensity and CO_2 concentration in culture tubes, and decreasing the sugar concentration. All were found to be beneficial for plantlet growth in the later stages of micropropagation

(Abdelmalek *et al.*, 1995). 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 vesicular-arbuscular mycorhizal formation during acclimatization of cultural plantlets (Abdelmalek *et al.*, 1995)

2.11 Somaclonal variation

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. Callus-derived strawberry cv. Redcoat plants differ significantly from standard runner plant with several vegetative characteristics (Nehra *et al.*, 1992). These variants pose a problem for production of uniform, true-to-type plants. Nehra *et al.*, (1994) observed 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 regeneration from leaf disk. Some studies have shown that modified characteristics are epigenetic and disappear over time. Numerous authors have reported that genetic changes including insertions, deletions, point mutations and rearrangements occur during tissue culture, but few of the phenotypic symptoms found are heritable (Karp, 1995 and Kumar *et al.*, 1999). Damiano *et al.*, (1997) reported the somaclonal variation and *in vitro* regeneration of strawberry cultivar "Teodora' and 'Clea' on MS medium. 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. 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 (Tian *et al.*,2003). Yonghua *et al.*, (2005) 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. Plant off-types, i.e. non true-to-type and genetically not identical to the mother plant (Gaafar and Saker,2006). 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. Different changes include leaf color variants and dwarf plants, among others (Adel El-Sawy Mohammed., 2007). Presently, there are various methods available which can be used to detect and monitor tissue culture-derived plants and varietal 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. ISSR (Inter Simple sequence repeats) and RAPD (Random amplified polymorphic DNAs) are the molecular 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 (Adel El-Sawy, 2007). Polymorphisms also appear in acid-phosphatase, glutamate-dehydrogenase and peroxidase of regenerated plants.

Adel El-Sawy (2007) 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. DNA extracted from *in vitro*-derived plantlets and conventionally propagated plants were analyzed by RAPD to detect possible drift in genetic stability of micro-propagated plants. He observed that the banding pattern of *in vitro*-derived plantlets were similar with the fingerprints of mother 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. Ten deca-nucleotide primers (among 48 tested) were chosen for RAPD analysis, all of them indicating genetic stability for micropropagated plants of the investigated varieties of ornamental strawberry. A comparative study was conducted based on morphological parameters as well as genetic assessments using ISSR markers. The *in vitro* generated strawberry plants exhibited significantly vigorous morphological growth and earlier flower induction when compared to the plants propagated through planting of runners. Genetic assessment through ISSR showed no polymorphism in banding pattern and thus it was revealed that, there was no significant variation between micropropagated and conventional propagated plants at molecular level.

Sutan *et al.* (2009) studies the genetic stability of plants obtained from tissue culture propagation of ornamental strawberry "Serenata". Ten primers (from 48 previously tested) were selected and used in RAPD analysis to prove the clonal fidelity of the tissue culture-derived ornamental strawberry plants. They observed that there are similar banding patterns of the RAPD profiles obtaining from in *vitro* plants, regenerated via organogenesis or meristem culture. There are no differences with regard to different explants sources, number of subcultures or use of different basal medium with growth regulators.

2.12 Genetic fidelity study

Somaclonal variation has been reported in berry plants and concern has been expressed about the genetic stability of micropropagated plants (Debnath , 2013). Discrete morphological variants also found in micropropagated strawberries (Swartz *et al.*, 1981). Strawberry regenerants produced from anther culture have been demonstrated to vary with respect to earliness, calyx separation, rate of ripening, and mildew (*Sphaerotheca macularis* L.) tolerance (Simon *et al.*, 1987). The introduction of DNA-based markers allows direct comparisons of different genetic material, independent of environmental influences (Weising *et al.*, 1995). Somaclonal variants with fungal resistance in strawberry have been reported (Damiano *et al.*, 1997).Sporadic occurrences of abnormal fruit setting and a hyper-flowering habit that might be due to DNA methylation, have been reported in micropropagated strawberryplants (Boxus *et al.*, 2000). Somaclonal variation can result in a range of

genetically stable variations useful in crop improvement (Jain, 2001). ISSR markers cost less and are easier to use than AFLPs and do not require prior knowledge of flanking sequences, like SSRs (Reddy *et al.*, 2002). Although there are advantages for the use of micropropagation, there are concerns about genetic changes resulting from the process (Dale *et al.*, 2008). It is unpredictable in nature, and can be both heritable (genetic) and non-heritable (epigenetic). A number of molecular markers, including restriction fragment length polymorphism (RFLP), random-amplified polymorphic DNA (RAPD), arbitrary primed polymerase chain reaction, DNA amplified fingerprinting, simple (short) sequence repeat (SSR), short tandem repeat, sequence characterized amplified region, sequence-tagged sites (STSs), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), expressed sequence tag (EST)-PCR, and cleaved amplified polymorphic sequences derived from EST-PCR markers are available for genetic analysis of tissue culture-raised plants (Debnath, 2008a). The degree of similarity between banding patterns can provide information about genetic similarity and relationships between the samples studied. RAPD and ISSR marker analyses have been developed in the author's laboratory to identify genetic diversity in strawberry (Debnath *et al.*, 2008), and can be used to verify trueness-to-type of micropropagated strawberries. These markers have proved to be more reproducible than RAPD markers.

Debnath (2009) compared bioreactor-derived tissue culture (BC) 'Bounty' strawberry plants obtained from sepal explants grown ex vitro with those propagated by tissue culture on gelled medium (GC) and by conventional runner cuttings (RC), for growth, morphology, anthocyanin content, and antioxidant activity over three growth seasons. The BC and GC plants produced more crowns, runners, leaves, and berries than the RC plants although berry weight per plant did not differ significantly. BC and GC plants produced berries with more anthocyanin contents and antioxidant activities than those produced by the RC plants. But ISSR marker assay produced a homogenous amplification profile in the tissue culture and donor control plants confirming the clonal fidelity of micropropagated plants. However, it is imperative to regularly check the genetic purity of the micropropagated plants in order to produce clonally uniform progeny (Debnath, 2007). *In vitro* culture on nutrient media apparently induces the juvenile branching characteristics that favored enhanced vegetative growth with more crown, runners, leaf, and berry production. Whether the

useful agronomic traits observed in the first three seasons of these plants are stable has to be ascertained in subsequent years and in field trials.

Rout *et al.* (2009) reported that homogenous amplification of DNA profile for all micropropagted plant of banana variety Bantala as compared to mother plant. But in their experiment some plant of Grand Naine observed to be varied from the mother plant in ISSR primer IG-13.

Gantait (2010) reported that micropropagated and conventionally propagated plantlets of strawberry (*Fragaria* × *ananassa* Duch. cv. Chandler) were transferred to the similar field condition and growth stage. A comparative study was conducted based on morphological parameters as well as genetic assessments using ISSR markers. The *in vitro* generated strawberry plants exhibited significantly vigourous morphological growth and earlier flower induction when compared to the plants propagated through planting of runners. Genetic assessment through ISSR showed no polymorphism in banding pattern and thus it was revealed that, there was no significant variation between micropropagated and conventional propagated plants at molecular level.

Sultan *et al.* (2012) evaluated the genetic stability and uniformity of intergeneric hybrids 'Pink Panda' and 'Serenata' (ornamental strawberry plants) by RAPD markers. Micropropagated shoots selected at random from four subcultures onto either Murashige & Skoog or Lee & Fossard media, each of them supplemented with 6-benzylaminopurine (BAP) at 1.0 mg/l Indol-3-acetic acid (IAA) at 1.0 mg/l and gibberellic acid (GA_3) at 0.1mg/l, were subjected to molecular analysis. Ten deca-nucleotide primers (among 48 tested) were chosen for RAPD analysis, all of them indicating genetic stability for micropropagated plants of the investigated varieties of ornamental strawberry.

Table. 1. ***In vitro*** **studies of strawberry (*Fragaria* × *ananassa* Duch.)**

Source of Explant	Culture Medium	Response	References
Nodal segment	MS containing 1.5 mg/l BAP + 0.5 mg/l Kinetin	Shoot multiplication	Shaila harugade *et al.*, 2014
	MS + 1.0 mg/l IBA	Induction of rooting	
Runner segment,	MS + 0.5 mg/l BAP	Shoot multiplication	Ashrafuzzaman *et al* ., 2013
	MS+ 0.5 mg/l IBA	Root intiation	
Leaf disk, shoot tips, hypocotyls	MS + 2 mg/l of BAP + 2 mg/l IAA.	Shoot bud initiation	Madani *et al.*, 2013
Runner tip	MS+1.5 mg/l BA with 0.5 mg/l Kinetin.	Shoot multiplication	Ara *et al.*, 2013
Meristem	N6 medium + 1.0 mg/l BAP + 0.05 mg/l IBA + 0.05 mg/l GA_3,	Shoot multiplication	Ali-Akbar *et al.*, 2012
Fresh nodes	MS+ 1.5 mg/l BAP + 0.1 mg/l Kinetin	Shoot bud, shoot multiplication, induction of rooting, field establishment	Bhat .R. *et al.*, 2012
Shoot bud	MS + 0.5 mg/l Kinetin + 1.0 mg/l BAP + 2.0 mg/l GA_3 + commercial sugar	Root induction	Kaur *et al.*, 2012 Kaur *et al.*, 2005;
	MS + 1.0 mg/l IBA + 200 mg/l Charcoal + 20g/l commercial sugar	Root induction	
Leaf disc of mature leaf	MS+ 1.0 mg/l 2,4-D + 0.1 mg/l BAP	Induction of callus	Ara *et al.*,2012
Immature leaves	MS + 3.0 mg/l 2,4-D + 0.5 mg/l BAP	Callus induction	Karim *et al.*, 2011
	MS+ 1.5 mg/l BAP + 0.75 mg /l NAA	Shoot bud initiation, multiplication, rooting	
leaf blade, nodal explant, runner segments	MS + 4.0 mg /l NAA+ 1.5 mg/l IBA	Callus proliferation	Biswas *et al.*, 2010
	MS+3 mg/L BA.	Shoot regeneration	
Nodal segment	1.0 mg/l 2,4-D + 0.5 mg/l BAP + 50% Proline.	Development of somatic embryo	Biswas *et al.*, 2007
Nodal se gment	MS + 1.5 mg/l BAP + 0.5-0.1 mg/l Kinetin	Shoot multiplication	Sakila *et al.*, 2007
	MS+1.0 mg/l IBA	Root multiplication	
Leaf, petiole, Stolon segment	MS + 0.11 µM BA + 0.011 µM 2,4-D + 1 µM TDZ	Adventitious shoot bud regeneration	Folta *et al.*, 2006
leaf disk,	MS + 0.44-0.88 mg/l	Adventitious shoot bud	Debnath, 2005

Petiole segment	TDZ	regeneration	
Leaf segment	MS + 2 mg/l TDZ + 0.5 mg/l IBA	Adventitious shoot bud regeneration	Hanhineva *et al*,. 2005
Adventitious shoot	MS + 0.5 mg/l BAP + 0.1 mg/l GA_3 + IBA 0.1 mg/l + 40 g/l sucrose	Proliferation of axillary shoot, development of runner	Litwinczuk & Zubela, 2005
Leaf disk segment	MS + 1.5 mg/l TDZ + 0.4 mg/l IBA	Adventitious shoot bud regeneration	Qin *et al.*, 2005
Leaf disk segment	MS + 1.0 mg/l $AgNO_3$ + 1.5 mg/l TDZ + 0.4 mg/l IBA	Adventitious shoot bud regeneration	Qin *et al.*, 2005
Leaf, Petiole segment	MS + 0.5 mg/l BA + 0.1 mg/l GA_3 + 0.1 mg/l IBA	Adventitious shoot bud regeneration	Litwinczuk *et al.*, 2004
Leaf disk	½MS+ 1.0 mg/l BAP + 1.0 mg/l IAA	Adventitious shoot bud regeneration	Singh *et al.*,2004
Leaf, Petiole segment	MS + B5 vitamins + 2.2 µM TDZ + 0.3 uM IBA	Adventitious shoot bud regeneration	Zhao *et al.*, 2004
Leaf disc, Petiole, Stipule, Root segment	MS+ 1.0 mg/l BAP+ 0.1 mg/l IBA+ 0.2 mg/l GA3	Callus Proliferation	Passey *et al.*, 2003
	MS+ 3.0 mg/l IBA	Induction of rooting	
	MS+ 2.0 mg/l BAP + 0.5 mg/l TDZ+ 0.2 mg/l 2,4-D	Shoot bud regeneration	
Leaf disk, petiole, root, stipule explant	MS + 1 mg/l TDZ + 0.2 mg/l 2.4-D, MS + 2 mg/l BA + 0.5 mg/l TDZ + 0.2 mg/l 2,4-D, 1 mg/l TDZ + 0.2 mg/l NAA, 2 mg/l BA + 0.2 mg/l 2,4-D	Adventitious shoot bud regeneration	Passey *et al.*, 2003
Nodal segments	MS + 4.0µM 6-benzyladenine + 0.1 µM NAA	Shoot multiplication	Bhatt & Dhar, 2000
Leaf disk	N30K macrosalts + MS microsalts and vitamins (2 BA + 0.5 IBA)	Adventitious shoot regeration	Barcelo *et al.*, 1998
Leaf disk	MS + B5 vitamins + 2.3 mg/l BAP + 1.8 mg/l IAA	Adventitious shoot bud regeneration	Nehra *et al.*, 1989
Leaf tissue	LS + 3 mg/l BAP + 0.1 mg/l IBA + 400 - 600 mg/l Casein hydrolysate	Shoot bud regeneration	Liu *et al* .,1988

CHAPTER-3

MATERIALS AND METHODS

MATERIALS AND METHODS

The present study was carried out at Department of Agricultural Biotechnology, College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar, Odisha.

3.1 Selection of plant: Strawberry plant (*Fragaria* × *ananassa* Duchesne) cv. Chandler was selected for the experiment .

3.2 Taxonomy of Plants:

Kingdom	Plantae
Unranked	Angiosperms
Unranked	Eudicots
Unranked	Rosids
Order	Rosales
Family	Rosaceae
Sub- family	Rosoideae
Genus	*Fragaria*
Species	*F.* × *ananassa*
Botanical name:	*Fragaria* × *ananassa* Duchesne

3.3 MATERIALS

3.3.1 Plant material

The explants of strawberry cv. Chandler were collected from the garden of Dr. A.K. Das, Orissa University of Agriculture & Technology, in the month of August – September during the year 2014.

3.3.2 Explants source

Leaf disces, runner tips, roots, stem cuttings, petioles, embryoes, flower buds, meristems and nodal explants were collected from the potted plants of Strawberry (*Fragaria* × *ananassa* Duchesne) cv. Chandler were used for *in vitro* studies. The experiment was carried out in the laboratory of Department of Agricultural Biotechnology, College of Agriculture, Bhubaneswar.

3.4 Plant nutrient media

For *in vitro* studies, the common basal media MS (Murashige and Skoog, 1962) was used to carry out the different *in vitro* experiments (Appendix-II). The inorganic and organic constituents of the MS media are listed in Table 2. Three percent sucrose was routinely used as the carbon source. The growth regulators and other additives were included in the basal media, either alone or in combinations, to test their efficiency for inducing, promoting or regulating the process of growth and differentiation.

3.4.1 Plant growth regulators

For standardization of *in vitro* shoot multiplication, rooting and plant regeneration, different auxins like Indole-3-acetic acid (IAA),Indole-3- butyric acid (IBA),1-Naphthaleneacetic acid (NAA) and 2, 4-dichlorophenoxy acetic acid (2,4-D) and cytokinins like 6-benzylaminopurine (BAP), Adenine Sulphate (Ads) and kinetin (Kn) at different concentration were used. The solution was prepared as given below.

3.4.2 PREPARATION OF THE PHYTOHORMONE STOCK

a) **1 mM NAA stock solution 100 ml (MW: 186.2g)**

18.62 mg of NAA was dissolved in 0.5ml DMSO (Dimethyl Sulfoxide) and sterile double distilled water was added and stirred. Finally the volume made-up to 100ml and stored at 4°C.

b) **1 mM IAA stock solution 100 ml (MW: 175.2g)**

17.5 mg of IAA was dissolved in 0.5ml 1 N NaOH and sterile double distilled water was added and stirred. Finally the volume made-up to 100ml and stored at 4°C.

c) **1 mM IBA stock solution 100 ml (MW: 203.2g)**

20.3 mg of IBA was dissolved in 0.5ml 1 N NaOH and sterile double distilled water was added and stirred . Finally the volume made- up to 100ml and stored at 4°C.

) **1 M 2,4-D stock solution 100 ml (MW: 221mg)**

22.1 mg of 2,4-D was dissolved in 0.5ml 1N NaOH and 10 ml water by vortexing and the volume made-up to 100ml by adding sterile double distilled water. Stored at 4°C.

) **1 mM BAP stock solution 100 ml (MW. 224.2mg)**

22.5 mg of BAP was dissolved in in 1N NaOH (0.3-0.5 M) and double distilled water was added slowly with stirring and volume made-up to 100ml and stored at 4°C

d) **1 mM Kinetin stock solution 100 ml (MW: 215.2mg)**

21.5 mg of Kn was dissolved in 0.5ml of 0.5N NaOH and double distilled water was added slowly while stirring, and the volume made-up to 100ml. Stored at 4°C.

e) **Ads**: Adenine sulphate was first dissolved in a little quantity of hot water then added to the medium.

All the prepared solution was filter sterilized and kept in the freeze for further use.

3.4. 3 Antibiotics (source: M.P. Biomedical)

Reframycin, used for the experiment was purchased from EMERK, Biosciences, India and appropriate stocks were prepared. Stocks of antibiotic were prepared as given below:

Reframycin: 1g of reframycin was dissolved in 50% ethanol. Filter sterilized by using syringe filter into sterile eppendorf tube aseptically and stored at 4^0c.

3.5 Sterilization

3.5.1 Sterilization of glassware and Media

Sterilization refers to any process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses, spore forms, etc.) from the working surface, equipments or biological culture medium. Sterilization can be achieved through application of heat, chemicals, high pressure or filtration.

3.5.2 Steam sterilization

A widely-used method for heat sterilization is the autoclave. Autoclaves commonly use for steam sterilization at 121^0C. To achieve sterility, a holding time of at least 15 minutes at 21^0C. Additional sterilizing time is usually required for liquids and instruments packed in layers of cloth, as they may take longer to reach the required temperature. After sterilization, autoclaved liquids kept in the media preparation room for normal temperature for further use.

3.5.2 Filter sterilization

Hormones that would be damaged by heat irradiation can be sterilized by mechanical filtration. This method is commonly used for sensitive pharmaceuticals and protein solutions in biological research. A filter with pore size 45μm will effectively remove bacteria. The filtration equipment and the filters themselves may be purchased as pre-sterilized disposable units in sealed packaging, or must be sterilized by the user, generally by autoclaving at a temperature that does not damage the fragile filter membranes.

3.6 Glassware's used

Glassware's like culture tubes (25×150 mm) , conical flasks (250 ml), petriplates (80 mm) and beakers (500ml) etc., were purchased from Borosil (India) and used for the experiments. All the chemicals and plant growth regulators were of analytical grade and are procured from standard chemical manufacturing companies.

3.6.1 Cleaning of glassware's

Glassware's were rinsed in water and then soaked in 0.15% chromic acid overnight. The chromic acid was drained out and the glassware's were washed with clean soap solution. The thoroughly washed glassware's are rinsed in distilled water and dried in a hot air oven. The instruments like forceps, scalpels etc., were also cleaned and dried.

3.6.1 Sterilization of glassware's

Clean glassware's were rinsed in double distilled water and dried in oven at 80^0C and sealed with aluminium foil, petriplates placed in autoclavable covers small instruments like scalpel, forceps, and blade holders wrapped in aluminium foil were autoclaved at 121^0C in 17 lbs pressure for 15 minutes. The glassware's were then transferred to sterile inoculation chambers.

3.7 Disinfection of laminar Air flow chamber

All steps in this experiment like the sterilization of explants preparation, inoculation of the explants, sub culturing were conducted under aseptic condition in the laminar air flow cabinet. Before the laminar air flow cabinet was used, the working surface of the chamber is sterilized by swabbing with 70% alcohol. The chamber was then exposed to UV light for 15 minutes. The walls of the chamber were also swabbed with 70% alcohol to ensure total sterility. Before taking the materials into cabinet, they were swabbed with 70% alcohol. In case of glassware's, the mouth of the bottles, flasks etc. are flamed before and after use. Before starting the experiment, the hands are swabbed well with alcohol.

3.8 Preparation of explants

For the surface sterilization, the explants first were washed thoroughly in running tap water for 30 minutes. After that they were again washed with liquid detergent (Labolene, Qualigen, India) or Tween 20 (Himedia Laboratories, India) for 10 minutes with vigorous shaking. After washing with detergent, the explants were again washed

with running tap water to remove any traces of detergent for 30 minutes and kept in 1% w/v solution of Bavistin (BASF India Limited) for 15 min. Further, the explants were taken out and dipped in 70% ethyl alcohol for 30 seconds. After alcohol dip, explants were surface sterilized with freshly prepared 0.1% (w/v) aqueous solution of mercuric chloride for 7 minutes. Subsequently, the explants were thoroughly washed with sterile distilled water for 2 - 3 times to remove any traces of mercuric chloride.

Table 2 . Composition of basal MS medium

MACRO SALT	(mg/l)
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2o$	440
KH_2PO_4	170
MICRO SALTS	**(mg/l)**
$MnSO_4.\ H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025
Na_2Fe-EDTA	37.24

ADDITIVES	(mg/l)
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	30000

Amount of stock solutions added to the media

S no	Stock solution	Strength	Amount to be added (ml)
1.	Macronutrients	20X	50
2.	Micronutrients	1000X	1
3.	Iron source	200X	5
4.	Organic supplements	1000X	1

Agar (8g/l) and Myoinositol (100mg/l) were added separately

3.9 Nutrient concentration of the media

The strength of basal culture medium was modified to examine the effect of concentrations of organic and inorganic nutrients. Half strength MS medium was prepared by reducing all the salts into half except iron, glycine, vitamin. Half strength, one fourth strength, one eight strength, doubled and 2.5 times MS medium was prepared by adjusting all the salts into half /quarter except iron, glycine and vitamin. All the basal media mentioned above were supplemented with usual dosage of sucrose, inositol, cytokinins and auxins for shoot multiplication and callus induction.

3.10 Gelling agent

Agar, the widely used gelling agent was used to solidify the medium in all the experiments. Six gram/litre of agar was added to the medium after adjusting the pH (5.8), prior to autoclaving. In specified experiments, different concentrations of agar (4, 5, 6, 7, 8 or 10g/l) was used to optimise the medium for plant production.

3.11 pH of the media

After mixing all the salts and hormones the volume of the media was made up using distilled water. The pH of the media was adjusted to 5.8 in all experiments. In specified experiments the pH of the media was adjusted to different levels (5.0, 5.5, 5.8, 6.0, 6.2 and 6.5) to study the optimum level of pH suitable for strawberry.

3.12 Source of carbohydrate and its concentration

A carbohydrate supplement to the medium was essential to maintain the osmoticum for normal growth of explants. To determine the most suitable source

of carbohydrate, the medium was supplemented with D-glucose, sucrose, fructose, maltose, mannitol and commercial sugar. To determine a suitable concentration of sucrose as a carbon source another experiment was laid with different concentrations of sucrose (0, 10, 20, 30, 40 and 50g/l) in the shoot multiplication medium in addition to growth regulators.

3.13 Culture condition

In the initial experiments, all the cultures were incubated under 16h photoperiod at a light intensity of 3000 lux provided by cool, white, fluorescent lamps (Phillips, 1994). The temperature and humidity of the incubation room was maintained at 25±2^{0}C and 65-70% RH (Relative Humidity) respectively.

3.14 Light intensity and Photoperiod

In separate experiments in an environmental growth cabinet, different light intensities (1000, 2000, 3000, 4000, 5000 and 6000 lux) and different photoperiods (0, 8, 12, 16, 20 and 24 h) were used; the light intensities were regulated by adjusting the distance between the light source and selves on which the cultures rested. The photoperiod was maintained by an in-built timer in the growth cabinet. While doing experiments on light intensity, the photoperiod was kept constant at 16 h. The light intensity was maintained at 3000 lux while studying the effect of photoperiod. In all the cases temperature was maintained at 25±2^{0}C.

3.15 Temperature

In most cases the incubation temperature was maintained at 25±2^0 C. But for studying the effect of temperature on shoot multiplication, the cultures were incubated under different temperature (18, 20, 22, 25, 28 and 30^{O}C) with a light

intensity of 3000 lux under 16 h photoperiod. The experiment was carried out in an environment growth cabinet.

Different equipments used during the course of experiment has been mentioned in Appendix IV.

3.16 Standardization of regeneration protocol

3.16.1 Culture conditions

All the *in vitro* culture experiment was carried out aseptically in a laminar airflow chamber and the culture were incubated in the culture room maintained at 25±2^{0}C, under 3000 lux intensity with fluorescent tubes over 16 h photoperiod.

3.16.2 Inoculation of explants

The sterilized explants (leaf, stem, runner tip , root and internodal cuttings) were inoculated in culture tubes and bottles aseptically with the help of sterile forceps under aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed to make them in suitable sizes. After cutting explants into suitable size (2-6 mm), explants are transferred to culture bottles containing MS medium with various concentration of auxins and cytokinins. After vertically inoculating the explants in the culture tubes, the mouth of tubes was quickly flamed and tightly capped to avoid entry of external air. After proper labelling, clearly mentioning media code, date of inoculation etc. the tubes were transferred to growth room.

3.16.3 Direct Organogenesis

The meristem tips were inoculated into the shooting media supplemented with different concentration of auxins and cytokinins . After formation of sufficient multiple shoots, these shoots were separated and transferred to the rooting media where different concentration of auxins (IBA, NAA) used for observing the efficient rooting response in *in vitro* regenerated shoots.

3.16.4 Callus induction

The different explants (internodal cuttings, matured leaves, young leaves, flower buds) were cultured on MS medium supplemented with different growth regulators and incubated in culture room for callus induction. Observations were recorded on callus weight after 30 days, callus induction frequency, colour and nature of callus. Response of explants to callus initiation was assessed by calculating number of explants responded for callus initiation and expressed in percentage.

$$\text{Percent callus induction} = \frac{\text{No. of explants with callus initiation}}{\text{Total no. of explants cultured}} \times 100$$

3.16.5 Regeneration via callus

Healthy calli were taken in a sterile petridish and chopped into small pieces with a fresh weight of approximately 100 - 120 mg, then inoculated on shoot regeneration medium containing different concentration of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) and NAA (0.2 and 0.5 mg/l). Green shoot primordia developed on the surface of calli within 4-5 weeks. The leaves and shoots elongated subsequently, so the number of shoots formed per callus were investigated after 4 weeks of culturing. Regenerated shoots were then transferred to MS medium with BAP and NAA for shoot multiplication and subsequent induction of best roots.

$$\text{Regeneration frequency} = \frac{\text{No. of regenerated from calli}}{\text{Total no. of callus inoculated}} \text{ X } 100$$

3.16.6 Rooting of microshoots

Newly grown shoots measuring about 1 – 2 cm in length were excised individually from the parent culture and transferred to different rooting media. The microshoots were transferred to MS basal media without growth regulators and with growth regulators like IBA or NAA with 2-3% sucrose. Full strength and half strength MS medium was also tested for this experiment.

3.17 Acclimatization

After 4 weeks of culture on rooting media, the plantlets were shifted to plastic pots for their primary hardening prior to final transfer to soil under natural conditions. For hardening of plants, plants with newly formed roots were taken out from the culture tubes with the help of forceps with utmost care to prevent any damage to newly formed roots and washed in tap water to remove the agar gelled medium. The rooted plantlets were carefully planted in plastic pots containing 1:1:1 mixture of sand, soil and farmyard manure. After planting the plants were thoroughly watered and kept under greenhouse with 85% humidity and 30°C temperature for ten days. There after the plants were shifted to shade house with less humidity level and indirect sunlight. In shade house, watering was made in everyday two times to prevent wilting.

3.18 DNA Profiling

Mother plant as well as the *in vitro* raised plants of strawberry (*Fragaria* × *ananassa* Duchesne) were taken for genetic fidelity test .

3.18.1 Sample collection

Four to five young, fresh leaves were collected from both the tissue culture grown regenerates and mother plants, grown in the greenhouse of Dept. of Agriculture Biotechnology, C.A., OUAT, Bhubaneswar. The leaves were cut, wrapped in aluminium foil and brought to the laboratory.

3.18.2 DNA extraction and purification

The leaves were first washed, air dried weighed to 2 gram and then crushed on to a sterilized pestle and mortar in liquid nitrogen to obtain fine powder. Care was taken to avoid thawing of the material. Then fine powder was transferred into 50 ml Falcon tube with 10 ml extraction buffer (Appendix- III) maintained at 65°C in a water bath. The Falcon tube was incubated at 65°C for 2 hours in hot water bath. Intermittent shaking (4-5 times) was given for complete mixing and formation of emulsion. After that equal

volume of choloform: isoamyl alcohol (24:1) was added and mixed them by inverting for 7-8 minutes. After complete emulsion formation, centrifugation was done at 10,000 rpm for 20 min at 4°C. The aqueous phase was transferred to a fresh centrifuge tube and then isopropanol (twice the volume of aliquot) and 300µl of 3M sodium acetate was added. DNA was allowed to settle down for overnight. Then, DNA was spooled out and the excess chemicals were drained out with a pipette. To wash the salt impurities and some other residues present in the isolated DNA, 0.5 ml of 70% ethyl alcohol was added. The contents were mixed gently and incubated for 30 min. The contents were decanted by gentle spinning and then added 70% ethyl alcohol and kept for 2 hours. The alcohol was decanted and the pellets were dried under vacuum dryer (lyophilizer) at -40°C for 45 min. The DNA was dissolved in 300 µl of TE (10:1) buffer (pH 8.0) and kept overnight for complete dissolution. Then 7µl RNase solution was added to the tube containing dissolved DNA and incubated at 37°C for 1 hour. Equal volume of phenol: chloroform: isoamyl alcohol (1:2:1) was added and mixed properly for 2 min and stirred for 5 min. The DNA supernatant was taken out and again added equal volume of chloroform: isoamyl alcohol and mixed well and centrifuged for 10 min. The aqueous layer was removed and repeated to produce creamy colour solution. Then $1/10^{th}$ amount of 3M sodium acetate and 2.5 volume of absolute chilled alcohol were added to remove aqueous layer, mixed it gently so that DNA could precipitate down and kept overnight or for an hour at -20°C when precipitation was not observed. The solution was then centrifuged at 8000 rpm for 5 min. and the supernatant was decanted off. vacuum, dissolved in TE (10:1) buffer at room temperature and stored frozen at -20°c.

3.18.3 Measurement of DNA concentration and quality checking

The concentration of DNA was estimated by the measurement of the UV irradiation absorbed by nucleic acid bases. First the spectrophotometer was calibrated using 2000 µl of TE in a quartz cuvette at 260 nm and 280 nm. Then 5 µl of DNA sample was added to 995 µl of TE, mixed well and absorbance (OD) was taken at 260 nm and 280 nm. The concentration of the DNA in the sample was estimated as follows,

Concentration of DNA (µg/ml) = OD at 260 x Dilution factor x 50.

The ratio between readings at 260 nm and 280 nm (OD 260 / OD) provided an estimate for the purity of nucleic acid. Any sample showing the ratio below 1.8 or above 2.0 was further subjected to purification. Further, in order to know the concentration and intactness of genomic DNA, an aliquot (2 µl) of each sample was subjected to agarose gel (0.8 % w/v) electrophoresis for about 2 hours along with 500 ng of molecular weight marker (Lambda / *EcoR*I digest). The gel was stained with ethidium bromide (0.5µg/ml), viewed under Gel-Doc system (UVITECH) and photographed immediately for further interpretation. By comparing the fluorescent intensity of the bands of genomic DNA with the standard molecular weight marker (Lambda / *EcoR*I digest), DNA concentration was estimated following the method described by Sambrook *et al.* (1989). Part of the stock DNA samples was diluted with appropriate amount of TE buffer to obtain a working concentration of 10ng/µl. The diluted DNA samples were stored at 4°C for further analysis.

Optimisation of PCR employing ISSR primer

Component Stock	Final conc.	Per reaction
10x buffer	1x	2.5 µl
dNTPs (10 mM)	200 µM	2 µl
Primer	5 µM	2 µl
Taq polymerase	1 unit	1 µl
Deionised water	-	15.5 µl
Total	-	23 µl
DNA	20-50 ng	2 µl

Table 3. List of 12 Primers used for accessing the genetic fidelity of Tissue culture derived plantlets of Strawberry cv. Chandler

Primer	Primer sequence
UBC 808	AGA GAG AGA GAG AGA GC
UBC 811	GAG AGA GAG AGA GAG AC
UBC 812	GAG AGA GAG AGA GAG AA
UBC 813	CTC TCT CTC TCT CTC TT
UBC 815	CTC TCT CTC TCT CTC TG
UBC 825	ACA CAC ACA CAC ACA CT
UBC 834	AGA GAG AGA GAG AGA GYT
UBC 840	GAG AGA GAG AGA GAG AYT
UBC 842	GAG AGA GAG AGA GAG AYG
UBC 857	ACA CAC ACA CAC ACA CYT
UBC 860	TGT GTG TGT GTG TGT GRA
UBC 864	ATGATGATGATGATGATG

Twelve customs synthesized (M/s Bangalore Genei, Bangalore) ISSR primers were choosen for the study. Polymerase chain reactions (PCR) with different primers were carried out by using 20ng template DNA.

The optimum thermocycler conditions employing ISSR primers were standardized as follows:

I. Pre-denaturation at 94°C for 5min.

II. Fourty cycles of denaturation, annealing and extension was as follow

PCR step	Temp ^{0}C	Duration
Denaturation	94 ^{0}C	1min
Annealing	AT ^{0}	1min
Extension	72 ^{0}C	2min

Note: AT°= Annealing temperature which varies with primer to primer.

III. Followed by final extension at 72°C for 15 min.

3.18.4 Agarose gel Electrophoresis and gel documentation

Agarose gel (1.5%) was prepared by mixing 3.0 g agarose in 200ml of 1 × TBE buffer and boiled in micro-oven till it completely dissolved. During warming intermittent shaking were done 4-5 times to prevent formation of clumps of agarose. After complete dissolve the molten agarose was kept for cooling at 50-60°C. The gel casting tray was cleaned by 70% ethyl alcohol and was levelled. Staining dye ethidium bromide (5µl/100ml) was thoroughly mixed in molten agarose and then was poured in the casting plate containing comb and kept for solidification. Then the casted gel was transferred to electrophoresis unit containing 1X TBE buffer. PCR amplified products were mixed with 5.0µl of 6x loading dye and then loaded into the wells of the gel along with DNA ladder. Gel was run at 100 volts for 2 hours. The run was stopped when bromophenol blue dye has travelled 2/3 of the length of the gel. Stained DNA/gels were placed on the Gel Doc. system (UVITECH, Cambridge, UK) and was photographed under U.V. light.

3.19 Statistical analysis

As all the studies were done in laboratory under controlled condition with maintaining temperature, light intensity and photoperiod. All the experiment is completely randomized design (CBD). For accurate statistical analysis, each experiment was repeated. All the data were analyzed using ANOVA test, used for comparisons among means.

CHAPTER-4

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

The present investigation was carried out for standardization of *in vitro* protocol on plant regeneration of *Fragaria* × *ananassa* Duch. cv. Chandler and its evaluation of genetic fidelity through molecular marker. The results of different experiments were presented in this chapter.

In vitro plant development is considerable to be completely dependent upon the species. The tissue can be regenerated into whole plant (totipotency) through shoot tip, leaf, roots and axillary buds or stem segment. Both intrinsic and extrinsic factors that influence the morphogenic response of explanted tissue under culture condition. These factors were either physical or physiological in nature depending upon the type of explants used, media composition and culture environment. The culture media contains inorganic and organic salts (macronutrients, micronutrients and vitamins) as well as energy source (sucrose), growth regulators and agar-agar are used as nutrient source to plant. In most of the cases, Murashige and Skoog (1962) basal medium was used for plant growth and development. Growth regulators like auxins and cytokinins were used for shoot proliferation, adventitious shoot development, root induction and callus proliferation. Shoots produced through *in vitro* are generally easy to induction of root by manipulating the growth regulators, nutrient media and culture condition. The resulting rooted shoots are transfer to greenhouse for acclimatization and subsequently transferred to field condition. *In vitro* culture systems are also been adapted for mass multiplication of various plant species including many horticultural crops like banana, strawberry, orchids, pomegranate, pineapple as well as the medicinal and aromatic plants.

4.1 *In vitro* plant regeneration of *Fragaria* × *ananassa* Duch. cv. Chandler

The standardization of protocol on an efficient plant regeneration is utmost important for successful commercialization of plant and also crop improvement through genetic transformation. The present investigation deals with the induction of shoot multiplication either from meristem tip or internodal segments by manipulating growth regulators and physiological condition. Various concentration of plant growth regulators

in the culture medium and additional media amendments also play a determining role in morphogenesis.

4.1.1 Effect of sterilizing agents

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 exudates of bacteria was observed around the base of the explants after 2-3 days. The problem was complicated further by the latent nature of the contaminants.

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 explants and endogenous microflora present in the explants.

The inoculated runner tips were proliferated within 2–3 weeks. But after some days it was found that some explants are contaminated by fungi and bacteria. For this contamination problem , the shoot tips were surface sterilized with 0.5% carbendazim (Bavistin) for 15 minutes then surface sterilization was done by taking mercuric chloride ($HgCl_2$) at a concentration of 0.1% for 7 minutes, then these explants were rinsed for 5 times with doubled distilled water to run out excess mercuric chloride. Tween-20 @ 1% was also used for ten minutes before surface sterilization with $HgCl_2$. With addition to it reframycin 50 mg/l was added to the culture media. Finally the excised shoot tips were put on a Petri disc on filter paper to remove excess water to avoid contamination just before inoculation.

4.1.2 Direct regeneration

Generally, direct regeneration achieved by shoot proliferation from runner tips. Various explants like cotyledonary leaf, shoot-tip and meristem are used for direct regeneration by formation of multiple shoots. Different concentrations of growth regulators (BAP and NAA) along with MS medium were used for shoot induction and multiple shoots per explants. Stages of direct regeneration from incubated runner tips of

Fragaria × *ananassa* Duch. cv. Chandler on MS medium fortified with plant growth hormone were presented in figure 1.

4.1.2.1 Effect of cytokinins on shoot multiplication

Three cytokinins (BAP, Kn and Ads) have been tested for shoot multiplication from apical meristem, runner tips, and first node of runner shoots of *Fragaria* × *ananassa* Duch. cv. Chandler and the experimental findings have been given in Table 4. Among the three cytokinins, 6-benzylaminopurine and kinetin favoured maximum rate of shoot proliferation. Without cytokinin, the shoots did not show any positive response. The medium supplemented with BAP, Kinetin and adenine sulphate observed to provide the maximum rate of shoot growth and multiplication. Among the different concentrations of nutrient tried, the full strength MS medium supplemented with BAP, Kinetin and adenine sulphate showed significant response as compared with other treatment. The explants initially exposed to tap water for longer period and subsequently transferred to culture medium showed the positive impact on shoot growth. The moderate concentrations of BAP (1.0 mg/l) along with 0.25 mg/l kinetin and 50 mg/l adenine sulphate showed higher response in case of *Fragaria* × *ananassa* Duch. cv. Chandler. Low concentration of BAP was found to be ineffective for shoot induction. The shoot of *Fragaria* × *ananassa* Duch. cv. Chandler showed maximum percentage of multiplication (85.8%) on MS medium supplemented with BAP 1.0 mg/l along with 0.25 mg/l kinetin and 50mg/l of adenine sulphate . Increase the concentration of from 0.25 to 3.0 mg/l BAP showed increase in the percentage of response as well as the number of multiple shoots per culture was also increased. The range of shoots varied from 1.1 to 3.7 and the average number of shoots was 1.8 per culture.

The combinations of cytokinins favour the shoot proliferation and elongation. It indicates that cytokinin/auxin ratio being the principal players in the induction of shoot multiplication from explants. The present findings suggest a high frequency of shoot production from organogenic calli could be obtained by manipulating the growth regulators and culture condition. There were differences between treatments both in the percentage of cultures with response and in the mean number of shoot buds per culture.

However, the molecular mechanisms through which auxin-cytokinin crosstalk act in concert to exert the shoot meristem induction are still poorly understood. A lower concentration of BAP 0.25 mg/l in the culture medium inhibited the growth of the shoot buds. Percentage of response per culture varied from 13.6 to 85.8 % in case of runner tips in different treatments. The rate of shoot bud regeneration increased as the number of subcultures increased.

Table. 4. Effect of cytokinins on shoot multiplication of *Fragaria* × *ananassa* Duch. cv. Chandler

MS+ Growth regulators(mg/l)			Percentage of plants developed multiple shoot (Mean±S.E.)*	Number of shoots/ culture (Mean±S.E.)*
BAP	Kn	Ads		
0	0	0	0	0
0.25	0	0	13.6±0.3a	1.1±0.1a
0.5	0	0	21.5±0.9b	1.2±0.03a
1.0	0	0	65.7±0.6h	1.3±0.04a
1.5	0	0	68.9±1.1i	1.6±0.1a
2.0	0	0	77.1±1.3j	2.6±0.2b
3.0	0	0	78.2±1.3j	2.4±0.1b
0	0.25	0	21.5±0.9b	1.8±0.1a
0	0.5	0	39.9±0.8c	2.3±0.2b
0	1.0	0	41.6±0.7c	2.2±0.2b
0	1.5	0	50.7±1.0d	2.4±0.2b
0	2.0	0	52.5±0.6e	1.5±0.1a
0	3.0	50	61.3±0.6g	1.8±0.2a
1.0	**0.25**	**50**	**85.8±0.8 k**	**3.7±0.2c**
2.0	0.25	50	78.2±1.3 j	2.8±0.2b
3.0	0.25	50	77.1±1.3j	1.4±0.1a
1.0	0.25	50	68.9±1.1 i	2.6±0.2b
1.5	0.5	50	76.1±0.7 j	3.1±0.2c
2.0	0.5	50	65.7±0.6 h	2.2±0.2b
3.0	0.5	50	54.5±0.7 f	1.6±0.1a
1.0	0.5	50	60.8±0.8 g	1.4±0.1a
1.5	1.0	50	55.4±0.7 f	1.9±0.2a
2.0	1.0	50	42.3±0.7 c	1.3±0.04a
3.0	1.0	50	41.1±0.5 c	1.1±0.04a
3.0	1.5	50	43.7±0.8 c	1.1±0.04a

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05)

4.1.2.2 Effect of auxins on shoot induction

Three auxins (IAA, IBA and NAA) have been tested for shoot multiplication from apical meristem, runner tips, and first node of runner shoots of *Fragaria× ananassa* Duch. cv. Chandler. Among these three auxins NAA favoured maximum rate of shoot proliferation. Without auxins, the shoots did not show any positive response. The medium supplemented with NAA and IAA showed the maximum rate of shoot growth and multiplication. Among the different concentrations of nutrient tried, the full strength MS medium supplemented with NAA and IAA showed significant response as compared with other treatment. The lower concentrations of NAA (0.1 mg/l) along with 0.25 mg/l IAA showed higher response in case of *Fragaria×ananassa* Duch. cv. Chandler. The shoot of *Fragaria×ananassa* Duch. cv. Chandler showed maximum percentage of multiplication (80.7%) on MS medium supplemented with NAA @ 0.1mg/l along with 0.25 mg/l IAA . Increase the concentration of from 0.1 to 1.0 mg/l NAA showed increase in the percentage of response as well as the number of multiple shoots per culture was also increased. The range of shoots varied from 1.1 to 2.8 and the average number of shoots varied from 1.5 per culture. The lower percentage of shoot multiplication was obtained when we used only IAA @ 0.1 mg/l and the detailed result has been reported in the Table 5. Hence the result from this experiment clarified that combination of auxin and cytokinin is essential for better organogenesis of *Fragaria* × *ananassa* Duch. cv. Chandler.

Table 5. Effect of auxin on shoot multiplication of *Fragaria* × *ananassa* Duch. cv. Chandler

MS+ Growth regulators(mg/l)			Percentage of plants developed multiple shoot (Mean±S.E.)*	Number of shoots/ culture (Mean±S.E.)*
IAA	NAA	IBA		
0	0	0	0	0
0.1	0	0	10.2±0.06a	1.1±0.1a
0.25	0	0	25.4±0.7b	1.1±0.1a
0.5	0	0	25.4±0.7b	1.4±0.1a
1.0	0	0	25.6±0.8b	1.3±0.04a
0	0.1	0	50.8±0.9c	1.3±0.04a
0	0.25	0	53.7±0.9d	1.4±0.1a
0	0.5	0	61.6±0.8f	1.5±0.1a
0	1.0	0	66.8±0.5g	1.2±0.03a
0	0.25	0.25	71.1±1.3h	1.2±0.03a
0.25	0.25	0.25	77.4±1.2i	1.3±0.04a
0.25	**0.1**	**0**	**80.7±0.6j**	**2.8±0.2b**
0.5	0.1	0	74.9±1.1i	2.4±0.2b
1.0	0.1	0	76.4±0.8i	2.5±0.2b
0.25	0.25	0	70.1±0.9h	2.2±0.2b
0.25	0.5	0	75.2±0.6i	2.4±0.2b
0.25	0.5	0.25	74.8±0.9i	1.9±0.2a
0.5	0.5	0	76.1±0.8i	2.3±0.2b
0.5	0.5	0.5	77.6±0.7i	1.3±0.04a
1.0	0.5	0	78.3±0.9i	1.7±0.2a
0.25	1.0	0.25	79±1.0i	1.1±0.05a
0.25	1.0	0	75.3±0.6i	1.2±0.03a
0.5	0.1	0.1	58.4±0.9e	1.6±0.1a
0.5	0.1	0.5	61.6±0.8f	1.1±0.05a
1	1	0	66.8±0.5g	1.1±0.05a

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level.(Post-hoc multiple comparison test, p=0.05)

4.1.2.3 Effect of cytokinins and auxins in shoot multiplication

The combination of cytokinins and auxins also favoured positive impact on shoot proliferation and multiplication which has been represented in figure 2. In the present investigation, one best cytokinin and two auxins were tested for shoot proliferation and multiplication. Different concentration of these three plant growth regulators and their impact on shoot multiplication of strawberry has mentioned in the Table 6. Among the two auxins tested, BAP along with NAA favoured the maximum rate of shoot multiplication. The medium supplemented with BAP and IAA did not show any positive response. In medium having 1.5 mg/l BAP along with 0.1mg/l NAA showed the highest rate of shoot growth and proliferation in case of of *Fragaria* × *ananassa* Duch. cv.

Chandler. Increase the concentrations of either BAP or NAA, the cultures did not showed any more positive response. In case of of *Fragaria×ananassa* Duch. cv. Chandler, the maximum percentage (89.8%) of shoot proliferation was observed in MS medium supplemented with 1.5 mg/l BAP, 0.1 mg/l NAA and 3% sucrose within 4 weeks of culture .The average number of multiple shoots per culture was varied from 1.1 to 4.3 in different treatments. Low percentage of shoot multiplication (13.6%) was obtained when the medium having MS used with 0.25 mg/l BAP,1.5 mg/l IAA, 0.2 mg/l NAA with full strength MS medium. But in this same condition numbers of shoots are not significantly varied with some of the treatments. Highest number of multiple shoot 4.3 was also obtained from the same media supplemented with 1.5 mg/l BAP, 0.1 mg/l NAA and 3% sucrose.

Table.6 Effect of auxin and cytokinin on shoot multiplication of *Fragaria* × *ananassa* Duch. cv. Chandler

MS+ Growth regulators(mg/l)			Percentage of plants developed multiple shoot (Mean±S.E.)*	Number of shoots/ culture (Mean±S.E.)*
BAP	IAA	NAA		
0	0	0	0	0
1.0	0.1	0	65.7±0.6c	1.1±0.05a
1.25	0.1	0	68.9±1.1c	1.2±0.03a
1.5	0.25	0	76.1±0.7d	1.3±0.04a
2.0	0.25	0	76.1±0.7d	1.9±0.2a
3.0	0.5	0	77.4±1.0d	1.3±0.04a
1.0	0	0.1	78.2±1.3d	1.7±0.2a
1.25	0	0.1	78.3±0.9d	1.9±0.2a
1.5	0	0.25	80.7±0.6d	2.9±0.2b
2.0	0	0.25	80.1±0.7d	2.8±0.2b
3.0	0	0.5	79±0.9d	2.4±0.2b
0.25	1.5	0.2	13.6±0.3a	2.9±0.2b
0.5	1.5	0.2	52.5±0.6b	3.6±0.2c
1.0	1.5	0.2	61.6±0.8c	2.6±0.2b
2.0	0.5	0.2	70.1±0.9d	2.3±0.2b
1.5	2.0	0.2	75.3±0.6d	2.4±0.2b
2.0	2.0	0.2	76.1±0.7d	3.2±0.2c
2.0	2.0	0.2	78.3±0.9d	3.6±0.2c
1.5	**0**	**0.1**	**89.8±0.9e**	**4.3±0.4d**
1.5	0.1	0.1	84.9±0.7 e	3.6±0.2c
1.5	0.1	0.1	80.7±0.6d	3.1±0.2c
2.0	0.1	0.1	76.1±0.7d	1.4±0.2a
2.0	0	0.1	74.8±0.9d	1.1±0.05a
3.0	0	0.5	65.7±0.6c	1.4±0.1a
3.0	0.1	0.5	68.9±1.1c	1.4±0.1a

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level.(Post-hoc multiple comparison test, p=0.05)

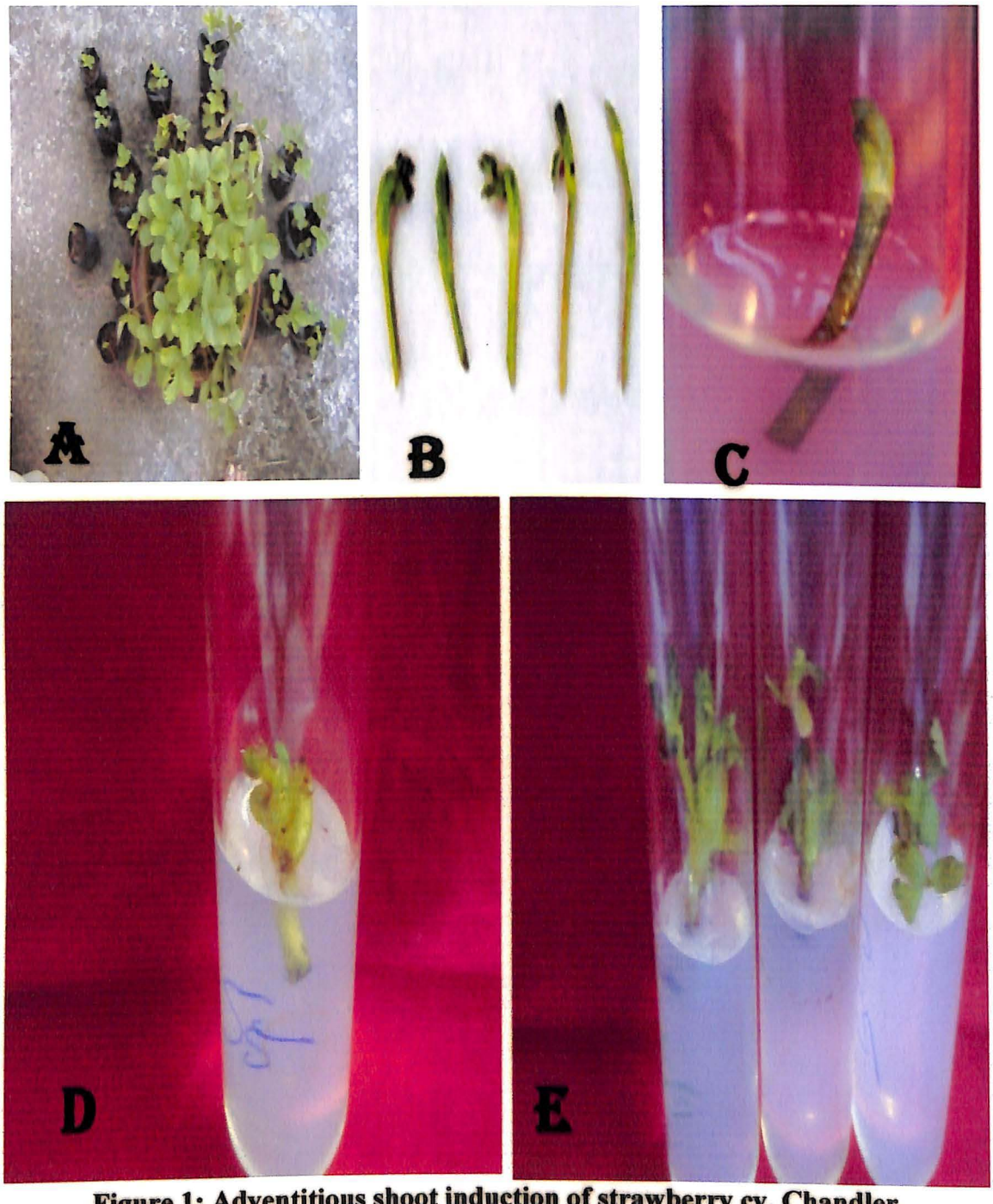


Figure 1: Adventitious shoot induction of strawberry cv. Chandler
A: Source of explant, B:Runner tips as explants ,C:Innoculation of runner tip
D: Shoot bud proliferation, E: Multiplication of shoot on MS media supplemented with 1.5 mg/l BAP, 0.1 mg/l NAA and 3% sucrose

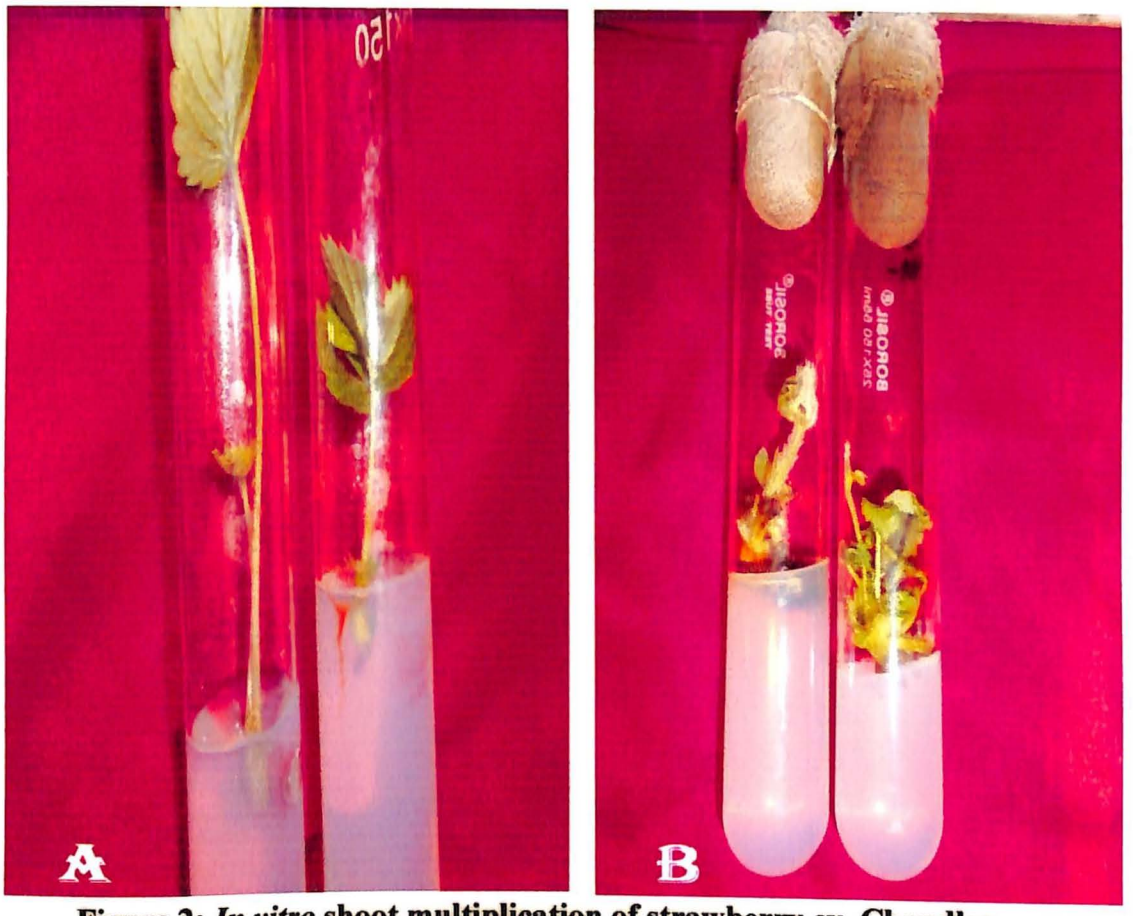
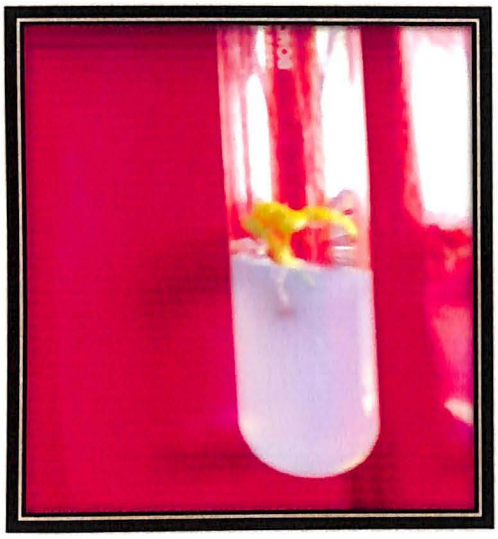


Figure 2: *In vitro* shoot multiplication of strawberry cv. Chandler

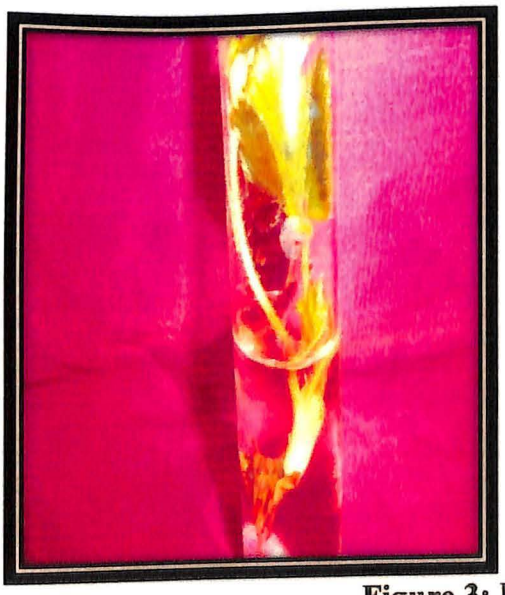
A: Growth of multiple shoot in MS media supplemented with 1.5 mg/l BAP , 0.1 mg/l NAA and 3% sucrose after 2 weeks of culture

B: Growth of multiple shoot in MS media supplemented with 1.5 mg/l BAP , 0.1 mg/l NAA and 3% sucrose after 4 weeks of culture



A: Initiation of rooting from microshoot after 1 week of transfer to1/2 MS medium supplemented with 1.0 mg/l IBA and 3% sucrose

B:Root growth after 2 weeks of root initiation



C:Rooted microshoot is ready to transfer to ex-agar meadium

Figure 3: Rooting of microshoots

4.1.3 Indirect regeneration

Callus refers to a mass of unorganized parenchymatous cells derived from plant tissue. The tissues used to initiate callus formation depend on plant tissues and growth hormone used. Generally, a higher auxins concentration in growth medium induces callus formation. The quality and quantity of callus mass depends on various factors like explants, plant growth regulators and light/dark incubation etc. The runner shoot cuttings, matured leaves and young leaves were cut into small parts in aseptic condition (4-5 mm) and cultured on MS medium supplemented with various concentrations of 2, 4-D , NAA and BAP . Expansion and swelling of explants at the cut surface were noted and soft crystal, greenish calli were emerged from the cut surface within 3-4 weeks culture MS medium supplemented with on 2, 4- D (1.0 mg/l) along with BAP (0.1 mg/l). Also same medium resulted the highest callus induction (170.6 mg) along with 90 % callus induction frequency. There was no response to callus induction on MS medium supplemented 2,4-D (0.5mg/l) along with BAP (0.5mg/l). The influence of different plant growth hormones on callus induction were presented in the Table 8. Different stages of callus induction and shoot regeneration from callus was presented in the figure 4.

Table. 8 Effect of plant growth regulators on callus induction of *Fragaria* × *ananassa* Duch. cv. Chandler

Treatments MS + Growth Regulators (mg/l)			Weight of callus after 30 days (mg)	Callus induction frequency (%)	Colour and nature of growth		
					Matured Leaves	Young Leaves	Internod-al cuttings
2,4-D	BAP	NAA					
0.5	0.5	0	0.0	00	Light Green Compact	Light Green Compact	Light Green Compact
1.5	0.5	0	29.0±0.2 a	30.0±0.1a	Green Compact	Green Compact	Light Green Compact
2.0	0.5	0	50.7±0.6 b	50.0±0.4b	Green Compact	Green Compact	Light Green Compact
2.5	0.1	0	92.4±0.5c	60.0±0.6c	Green Loosely Compact	Light Green Loosely Compact	Light Green Compact
1.0	**0.1**	**0**	**170.6±0.4f**	**90.0±0.2e**	**Green Compact**	**Green Compact**	**Green Compact**
0	0.5	2.0	142.9±0.9e	70.0±0.7d	Green Friable	Creamy Green Compact	Light Green Compact
0.5	0	1.0	112.6±0.2d	60.0±0.8c	Creamy Friable	Creamy Friable	Creamy Friable

N.B.- Medium used- Murashige and Skoog (MS) medium
*20 cultures per treatment; replicated thrice
Mean followed by different letters are significantly different at the 5% level.
(Post-hoc multiple comparison test, p=0.05)

Among the three explants sources taken for callus regeneration best response was obtained from internode explants. Young leaves and matured leaves observed to give varied poor performance in callus regeneration. So, shooting and rooting of regenerated calli was taken from internode only. Healthy and 3-4 weeks old well grown calli were transferred to MS basal media supplemented with BAP and NAA of different concentration. Shoot buds were started to regenerate from calli cultured on MS media containing different concentration of growth hormones. MS basal medium fortified with 2.5mg/l BAP and 0.5 mg/l NAA showed the maximum response to regeneration via calli inducing an average of 5.1 shoot buds per culture within 4-5 weeks. Thus it can be inferred from the table 9 that the given concentration of phytohormones in the media played a vital role in regeneration of plantlets.

Table.9 Effect of plant growth regulators BAP and NAA on the indirect regeneration of *Fragaria* × *ananassa* Duch. cv. Chandler after 4 weeks of culture

Treatments MS + Growth Regulators (mg/l)		No. of shoots regenerated per callus	Regeneration frequency (%)
BAP	NAA		
0.5	0.2	2.3±0.2 b	30±0.2a
1.0	0.2	1.9±0.5a	35±0.2b
1.5	0.2	2.7±0.8b	44±0.2c
2.0	0.2	3.0±0.1b	48±0.5c
2.5	0.2	3.5±0.5c	55±0.3d
3.0	0.2	2.4±0.3b	50±0.4c
0.5	0.5	2.8±0.6b	45±0.1c
1.0	0.5	3.0±1.1b	48±0.6c
1.5	0.5	3.5±1.2c	55±0.1d
2.0	0.5	3.5±0.9c	55±0.2d
2.5	**0.5**	**5.1±0.7d**	**68±0.5e**
3.0	0.5	3.0±0.3b	48±0.1c

N.B.- Medium used- Murashige and Skoog (MS) medium
*20 cultures per treatment; replicated thrice
Mean followed by different letters are significantly different at the 5% level.
(Post-hoc multiple comparison test, p=0.05)

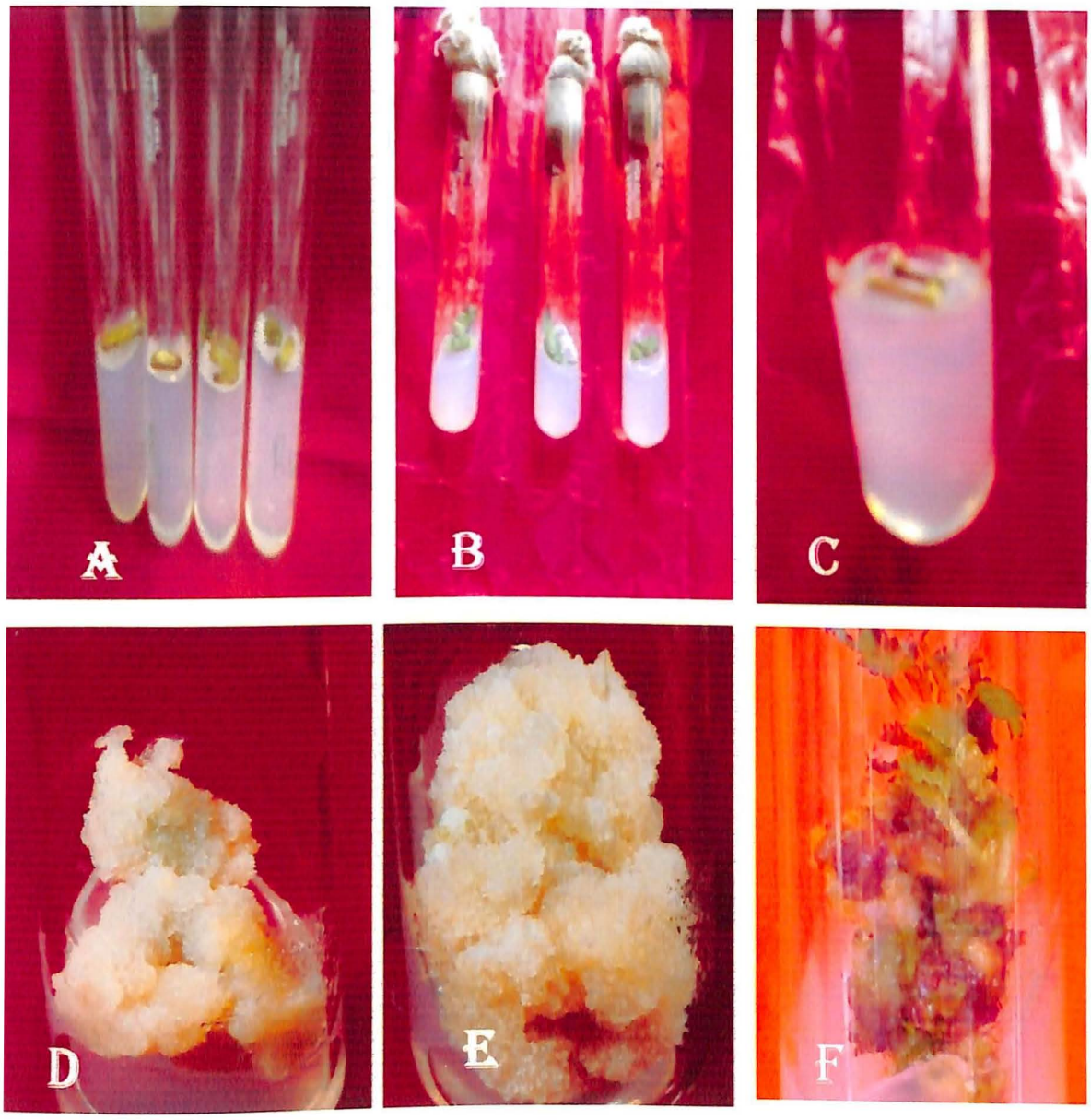


Figure 4: **Indirect organogenesis of strawberry cv. Chandler**
A: Innoculated internodal explant for callus induction
B: Innoculated leaf explant for callus induction
C: Initiation of callus from internodal explant after 1 week of culture
D: Callus induction after 4 weeks of culture
E: Callus induction after 5 weeks on MS medium supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l BAP
F: Shoot regeneration after 4 weeks of inoculation of callus in MS medium supplemented with 2.5 mg/l BAP and 0.5 mg/l NAA

4.1.3.1 Effect of different concentrations of auxins on root induction from microshoots regenerated from callus of *Fragaria ×ananassa* Duch. cv. Chandler

The process of *in vitro* root initiation, development and elongation normally require medium supplemented with auxin. The healthy microshoots were separated from the clump and transferred to different medium having various concentrations of auxins with 2% (w/v) sucrose for induction of rooting. No rooting was observed in basal full strength or half strength MS media without growth regulators. The maximum percentage of rooting was observed in half strength MS media supplemented with 0.25 mg/l IBA and 2% sucrose. The medium supplemented with NAA also favoured the induction of rooting but the efficiency was more in IBA containing medium. The higher percentage of rooting was 73.3% which was presented in the Table. 10. Rooting response was better when observed under dark incubation.

Table. 10 Effect of plant growth regulators (NAA & IBA) on rooting of microshoots

½ MS+ growth regulator (mg/l) + 2% (w/v) sucrose		% of response (Mean ± SE)*	No. of roots/ microshoots	Days to rooting
NAA	**IBA**			
0	0	0.0	-	-
0.25	0	23.3±0.6 a	2.2±0.5 a	28
0.50	0	26.6±0.5 b	2.3±0.4 a	26
1.0	0	43.3±0.4 d	3.4±0.3 a	25+
0	**0.25**	**73.3±0.6 g**	**4.5±0.6 b**	**20**
0	0.50	56.6±0.7 f	4.2±0.5 b	22
0	1.0	43.3±0.6 c	3.4±0.6 a	25+
0.25	0.25	46.6±0.8 e	3.8±0.7 a,b	26+

*12 replicates/treatment; repeated thrice.
+ - Callusing at the basal end
Mean followed by different letters are significantly different at the 5% level.
(Post-hoc multiple comparison test, p=0.05)

4.1.4 Effect of light intensity on shoot multiplication

The effect of light intensities (1000, 2000, 3000, 4000, 5000 and 6000lux) on shoot growth and multiplication were studied which had been clearly represented in the Figure 5.The rate of shoot multiplication (84.3%) was highest at 3000 lux. Under low light intensity the rate of shoot proliferation declined significantly. The multiple shoots obtained from the cultures grown under 3000 lux were healthy, and those from culture 1000, 2000 lux were thin and lanky. The number of shoots per culture (4.0) were the maximum at 3000 lux in *Fragaria* × *ananassa* Duch. cv. Chandler. Lower intensities of light did not favour shoot multiplication. Multiplication rate is also lower when higher intensities of light were used.

4.1.5 Effect of photoperiod on shoot multiplication

Basing on the results of experiment on light intensities, the effect of different photoperiods (0, 8, 12, 16, 20 and 24 h) on shoot growth and multiplication were examined at 3000 lux. The multiplication of shoot varied significantly with different photoperiods. The maximum percentage of shoot multiplication (86.3%) was achieved under 16 h photoperiod. Continuous dark did not favour shoot multiplication rather the plants become chlorotic. Shoot multiplication at 16 h photoperiod was proved to be higher than continuous light, 8 h, 12 h, 20 h, 24 h photoperiod. The maximum number of shoot per culture (4.1) was recorded under 16 h photoperiod (Figure 6).

4.1.6 Effect of different incubation temperature on shoot multiplication

The effect of different incubation temperatures (18±2^{O}C, 20±2^{O}C, 22±2^{O}C, 25±2^{O}C 28±2^{O} and 30±2^{O} C) was studied on shoot multiplication. The percentage of shoot multiplication was the minimum at 18 ± 2^{O}C in *Fragaria* × *ananassa* Duch. cv. Chandler. The maximum percentage of shoot multiplication (88.3) was achieved when the cultures were incubated at 25 ± 2^{O}C. The maximum number of shoots per culture (4.3) was also observed when the cultures were incubated at 25 ± 2^{O}C (Figure 7).

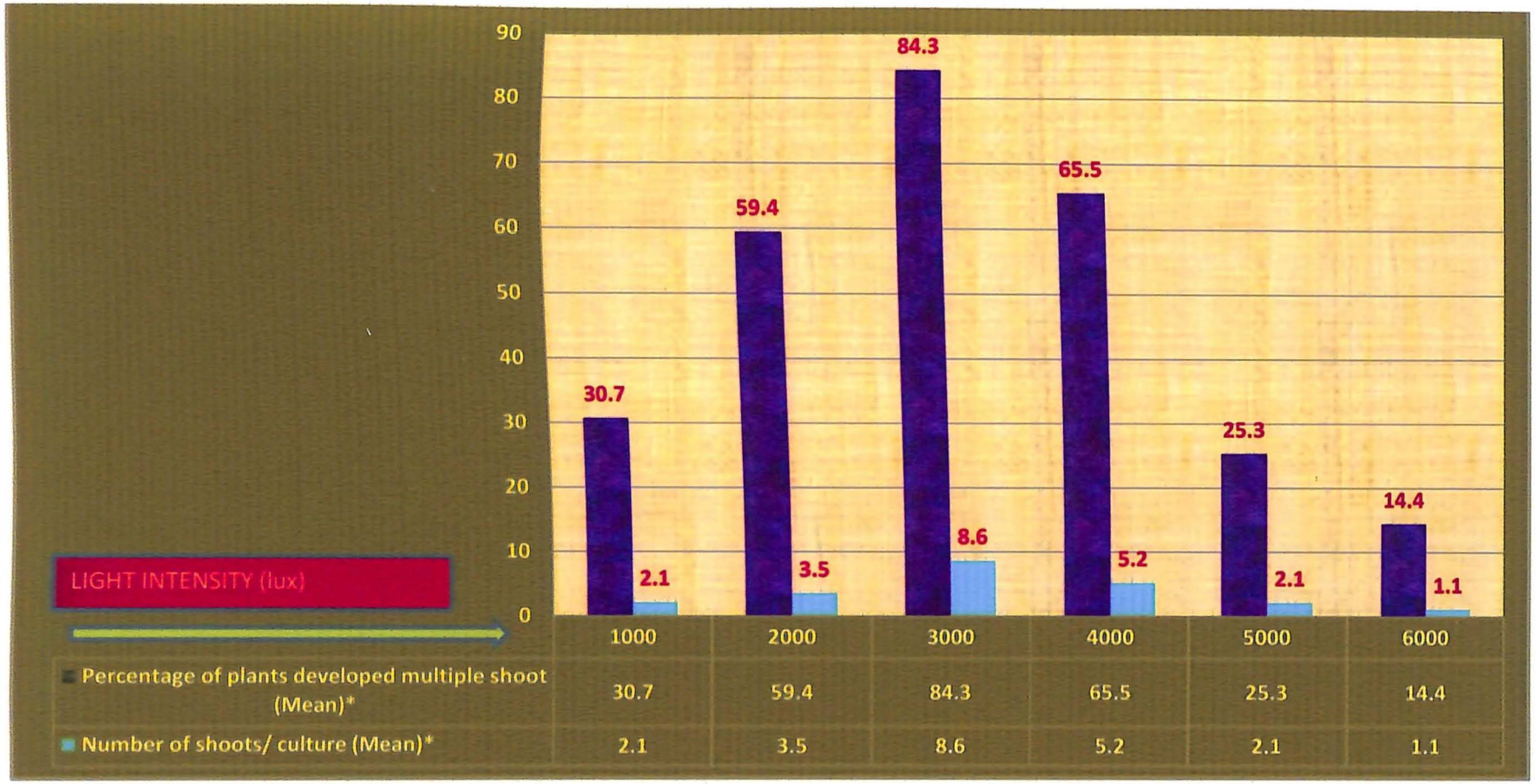


Figure 5: Effect of different light intensities on shoot multiplication of *Fragaria×ananassa* Duch. cv.Chandler on MS basal medium supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA incubated under 25±2°C temperature and 16 h photoperiod after 4 weeks of culture

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05).

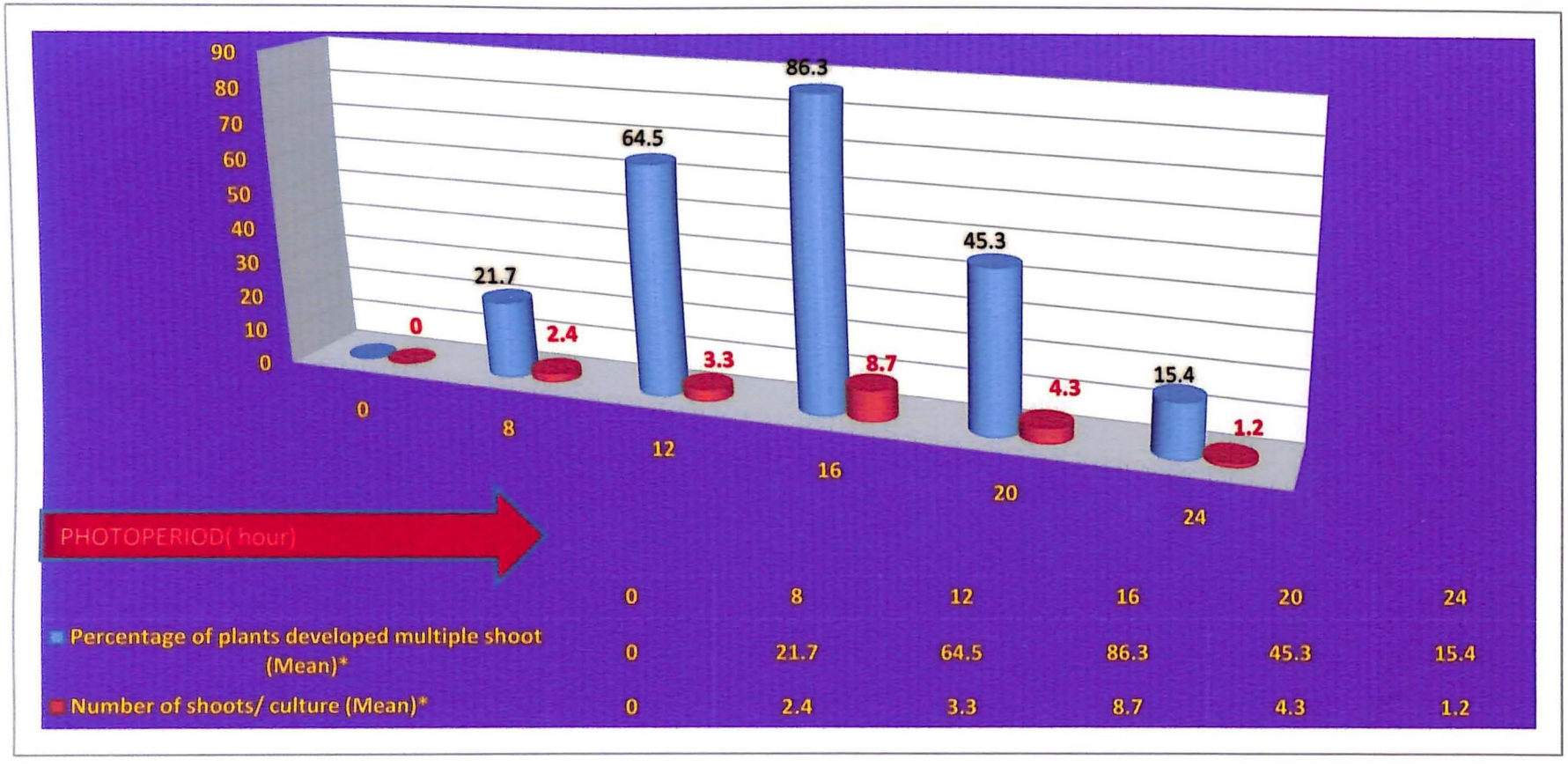


Figure 6: Effect of photoperiods on shoot multiplication of *Fragaria* × *ananassa* Duch. cv. Chandler on MS basal medium supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA incubated under 25±2°C temperature at 3000 lux light intensity after 4 weeks of culture

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05).

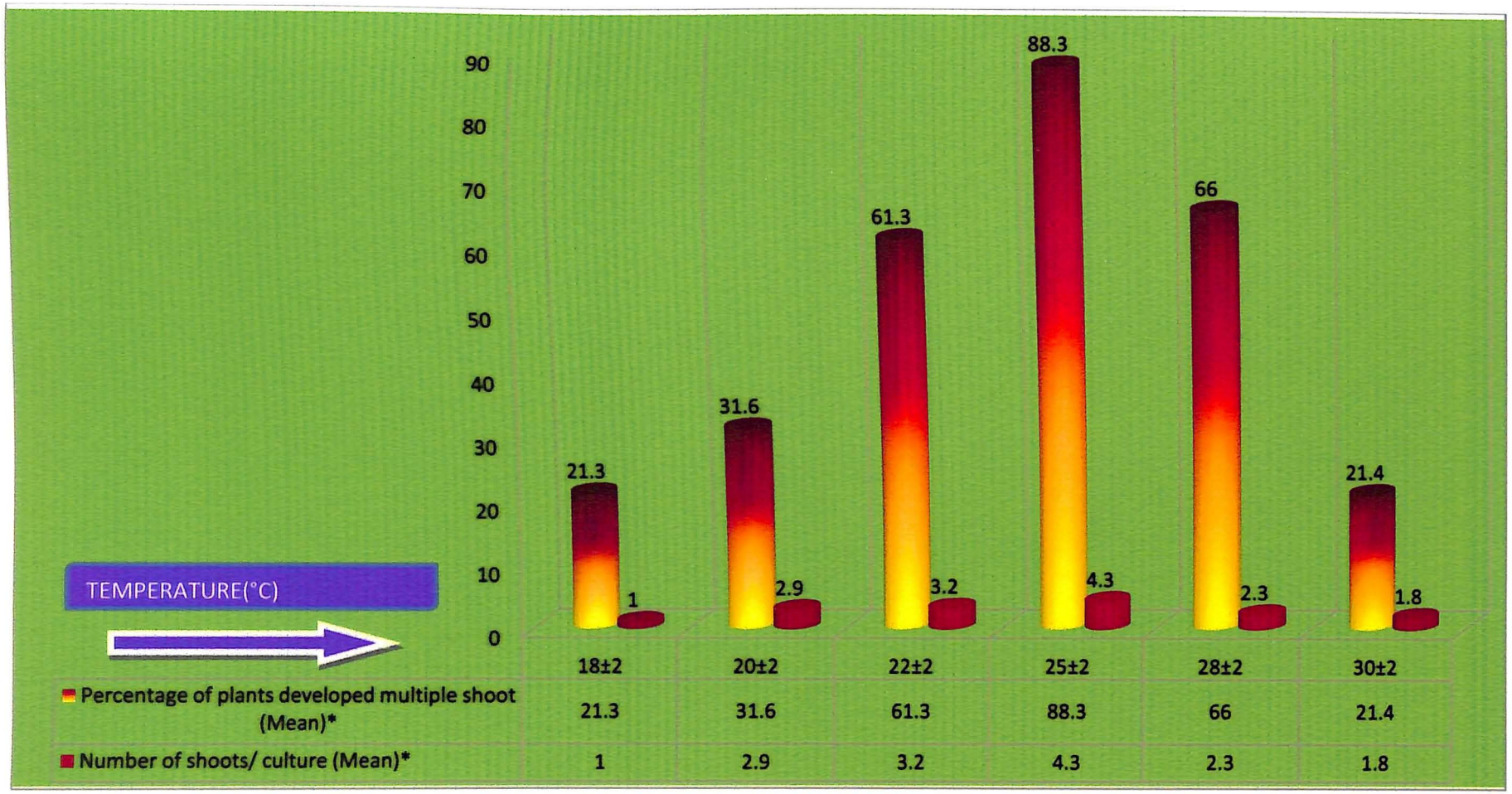


Figure 7: Effect of different incubation temperature on shoot multiplication of *Fragaria×ananassa* Duch. cv. Chandler on MS basal medium supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA incubated under 16 h photoperiod and 3000 lux light intensity after 4 weeks of culture

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05).

4.1.7 Effect of pH of the media on shoot multiplication

The pH of the media played an important role on shoot multiplication as it affected the availability of nutrients in the medium. The pH of media was adjusted at different levels (5.0, 5.5, 5.8, 6.0, 6.2 and 6.5) by adding NaOH or HCl prior to autoclaving. The shoot multiplication was the maximum (81.3) at the pH of 5.8 in *Fragaria* × *ananassa* Duch. cv. Chandler .The rate of shoot multiplication and number of shoots per culture were at the minimum at pH 6.5 that is 5.4 and 1.1 respectively. The plants were healthy and produced maximum number of shoots per culture at pH 5.8, hence it was considered as the optimum pH of the medium for shoot multiplication (Figure 8).

4.1.8 Effect of nutrient concentration on shoot multiplication

To study the effect of nutrient concentrations on shoot multiplication, the medium with full strength MS nutrients was compared with the medium having different nutrient strength (½, ¼ , $^{1}/_{8}^{th}$, 2, 5/2 th) along with the usual doses of BAP (1.5 mg/l) and NAA (0.1mg/l) and sucrose (30g/l) . The percentage of explants producing multiple shoots varied significantly between the six concentrations of basal MS media. The percentage of shoot multiplication ranged from 8.6 to 86.6 in *Fragaria*×*ananassa* Duch. cv. Chandler which was presented in Figure 9. The rate of shoot multiplication was the minimum (8.6) at 5/2 strength of MS nutrients. The highest rate of multiplication (86.6) and highest numbers of multiple shoot (4.1) were obtained in full strength MS supplemented with BAP (1.5 mg/l) and NAA (0.1 mg/l).

4.1.9 Effect of carbon source on shoot multiplication

Since, the explants under *in vitro* conditions were not fully adopted to an autotrophic nutrition at the early stage of culture, a carbohydrate supplement to the medium was essential to maintain the osmoticum for normal growth of explants. However, it was important to determine the most suitable source of carbohydrate. Among the several source of carbohydrate tried, sucrose and commercial sugar performed better than D-glucose, fructose and maltose. The explants became black and died within three weeks of culture when manitol was used as source of carbohydrate in the medium. The maximum percentage of shoot multiplication (86.2) was achieved with sucrose followed by commercial sugar (76.9). Maximum numbers (4.0) of multiple shoot was obtained from sucrose. But no response was obtained when manitol was used as carbohydrate source (Figure 10).

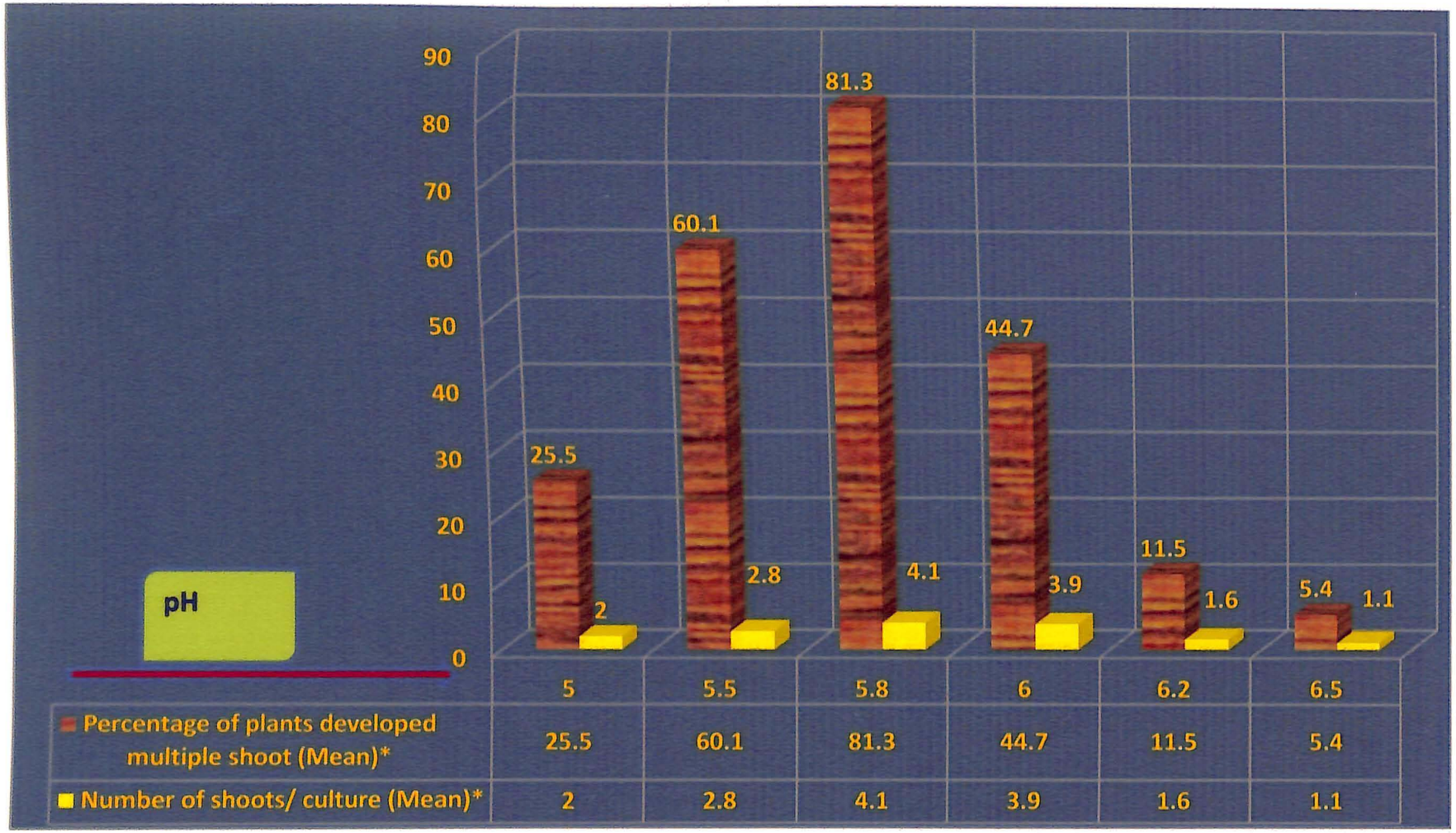
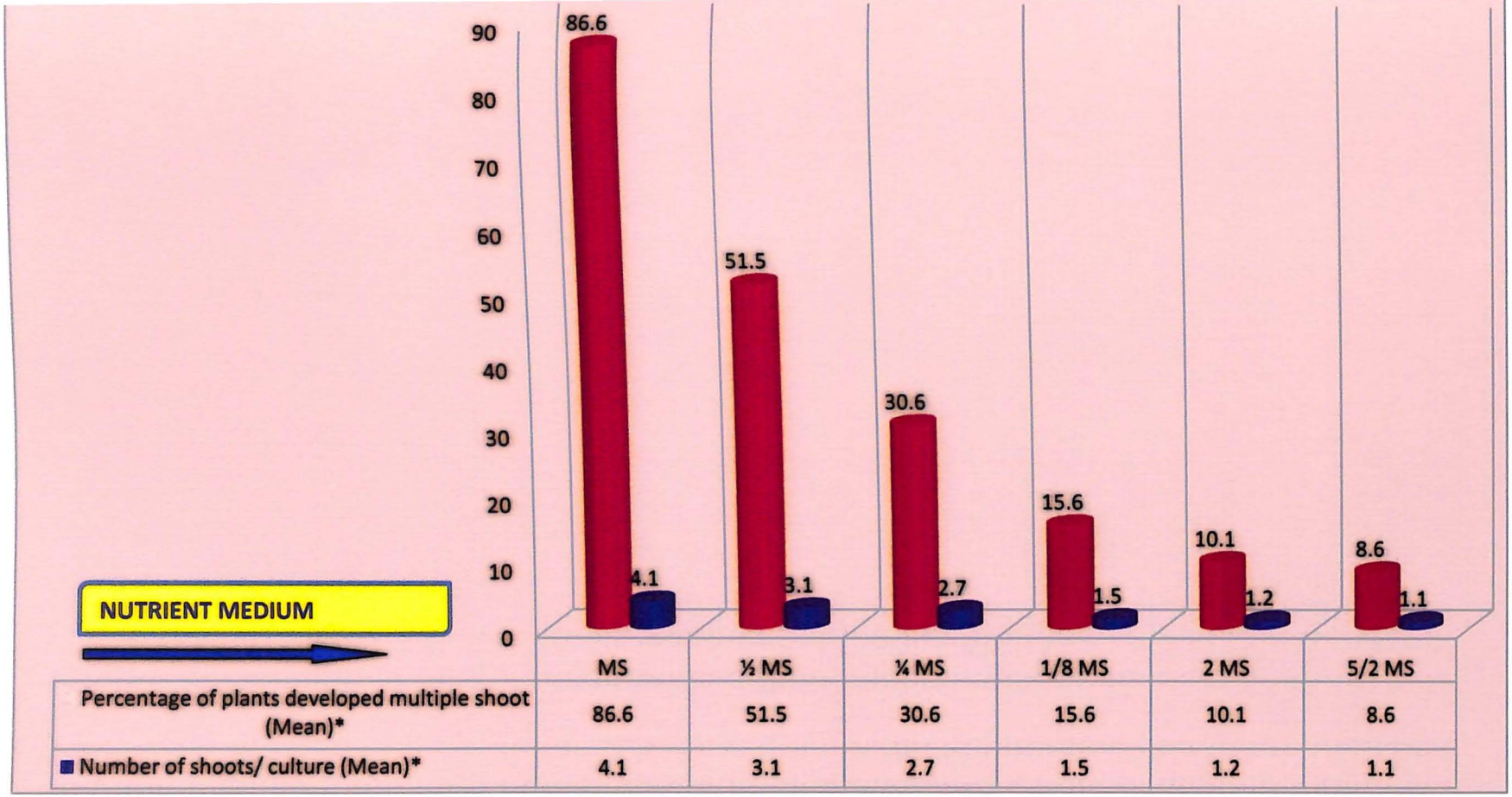


Figure 8: Effect of different pH on shoot multiplication of *Fragaria×ananassa* Duch. cv. Chandler on MS basal medium supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA incubated under 25±2 °C temperature , 3000 lux light intensity and 16 h photoperiod after 4 weeks of culture

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05).



NUTRIENT MEDIUM	MS	½ MS	¼ MS	1/8 MS	2 MS	5/2 MS
Percentage of plants developed multiple shoot (Mean)*	86.6	51.5	30.6	15.6	10.1	8.6
Number of shoots/ culture (Mean)*	4.1	3.1	2.7	1.5	1.2	1.1

Figure 9: Effect of different concentration of nutrient on shoot multiplication of *Fragaria ×ananassa* Duch. cv. Chandler supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA incubated under 25±2 ° C temperature , 3000 lux light intensity and 16 h photoperiod after 4 weeks of culture

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05).

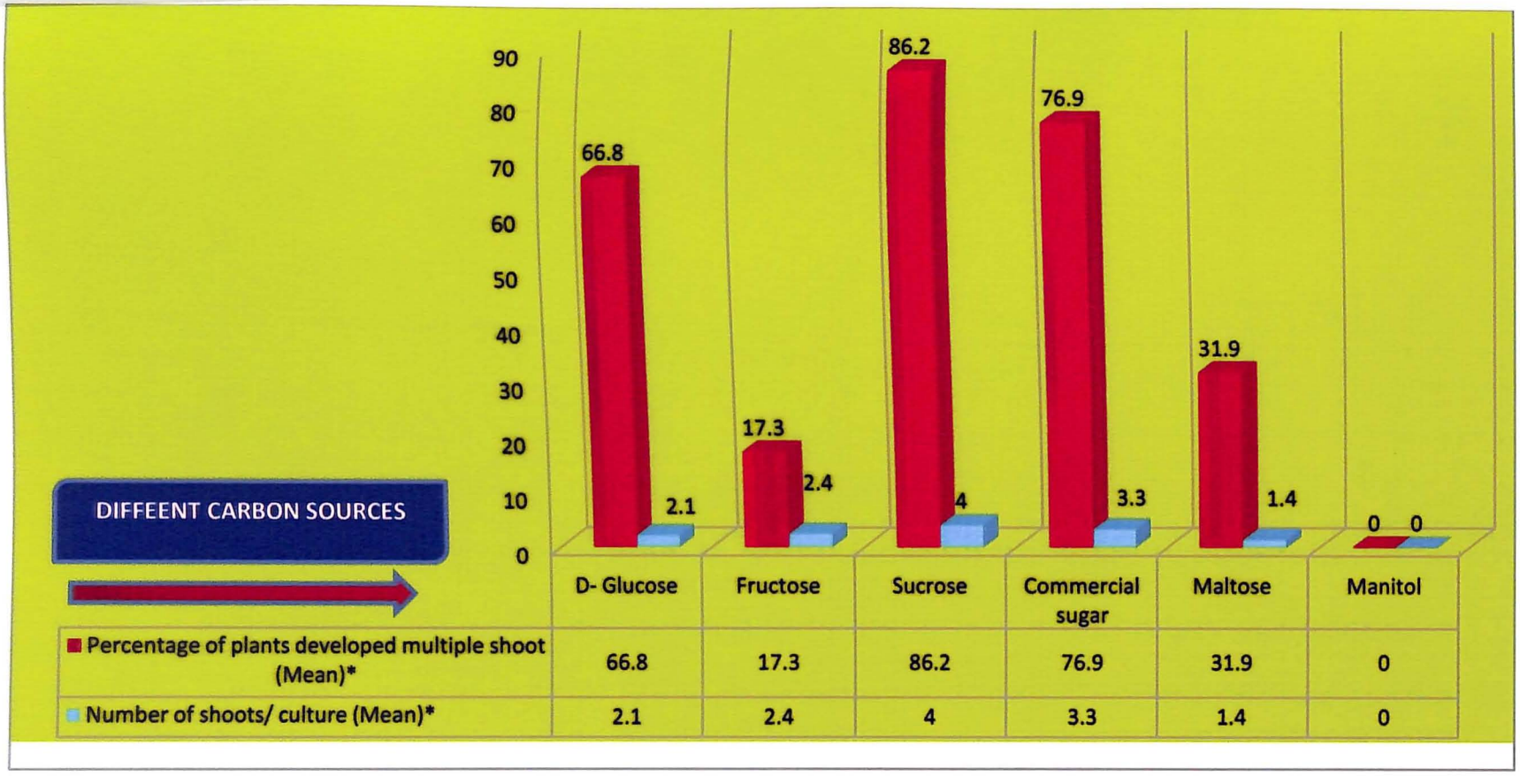


Figure 10: Effect of different types of carbohydrates on shoot multiplication of *Fragaria ×ananassa* Duch. cv. Chandler supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA incubated under 25±2 ° C temperature , 3000 lux light intensity and 16 h photoperiod after 4 weeks of culture

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05).

4.1.10 Effect of sucrose concentration on shoot multiplication

Different concentrations of sucrose (0, 1, 2, 3, 4 and 5%) were tested for shoot multiplication in *Fragaria* × *ananassa* Duch. cv. Chandler and represented in graphical manner in Figure 11. Shoot multiplication was not observed in the media devoid of sucrose. The percentage of shoot multiplication and average number of shoots per culture were the lowest at 1% and 5% of sucrose. The maximum percentage of shoot multiplication was recorded at 3% sucrose in *Fragaria* × *ananassa* Duch. cv. Chandler. The maximum percentage of shoot multiplication (88.5) was achieved with 3% sucrose and full strength MS medium supplemented with BAP (1.5 mg/l) and NAA (0.1 mg/l). The shoots were healthy and maximum in number (4.0) in the medium having 3% sucrose.

4.1.11 Effect of agar concentration on shoot multiplication

The effect of different concentration of agar (0.4%, 0.5% , 0.6%,0.7%, 0.8% and 1.0%) was studied on shoot multiplication and findings were presented in Figure 12. One percent agar was considered to be the poorest for shoot multiplication. The medium having 0.6% agar was considered to be the best for shoot multiplication. Although number of shoots per culture at 0.4 % was at par with 0.5%, the shoots were healthy and highest in number at 0.6% agar concentration. In the medium 0.6% agar concentration is considered as the best. The highest percentage of shoot multiplication (82.4) and the maximum number of shoots per culture (4.1) were observed in the medium having 0.6% agar in *Fragaria* × *ananassa* Duch. cv. Chandler.

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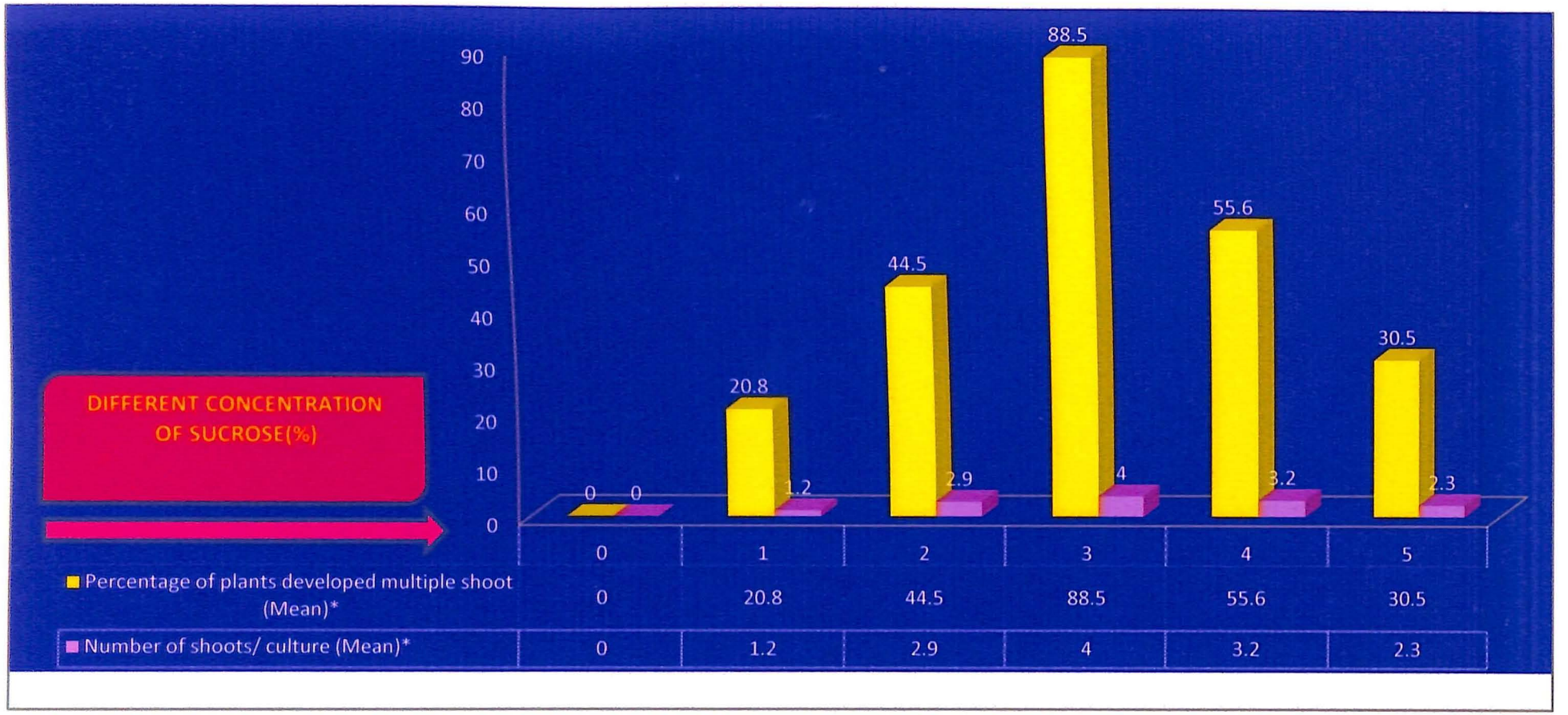

Figure 11: Effect of different concentration of sucrose on shoot multiplication of *Fragaria×ananassa* Duch. cv. Chandler cultured on MS basal medium supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA incubated under 25±2 ° C temperature, 3000 lux light intensity and 16 h photoperiod after 4 weeks of culture.

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05).

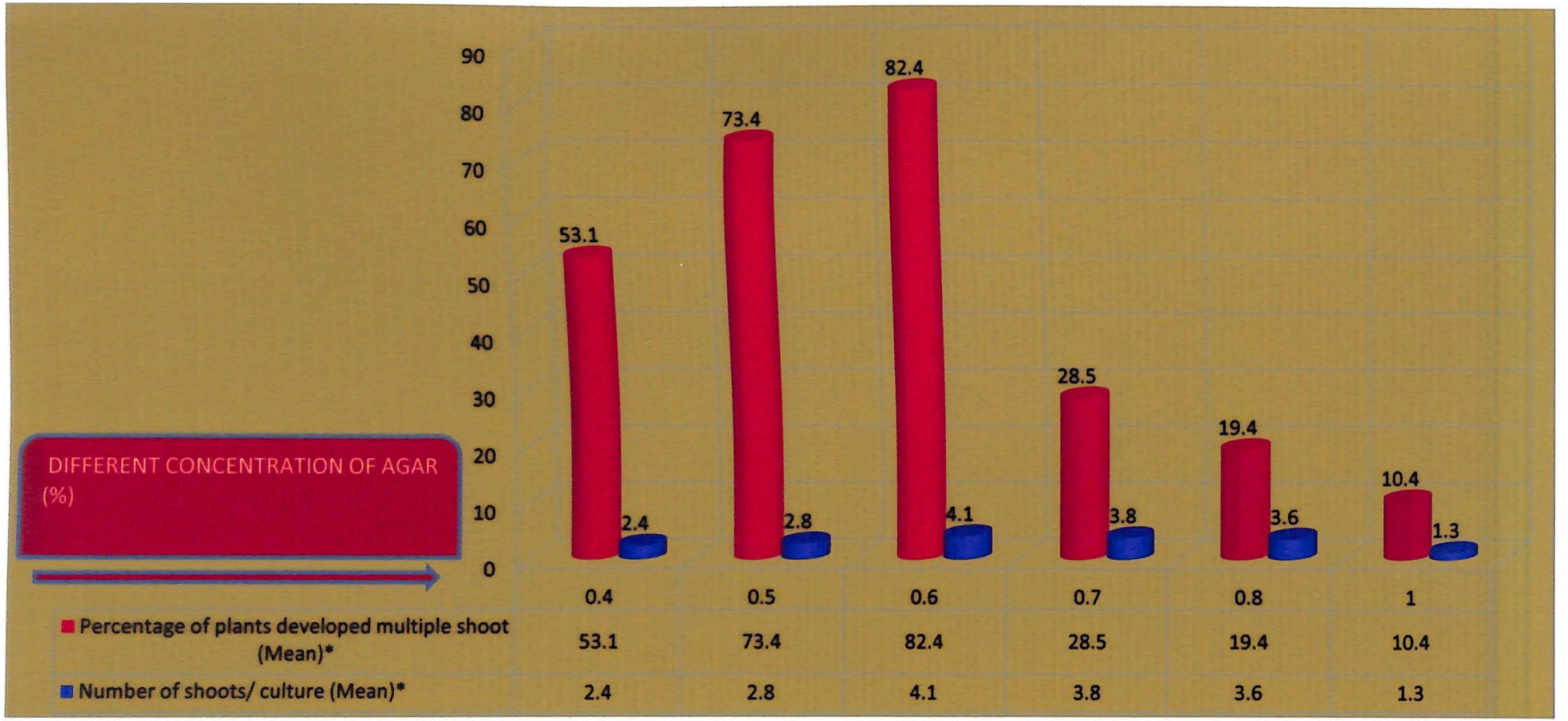

Figure 12: Effect of different concentration of Agar on shoot multiplication of *Fragaria×ananassa* Duch. cv. Chandler cultured on MS basal medium supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA incubated under 25±2 ° C temperature , 3000 lux light intensity and 16 h photoperiod after 4 weeks of culture.

*20 cultures per treatment; replicated thrice

Mean followed by different letters are significantly different at the 5% level (Post-hoc multiple comparison test, p=0.05).

4.2 Acclimatization of *in vitro* raised plantlets.

In plant tissue culture, the ultimate success of *in vitro* regenerated plantlets lies in its growth in the external environmental field conditions. The well elongated healthy plantlets with good roots were selected for hardening. These roots of healthy plantlets were washed gently with sterile distilled water to remove all traces of agar media. After one day these rooted plants were pre hardened to the black polythene pot containing autoclaved mixture of sand: soil: FYM (1:1:1) and kept in the greenhouse with 85% humidity for acclimatization (figure 13 and 14). About 90% of the plantlets were established in the green house within 1-2 weeks of transfer. The plant grew well and attained 6 – 8 inches within 4 weeks of transfer. The prevailing conditions (humidity and temperature) of transplanting season greatly influenced the initial survival of potted plantlets .The plantlets were transplanted to soil and acclimatized in the growth chamber under high humidity conditions. The healthy and vigorous rooted plants were transferred to field nursery for hardening.

4.3 Genetic fidelity analysis of *in vitro* raised strawberry (*Fragaria×ananassa* Duch.) cv. Chandler

The quality of *in vitro* derived regenerates were screened with 12 ISSR primers that have showed monomorphic among the plantlets. The banding pattern of PCR amplified product from micropropagted plantlets were found to be monomorphic for most of the primer tested. In tissue cultured plants, no polymorphism was detected in the DNA profile, however some weak bands were absent in some of the regenerates though their frequency was quite low and were not reproducible when repeated. The identical ISSR banding pattern of 19 *in vitro* raised plantlets and their mother plant have shown in the figure 15. Most of the primers showed identical DNA profiles as compared with mother plant. No somaclonal variation was detected. The 12 ISSR primers produced 48 distinct and scorable bands, with an average of 4 bands per primer. Each primer generated a unique set of amplification products ranging in size from 100 bp (UBC825) to 1500bp (UBC 860).In ISSR -21- (UBC-825), two bright bands were shown with a lower band of 650 bp and upper one having 850 bp which was revealed from figure 15. But in case of ISSR -27- (UBC 864) , the lower bright band is of 500 bp and upper one is of 1000 bp which was presented in table 11.

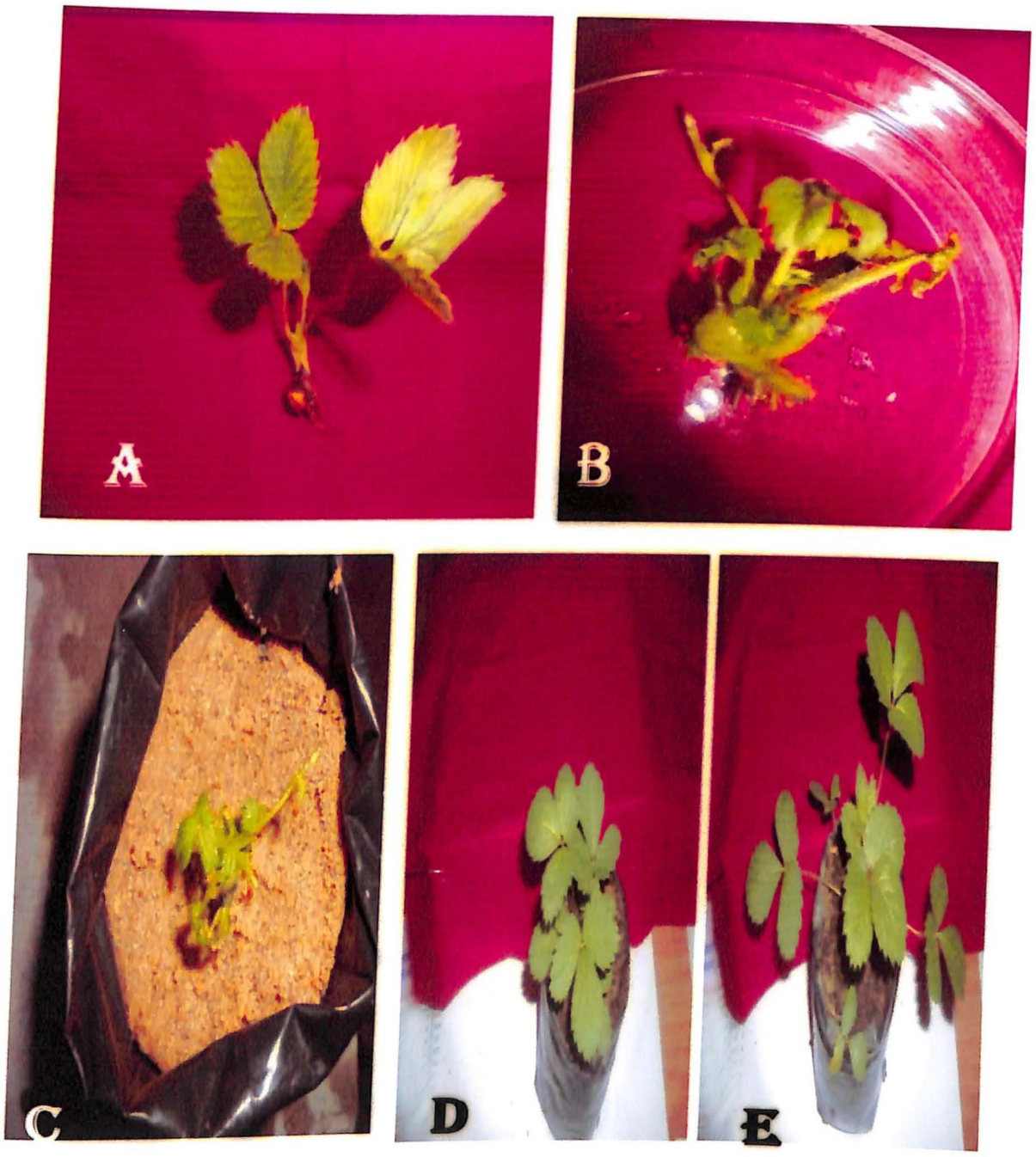

Figure 13: Hardening of *in vitro* propagated strawberry cv. Chandler
A:Ex-agar plant after 18 days of inoculation, B:Ex-agar rooted plant after 30 days noculation , C:Planting the ex-agar plant in sand medium, D:Plant growth in sand:soil: FYM(1:1:1) medium after 2 weeks of translplanting E: Plant growth sand:soil: FYM(1:1:1) medium after 4 weeks of translplanting

An *in vitro* regenerated plantlet of strawberry cv. Chandler after 7 days of weaning of agar gelled media

An *in vitro* regenerated plantlet of strawberry cv. Chandler after 15 days of weaning of agar gelled media

An *in vitro* regenerated plantlet of strawberry cv. Chandler after 30 days of weaning of agar gelled media

Figure 14: Successful establishment of *in vitro* regenerated plantlet of strawberry cv. Chandler

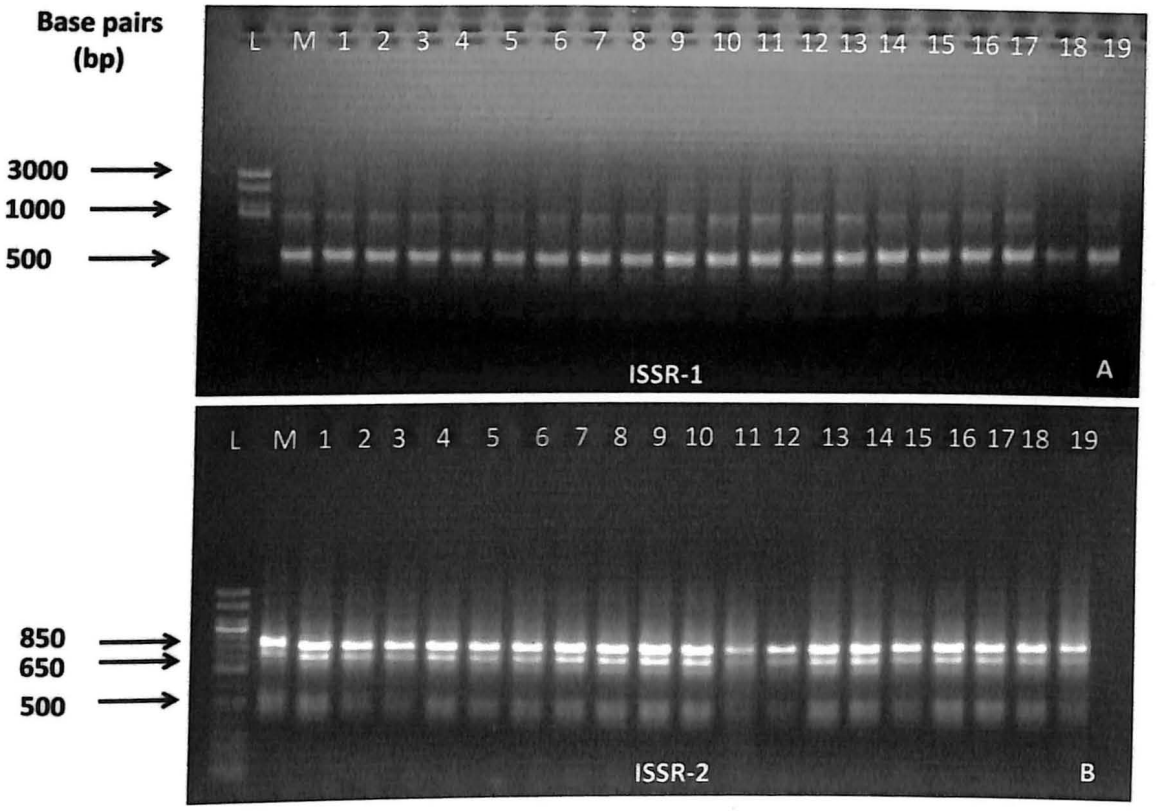

Figure 15: Analysis of genetic fidelity study of *in vitro* raised plants of strawberry var. Chandler by using ISSR markers A(UBC-864)* and B(UBC-825)**.

* UBC-864 : ATGATGATGATGATGATG
** UBC-825: ACACACACACACACACT

CHAPTER-5

DISCUSSION

DISCUSSION

5.1 Micropropagation

Plant tissue culture is a vital tool in both basic and applied studies in crop plants. The technology owes its origin to the ideas of Haberlandt (1902) who suggested possibility of culturing vegetative cells artificially. He introduced the concept of 'Cell totipotency'. Micro-propagation techniques are becoming important for mass cloning of many economical plant species because of the uniformity and potential for planned production. Several chemical and physical factors influenced the establishment of micropropagation of plants (Narayanaswamy, 1977). Significant information on the consistency of the rate of multiplication, the genetic stability of the *in vitro* raised plants and the success rate of establishment of the micropropagated plants in soil and their performance in the field was extremely important before adopting a technique on commercial scale. While, it has been recognized that the propagation through tissue culture must proceed through a sequence of steps such as, (1) establishment of aseptic culture, (2) multiplication of the propagules, (3) understanding the physical and chemical factors responsible for high frequency multiplication, (4) genetic uniformity of the micropropagated plants and (5) establishment of plantlets in the soil. In recent years, note-worthy contributions have been made by various workers on *in vitro* plant propagation and the findings have helped to standardize protocols for commercial propagation of a large number of plant species.

During the course of the present investigation, different aspects on *in vitro* mass cloning of *Fragaria* × *ananassa* Duch cv. Chandler and its genetic fidelity test by using ISSR marker were examined through a series of experiments.

5.2 *In- vitro* regeneration of strawberry

In-vitro plant regeneration system is very necessary for carrying out multiplication of strawberry for disease free and uniformity in harvesting. In case of strawberry, direct organogenesis of runner tips and indirect organogenesis via callus induction is the most common regeneration pathway. However, this technique can be very well employed for obtaining disease free plant materials as plantlets produced by

runner in case of strawberry carry latent virus which reduces yield and quality. It also helps in rapid multiplication of new clones and for crop improvement. With these points in views, the present study was undertaken to standardize the requirement of plant growth regulators for shoot multiplication and rooting using runner tips , leaves and internodal cuttings as explants.

5.2.1 Effect of surface strilants

In the present study, 0.1 % $HgCl_2$ was found to be effective for sterilization of runner tips, leaves and internodal cuttings. Treatment of with runner tips of 0.1% $HgCl_2$ for a period of seven minutes resulted better sterilization of explants and survival rate. The present finding is in congruent with the result reported by Ali-Akbar *et al.* (2012) and Ara *et al.* (2012) where 0.1% of $HgCl_2$ treatment for 4 minute duration also resulted higher aseptic culture with greater survival percentage. Tween-20 @ 1% was also used for ten minutes for surface sterilization and the same was reported by Ara *et al.* (2012) . Badawai *et al.* (1990 a, b) had tried different concentration of sodium hypochloride and mercuric chloride for disinfection of meristems of Pajaro, Tioga and Tufts cultivars, and had found that dipping meristems in 0.2% mercuric chloride for 20 minutes could result in lower contamination (5-15%) in all cultivars, and addition of sodium chloride to the medium adversely affected shoot proliferation and percentage of survival.

5.2.2 Direct regeneration and shoot multiplication

Perhaps, strawberry is the first fruit crop in which micropropagation technique has been standardized (Sharma and Singh,1999) and now its large scale commercial propagation is being done through tissue culture. This fruit plant requires very high number of plants /ha (50-60 thousand) and such high demand can be easily met only through micropropagtion. Different explants like meristem tips, anthers, immature embryos and first auxiliary buds of stolon have been produce millions of plants in a year (Waithaka *et al.*, 1980; Wang *et al.*, 1984; Jones *et al* ., 1988; Boxus *et al.*, 1989). Many culture media have been standardized, most widely used are Knop's solution, and Murashige and skoog's medium (MS) and Linemaier –Skoog (LS) with agar , sucrose and hormones at desired level (Jones *et al.*, 1988). The addition of growth regulators into the culture media has showed very simulative effect on

regeneration, growth and yield of tissue cultured plants. Three cytokinins (BAP, Kn and Ads) have been tested for shoot multiplication from apical meristems, runner tips, and first node of runner shoots of strawberry. Among the three cytokinins, 6-benzylaminopurine and kinetin favoured maximum rate of shoot proliferation . The moderate concentrations of BAP (1.0 mg/l) along with 0.25 mg/l kinetin and 50 mg/l adenine sulphate showed higher response (85.8%) on MS medium supplemented with 3% sucrose and the same was reported by Ali-Akbar *et al.* (2012). Among three auxins (NAA, IAA, IBA) , NAA favoured maximum rate of shoot proliferation. The runner tips showed maximum percentage of multiplication (80.7%) on MS medium supplemented with NAA @ 0.1mg/l along with 0.25 mg/l IAA. In case of of *Fragaria* × *ananassa* Duch. cv. Chandler, the maximum percentage (89.8%) of shoot proliferation was observed in MS medium supplemented with 1.5 mg/l BAP, 0.1 mg/l NAA and 3% sucrose within 4 weeks of culture. The number of multiple shoots were also highest (4.3) in that above cited medium. Similar observation was made by Sakila *et al.* (2007) whereas they used nodal segment as explants. IAA is less effective in inducing multiple shoot in combination with BAP. Bhat R. *et al.* (2012) has observed that the highest shoot multiplication was obtained by using MS medium supplemented with 1.5 mg/l BAP and 0.1 mg/l kinetin when they used the fresh nodes as the explants. Kaur *et al.* (2005) reported that the best response was obtained in MS medium with 0.5 mg/l kinetin and 1.0 mg/l BAP and 2.0 mg /l GA_3 with commercial sugar. Shaila *et al.* (2014) reported that MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l kinetin gave better shoot proliferation .

5.2.3 Rooting of direct regenerated plantlets.

The daughter shoots (3-4 cm length) were excised and transferred to root induction media. Both IBA and NAA were found to be effective for adventitious root induction and frequency of root induction ranged from 20.7 to 90.8%. Between two auxins IBA showed better performance which is congruent with Ara *et al.* (2013). In their experiment, Genotype AOG of strawberry produced highest number of roots per shoot with highest rooting frequency (90%) in medium fortified with 1.0 mg/l IBA. In the present study it was found that the medium supplemented with 1.0 mg/l IBA found to be provide maximum root induction frequency (90.8%) with highest root numbers (5.4).

5.2.4 Indirect regeneration

In direct regeneration passes through the way of callus and callus culture can be done from different vegetative organs such as the leaves, roots, nodes, stems, petioles, embryos and flower buds. Young vegetative organs are more effective for callus induction. Skoog *et al.* (1970) concluded that explant source is one of the most important parameters for successful long- term cell culture. Plant cell cultures provide an attractive route for producing high-value plant derived products, such as flavonoids, fragrances, alkaloids, colorants and pharmaceuticals that are very expensive to synthesize chemically and that occur naturally only at very low concentrations (Kim *et al.,* 2002). Successful callus culture also depends on the type of plants growth regulators. Cytokinin and auxins are known to promote callus formation in tissue culture (Popescu *et al.*, 1997, Letham *et al.,* 1974 and Akiyoshi *et al.* ,1983). Auxin has wide range of effects on plant growth and morphogenesis. A natural auxin of higher plants is involved in regulating cell elongation, cell division and differentiation (Dietz *et al.,* 1990). Cytokinin can promote cell enlargement in certain tissues as reported earlier (Rayle *et al.,* 1982; Ross and Rayle 1982).

Among the various hormonal supplements used, explants of strawberry showed high response to callus formation on MS medium supplemented with BAP and 2,4-D. The strawberry leaf explants and internodal explants induced to show callus development in most of the culture media formulations. However, the effect of different plant growth regulator formulations on the degree and types of callus formation were different (Karim *et al.,* 2011). Highest (90%) callus induction was noted in 1.0 mg/l 2,4-D + 0.1 mg/l BAP supplemented medium using internodal derived explants. The calli were green in colour and mostly compact in nature but few were loosely compact in texture. Akter *et al.* (2008) found similar result. Callus induction is a pre-requisite on the way to generate somaclonal variation. Because during callus division in artificial conditions different types of abnormalities occur in the genetic constituents that ultimately contributed to the regenerated plants (Larkin and Scowcroft, 1981; Shamima *et al.,* 2003). As a result, a lot of variation may be found in the plants regenerated from a single callus population. Callus formation is controlled by the level of plant growth regulators (auxins and cytokinins) in the culture medium. Concentrations of plant growth regulators can vary for each plant species and can

even depend on the source of explant or individual plant. Culture conditions (temperature, light and photoperiod etc.) are also important. Cultures will be used to study protoplast isolation, cell type, cellular selection, somatic embryogenesis, organogenesis and secondary metabolites production. Protocols in previous studies have shown that plant growth regulators concentrations and selections are vital for strawberry callus induction and regeneration. Various formulations of BAP, IBA, 2,4-D, Kn, NAA, TDZ, CH, and KNO_3, all have been reported using in callus induction and plant regeneration studies in strawberry (Liu and Sanford 1988; Nehra *et al.* (1990) and Goffreda *et al.,* 1995). Liu and Sanford (1988) reported the use of casein hydrolysate (CH) and potassium nitrate on leaf explants of 'Allstar' strawberry. Both stimulated the production of callus and shoot and reportedly had an additive effect. Best callus and shoot production in their study was achieved with a combination of BAP, IBA, CH, and KNO_3. Kartha *et al.* (1980) successfully regenerated 'Redcoat' using a combination of BAP, IBA and GA_3 as a precursor to a cryopreservation study of the cultivars.

In the present investigation auxin in combination with cytokinin was found the most effective for callus induction, which is concomitant with most of the previous report. Notable amount of callus induction was observed in media having 2.0 mg/l NAA + 0.5 mg/l BAP from internodal explants. The result illustrated that the internodal explants were best for maximum callus induction than young leaf and mature leaf explants. However Mature leaf explants were favourable for callus induction and shoot regeneration more than young leaves and internode in all case (Sultana, 2011). Hamnoudeh *et al.* (1998) achieved regenerated plantlets of Honeoye strawberry from the auxiliary buds by culturing for 4 weeks on MS medium supplemented with BAP (2.5 mg/l) and thiazuron (5.5 mg/l) with vitamins for shoot proliferation. Ara *et al.*(2012) found best response in callus induction of strawberry on MS media with 1.0 mg/l 2,4-D + 0.1 mg/l BAP, 2 weeks after culture . Maximum number of multiple shoots regenerated in MS medium supplemented with 2.5 mg/l BAP+ 0.5 mg/l NAA.

The healthy microshoots were separated from the clump and transferred to different medium having various concentrations of auxins with 2% (w/v) sucrose for induction of rooting. No rooting was observed in basal full strength or half strength

MS media without growth regulators. The maximum percentage of rooting was observed in half strength MS media supplemented with 0.25 mg/l IBA and 2% sucrose. The medium supplemented with NAA also favoured the induction of rooting but the efficiency was more in IBA containing medium. The higher percentage of rooting was 73.3% in case of *Fragaria* × *ananassa* Duch. cv. Chandler. For *in vitro* rooting of plantlets of Hofla and Tangi strawberry, Pereira *et al.*(1998) had reported that 3/4th to 1/2 strength salts of MS medium were better than full concentration.

Regenerated shoots (<4cm) started root initiation within 20 days of inoculation when they were cultured half strength of MS medium added with different concentrations of NAA and IBA individually . All treatments induced roots, but maximum rooting (73.3%) and vigorous growth of plantlets were observed on MS media within 20 days of inoculation. The highest mean number of roots (4.5±0.6) was recorded in the media containing 0.25 mg/l IBA in ½ MS medium. Addition of auxin in rooting media accentuates rooting but also microcuttings developed callus at their base that hamper their field establishment. Similar results on the rooting and subsequent field establishment were also reported by several researchers (Boxus ,1974; Owen and Miller ,1996; Jimenez- Bermudez and Redondo- Nevado 2002; Sultana , 2011). The protocol could be useful for successful induction of callus and regeneration of plants in strawberry to study somaclonal variation and improvement of variety.

5.2.5 Culture condition:

The success of micropropagation was greatly influenced by the composition of the culture medium besides the osmoticum and the nutrient medium in the culture. The results revealed that the ionic concentration and balance between different nutrient ions in the culture medium was crucial for optimization of shoot multiplication of strawberry. The multiplication rates and growth rates of shoots obtained with full strength and half strength MS medium varied significantly. Yellowing of leaves and slow rate of elongation were associated with the shoots cultured on MS medium at the lower nutrient concentration, particularly when the medium was either at 1/4th or 1/8th of the normal strength. The MS medium being a high mineral rich medium was often more effective (Skirvin, 1980). However Ali-Akbar *et al.* (2012) showed the better result in NN (Nitsch and Nitsch ,1969) medium

supplemented with 1.0 mg/l BAP, 0.5 mg/l IBA and 0.05 mg/l GA_3 then full strength MS medium. Litwinczuk *et al.* (2009) used medium recommended by Boxus (1999) supplemented with BAP (0.5 mg dm^{-3}), IBA (0.1 mg dm^{-3}), glucose (40.0 mg dm^{-3}) and GA_3(1.0-2.0 mg dm^{-3}), which give best result in increased numbers of auxiliary shoot of strawberry(*Fragaria* × *ananassa* Duch.) cv. Senga Sengana and Elsanta.

The osmotic potential of the nutrient medium which was dependent on the concentrations of the dissolved substances like major salts and sugar components affected the behaviour of the explanted tissues *in vitro*. The results indicated that 3% (w/v) sucrose was optimum for shoot multiplication and elongation of strawberry (*Fragaria* × *ananassa* Duch.) . The percentage of shoot multiplication and average number of shoots per culture were the minimum at 1% (w/v) and 5% (w/v) sucrose respectively. Similar observations were made in ginger and turmeric by Palai *et al.* (1997). The application of 3% (w/v) sucrose in the culture medium induced higher frequency of shoot multiplication and normal growth. Maximum *in vitro* multiplication of shoots was obtained on MS medium supplemented with kn 0.5 mg/l, BAP 1.0 mg/l and GA_3 2.0 mg/l and table sugar in place of sucrose (Kaur *et al* ., 2005). Apparently, the osmoticum like sucrose conjugates with growth regulator to make sugar alcohol, a form of compounds that could be transported to the cellular system quickly and could help in maintaining protein stability in the cell.

The shoot multiplication was the maximum (81.3%) at a pH of 5.8 in *Fragaria*×*ananassa* Duch. cv. Chandler. The medium having 0.6% agar was considered to be the best for shoot multiplication. Although the number of shoots at 0.4 % was at par with 0.5%, the shoots were healthy and highest in number at 0.6% agar concentration. Bhatt.I. *et al.* (2000) conducted the experiment on the micropropagation of Indian wild strawberry and used the MS medium solidified with 0.8% agar and pH adjusted to 5.8 prior prior to autoclaving. But Ara *et al.* (2013) found the best response by taking MS medium with 0.8% agar and pH adjusted to 5.7. Environmental factors such as light, temperature and humidity were the major requirements for production of multiple shoots (Mroginski *et al.*, 1999). The results of the experiment on the influence of different light intensities under 16 h photoperiod on shoot multiplication and growth were assessed. The results indicated that light intensities, in the range of 3000 lux was optimal for shoot multiplication of strawberry

(*Fragaria* × *ananassa* Duch.) through meristem cultures. Similar effects of light on *in vitro* morphogenesis were reported earlier in other species (Murashige, 1974; Seibert and Kadkade, 1980). As compared to different photoperiod cycles, the 16 h photoperiod at 3000 lux appeared to be the most favourable combination for both shoot multiplication as well as shoot growth of strawberry (*Fragaria* × *ananassa* Duch.). Incubation temperature was also an important factor for better shoot multiplication. Among the different temperature regimes, 25±2^{0}C was most favourable for the cultures *in vitro*. The result was same as Bhatt I. *et al.* (2000). However Shaila Harugade *et al.* (2014) mentioned the same 16 h photoperiod for best response but incubation temperature was 20±2°C in case of strawberry. Better performance was obtained by cultures when those were incubated at 26 to 28°C under light (2000 lux, 16 h photoperiod) provided by cool-white Philips fluorescent tubes (Kaur *et al.,* 2005) .

5. 3 Hardening of *in-vitro* regenerated plants

Hardening of regenerated plants is one of the important steps for achieving better field survival rate. In the present investigation, the regenerated plants were individually transferred on MS medium supplemented with BAP and NAA in different concentration for proper growth and development. Plantlets were fully grown within 3-4 weeks with spontaneously good rooting in rooting media. The well elongated healthy rooted plantlets were washed gently with sterile distilled water to remove all traces of MS media. After one day these rooted plants were pre hardened to the black polythene pot containing autoclaved mixture of sand: soil: FYM (1:1:1) and kept in the greenhouse with 85% humidity for acclimatization. About 90% of the plantlets were established in the greenhouse within 1-2 weeks of transfer. Similar findings were reported by Jofre-Garfias *et al.* (2010) and Azad *et al.* (2003). The rooted plants were transferred to field nursery for hardening (Anand and Rao, 2000).

5.4 Genetic fidelity test:

In recent years polymerase chain reaction (PCR) based molecular markers such as RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat) or AFLP (Amplified Fragment Length Polymorphism) were explored to study genetic diversity of many plant species (Anthony *et al.*,2004, Chen *et al.*, 2005,

Hagidimitriou *et al.*, 2005, He *et al.*, 2009). In the present investigation 12 Inter Simple Sequence Repeats (ISSR) primers were used to generate DNA fingerprint profile and to analyze the genetic fidelity of micropropagted and mother strawberry plants.

The quality of *in vitro* derived regenerates was screened with twelve ISSR primers that have showed monomorphic among the plantlets. The banding pattern of PCR amplified product from micropropagated plantlets was found to be monomorphic for most of primer tested. No polymorphism was detected in DNA profile, however some weak bands were absent in some the regenerates though their frequency was quite low and were not reproducible when repeated. The identical ISSR banding pattern was found from 19 *in vitro* raised plantlets and their mother control plant. Most of the primers showed identical DNA profile as compared with mother plant (*Fragaria* × *ananassa* Duch.) cv. Chandler.

Sen *et al.* (2010) ascertained the genetic uniformity of the nuclear genome of mother plant and its tissue cultured progenies of strawberry in order to establish true-to-type character through enhanced auxiliary branching micropropagation by employing ISSR-PCR molecular marker. The study yielded monomorphic bands in all the 12 UBC di-nucleotide motif primers. The results concluded that the tissue culture raised plantlets of strawberry are genetically identical and clonally uniform at least up to the 15th culture passage.

Although there are advantages for the use of micropropagation, there are concerns about genetic changes resulting from the process (Dale *et al.*, 2008). Somaclonal variation can result in a range of genetically stable variations useful in crop improvement (Jain, 2001). It is unpredictable in nature, and can be both heritable (genetic) and non-heritable (epigenetic). Somaclonal variation has been reported in berry plants and concern has been expressed about the genetic stability of micropropagated plants. Discrete morphological variants (Swartz *et al.*, 1981) and sporadic occurrences of abnormal fruit setting and a hyper-flowering habit that might be due to DNA methylation, have been reported in micropropagated strawberry plants (Boxus *et al.*, 2000). Strawberry regenerants produced from anther culture have been demonstrated to vary with respect to earliness, calyx separation, rate of ripening, and mildew (*Sphaerotheca macularis* L.) tolerance (Simon *et al.*, 1987). Somaclonal

variants with fungal resistance in strawberry have been reported (Damiano *et al.*, 1997). A number of molecular markers, including restriction fragment length polymorphism (RFLP), random-amplified polymorphic DNA (RAPD), arbitrary primed polymerase chain reaction, DNA amplified fingerprinting, simple (short) sequence repeat (SSR), short tandem repeat, sequence characterized amplified region, sequence-tagged sites (STSs), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), expressed sequence tag (EST)-PCR, and cleaved amplified polymorphic sequences derived from EST-PCR markers are available for genetic analysis of tissue culture-raised plants (Debnath, 2008a). While reviews of these techniques are plentiful (Varshney *et al.*, 2005) because of the rapidity with which relevant technology is proceeding, these may not remain compressive for long. PCR development has set the stage to overcome many of the shortfalls in the Southern blotting RFLP technique (Gantait *et al.*, 1985). PCR-based DNA marker systems can be divided into two basic classes: those that use primers designed from arbitrary or non-specific sequences, such as RAPD and AFLP, and those that use primers designed from a known sequence for targeting a single specific locus, such as SSRs and STSs. The introduction of DNA-based markers allows direct comparisons of different genetic material, independent of environmental influences (Weising *et al.*, 1995). The degree of similarity between banding patterns can provide information about genetic similarity and relationships between the samples studied. Each marker system has its own strengths and limitations, making the choice of marker an important decision. RAPD and ISSR marker analyses have been developed in the author's laboratory to identify genetic diversity in strawberry (Debnath *et al.*, 2008), and can be used to verify trueness-to-type of micropropagated strawberries. ISSR markers (Gupta *et al.*, 1994; Zietkiewicz *et al.*, 1994) are now being used in bioreactor micropropagated berry plants in the author's laboratory to verify clonal fidelity. ISSR primers target microsatellites that are abundant throughout the plant genome (Wang *et al.*, 1994). These markers have proved to be more reproducible than RAPD markers. They cost less and are easier to use than AFLPs and do not require prior knowledge of flanking sequences, like SSRs (Reddy *et al.*, 2002). Debnath (2009) compared bioreactor-derived tissue culture (BC) 'Bounty' strawberry plants obtained from sepal explants grown *ex vitro* with those propagated by tissue culture on gelled medium (GC) and by conventional runner cuttings (RC), for growth, morphology, anthocyanin content, and antioxidant activity over three growth seasons.

The BC and GC plants produced more crowns, runners, leaves, and berries than the RC plants although berry weight per plant did not differ significantly. BC and GC plants produced berries with more anthocyanin contents and antioxidant activities than those produced by the RC plants. But ISSR marker assay produced a homogenous amplification profile in the tissue culture and donor control plants confirming the clonal fidelity of micropropagated plants. However, it is imperative to regularly check the genetic purity of the micropropagated plants in order to produce clonally uniform progeny.

The present investigation was conducted to regenerate large scale disease free propagules of strawberry (*Fragaria* × *ananassa* Duch.) cv. Chandler through *in vitro* culture and to study the genetic uniformity of these regenerates, which is a pre-requisites for mass propagation. The result obtained from the investigation will be helpful for identification of duplications among the accession in tissue culture germplasm banks and also in the field. It is also beneficial to identify any adulteration or clonal variation which may bear desired traits and can be helpful for further crop improvement programme.

CHAPTER-6

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Strawberry is one of the important edible fruits in India. It is ranked high among small fruits (The wealth of India, 1970), esteemed as dessert and consumed for its flavour. The cultivated strawberry (*Fragaria* × *ananassa* Duch.) a hybrid between the Scarlet or Virginia strawberry (*F. virginiana* Duch.) and the pistillate South American *F. chiloensis* (L.) Duch. is a dicotyledonous, perennial low-growing herb which belongs to the family Rosaceae , traditionally propagated vegetatively by rooted runners but this method is not proved suitable due to incidence of many disease infections and environmental hazards and resulting in the gradual degeneration of cultivars performance. From different literature and present study it was observed that micropropagated strawberry plants were comparatively better in different characters (crown size, number of runners, flowering time and yield of berries) than conventionally propagated runner plants. To identify the variants created by means of *in vitro* propagation, which pose a problem for production of uniform, true-to-type plants, the genetic fidelity is carried out by using ISSR marker . So the aim of this study was to test the influence of media, hormone combinations on the regeneration and their genetic uniformity analysis.

The aseptic culture was obtained by treating the excised explants in Tween-20 @ 1% for 10 minutes and finally surface sterilized by 0.1% $HgCl_2$ for 7 minutes to avoid the contamination of culture. Three cytokinins (BAP, Kn and Ads) have been tested for shoot multiplication from apical meristem, runner tips, and first node of runner shoots of strawberry. Among the three cytokinins, 6-benzylaminopurine and kinetin favoured maximum rate of shoot proliferation. The moderate concentrations of BAP (1.0 mg/l) along with 0.25 mg/l kinetin and 50 mg/l adenine sulphate showed higher response (85.8%) on MS medium supplemented with 3% sucrose. Among three auxins (NAA, IAA, IBA) , NAA favoured maximum rate of shoot proliferation. The runner tips showed maximum percentage of multiplication (80.7%) on MS medium supplemented with NAA @ 0.1mg/l along with 0.25 mg/l IAA. In case of *Fragaria* × *ananassa* Duch. cv. Chandler, the maximum percentage (89.8%) of shoot proliferation was observed in full strength MS medium supplemented with 1.5 mg/l BAP, 0.1 mg/l NAA and 3% sucrose within 4 weeks of culture. Then the healthy

shoot are transferred to rooting media where the best response (90.8%) was observed in the media supplemented with 1.0 mg/l IBA.

Among the various hormonal supplements used, internodal explants of strawberry had been observed the high response to callus formation on MS medium supplemented with BAP and 2,4-D. The strawberry leaf explants induced to show callus development in most of the culture media formulations. However, the effect of different plant growth regulators formulations on the degree and types of callus formation were different. Highest (90%) callus induction was noted in the medium having 1.0 mg/l 2,4-D + 0.1 mg/l BAP using internode derived explants. The calli were green in colour and mostly compact in nature.

The healthy microshoots were separated from the clump and transferred to different medium having various concentrations of auxins with 2% (w/v) sucrose for induction of rooting. No rooting was observed in basal full strength or half strength MS media without growth regulators. The maximum percentage of rooting was observed in half strength MS media supplemented with 0.25 mg/l IBA and 2% sucrose.

This study also revealed that maximum percentage of multiple shoots per culture were achieved when cultured under 3000 lux light intensity and 16 h photoperiod at a incubation temperature of 25±2^{O}C. The pH of the media was maintained at 5.8 prior to autoclaving to achieve maximum percentage of multiple shoot. Among the carbohydrates tested, sucrose was found as the best source when used at a concentration of 3% for maximum shoot multiplication in *Fragaria* × *ananassa* Duch. cv. Chandler. The plants became white and died when mannitol was used as the source of carbohydrate. The medium having 0.6% agar proved to be the best for shoot multiplication for strawberry.

Plantlets were fully grown within 3-4 weeks with spontaneously good rooting in rooting media. The well elongated healthy rooted plantlets were washed gently with sterile distilled water to remove all traces of agar media. After two days, these rooted plants were pre hardened to the black polythene pot containing autoclaved mixture of sand: soil: FYM (1:1:1) and kept in the greenhouse with 85% humidity for

acclimatization. About 90% of the plantlets were established in the green house within 1-2 weeks of transfer.

The quality of *in vitro* derived regenerates was screened with twelve ISSR primers that have shown monomorphic among the plantlets. The banding pattern of PCR amplified product from micropropagated plantlets was found to be monomorphic for most of primer tested. No polymorphism was detected in DNA profile. The identical ISSR banding pattern was observed from 19 *in vitro* raised plantlets and their mother control plant. Most of the primers exhibited identical DNA profile as compared with mother plant (*Fragaria* × *ananassa* Duch) cv. Chandler.

In vitro propagation of strawberry provides significant opportunities for commercial cultivation. To overcome this challenge, careful optimization of protocol for each cultivar is utmost important. Suitable protocol can be used for automation, using a bioreactor, is one of the most effective ways to reduce the costs of micropropagation. The growth regulators like BAP, NAA and IBA proved to be effective for high scale of propagation. The *in vitro* raised plants produced maximum number of runners as compared with conventional propagated plants. Clonal fidelity is one of the main concerns in strawberry micropropagation. True-to-type propagules and genetic stability are pre-requisites for the application of micropropagation. The occurrence of somaclonal variation during micropropagation can be regulated by numerous factors including genotype, the presence of chimeral tissue, explant type and origin, media type, types and concentrations of growth regulators, cultural environment (temperature, light and photoperiod etc.) and duration of culture. *In vitro* organogenesis and somatic embryogenesis were alternatives means of mass scale propagation. Clonal fidelity of micropropagated plants can be monitored by their morphological, biochemical, physiological and DNA level. Molecular markers like ISSR, RAPD, RFLP are powerful tools in genetic identification of somaclonal variation. This protocol can be used for genetic transformation study for crop improvement programme . The development of a new "clean vector technology", the sequencing of gene controlling agronomic traits, under the control of their own promoters will allow to produce transformed plants with better consumer acceptance. Applications of this technology could support and complement the strawberry improvement program and competitiveness in the world market.

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APPENDICES

APPENDICES

Appendix I: Nutritional value of Strawberry

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

Appendix II:

1) Murashige and Skoog (MS) media (1962)

Component	Concentration in mediumMg/l	Concentration in stock solution (mg/l)
MS MAJOR SALTS		**20 X**
NH_4NO	1650.0	33000
KNO_3	1900.0	38000
$CaCL_2.2H_2O$	440.0	408800
MgSO4.7H2O	370.0	7400
KH_2PO_4	170.0	3400
MS MINOR SALTS		**200 X**
H_3BO_3	6.2	1240
$MnSO_4.4H_2O$	22.3	4460
$ZnSO_4.4H_2O$	8.6	1720
KI	0.83	166
$Na_2MoO_4.2H_2O$	0.25	5
$CoCl_2.6H_2O$	0.025	5
$CuSO_4.5H_2O$	0.025	50
MS VITAMIN		
Thiamine(HCL)	0.1	100
Glycine	2.0	400
Pyridoxine(HCL)	0.5	100
IRON SUPPLEMENT		**200 X**
Na_2 EDTA.H_2O	37.3	7460
$FeSO_4$.7H2O	27.3	5560
CARBON SOURCE	30000 mg/l	
Sucrose	Added freshly 100	
Myo-inositol	8000 mg/l	
Solidifying agent Agar	5.6-5.8	
pH		

Iron-EDTA (200X) 500 ml Stock

$FeSO_4.7H_2O$ (5560 mg) was dissolved in 500 ml of D.W. Na_2 EDTA (7460 mg) was added in 500 ml of D.W and boiled to dissolve it completely and it was then mixed with $FeSO_4.7H_2O$ solution.

Volume to be taken from stocks

Stock	Volume/lit
Macronutrients	50 ml
Micronutrients	5 ml
Iron EDTA	5 ml
Vitamins	10 ml
Myoinositol	10 mg
Glycine	2 mg
Ascorbic acid	100 mg
Sucrose	30 g
Agar	8 g
D.W	Make up 1000 ml
pH	5.8

Appendix III:

DNA Isolation and Purification

A. 1.0 M Tris-HCL (pH 8.0)

Dissolve in sterile de-ionized water, adjust pH to 8.0 with conc. HCL, and make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min.

B. 0.25 M EDTA (pH 8.0)

EDTA (dissolved salt; Mw = 372.3) = 9.31g

Dissolve, adjust pH to 8.0 with 5N NaOH, make up volume to 100 ml with de-ionized water and autoclaved at 15 psi for 20 min.

C. 0.5 M NaCl

NaCl = 29.2gm

Dissolve, make up volume to 100ml with de-ionized water and autoclave At 15 psi for 20 min

D. Extraction buffer

1M Tris–HCl (pH 8.0) - 19.8 ml

0.5 M EDTA (pH 8.0) - 8 ml

3M NaCl - 46.6 ml

2% CTAB(w/v) - 2gms

Dissolve, make up to 100 ml with de-ionized water and autoclave at 15 psi for 20 min.

E. 10 % working C-TAB

10% CTAB - 10 gm

5 M NaCl - 14 ml

Dissolve in water, make up to 100 ml and autoclave at 15 psi for 20 min.

F. 3M NaOAC (pH 6.8)

Sodium Acetate - 40.83 gm

Dissolve, adjust pH to with glacial acetic acid, make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min.

G. Choloroform: Iso-amyl alcohol Mixture (24:1)

Choloroform - 96 ml

Iso-amyl alcohol - 4 ml

H. 70% Ethanol (100ml)

Absolute alcohol - 70 ml

Double distilled water - 30 ml

I. RNase stock

1 M Tris- HCL (pH 8.0) - 100 µl

5 M NaCl - 300 µl

RNase - 10 mg

Adjust volume to 1 ml with de-ionized water, boil for 15 minutes and allow to cool slowly and stored at -20°C

J. TE (10:1)

1 M tris-HCL (pH 8.0) - 1 ml

0.25 M EDTA (pH 8.0) -0.4 ml

Dissolve; make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min

K. 10X TBE (pH 8.0)

Tris base - 108 gm

Boric acid - 55 gm

EDTA - 9.3 gm

Dissolve and make up volume to 100 ml with double distilled water.

Appendix IV: Equipments used

- Autoclave (Arch Tech)
- B.O.D. incubator (Remi)
- Deep freeze -20^{0}C (Blue star)
- Electronic balances (Sartorios)
- Hot air oven (Wiswo)
- Laminar flower (Clear)
- Microwave ovens (Samsung)
- Magnetic stirrer (Remi)
- Microscope (Zeiss)
- pH meter (EU-Tech)
- Refrigerated Centrifuges (Remi)
- Refrigerator (Whirlpool)
- UV transilluminator (UVI Tech)
- Vortex mixer (Geni)
- Gel Documentation Unit (UVI Tech)
- Horizontal Gel electrophoresis unit (Geni)
- Ice maker (orumsem)
- Incubator shaker (Pelican)
- Mini centrifuge (Biofuge)
- PCR (Peq Star)
- Spectrophotometer (BL-190)
- Soxhlet apparatus
- Water bath (GFL)
- Water purification system (Borosil)
- Lyophilizer (Christ)
- Rotary shaker (Remi)

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K. **10X TBE (pH 8.0)**

Tris base - 108 gm

Boric acid - 55 gm

EDTA - 9.3 gm

Dissolve and make up volume to 100 ml with double distilled water.

Appendix IV: Equipments used

- Autoclave (Arch Tech)
- B.O.D. incubator (Remi)
- Deep freeze -20^{0}C (Blue star)
- Electronic balances (Sartorios)
- Hot air oven (Wiswo)
- Laminar flower (Clear)
- Microwave ovens (Samsung)
- Magnetic stirrer (Remi)
- Microscope (Zeiss)
- pH meter (EU-Tech)
- Refrigerated Centrifuges (Remi)
- Refrigerator (Whirlpool)
- UV transilluminator (UVI Tech)
- Vortex mixer (Geni)
- Gel Documentation Unit (UVI Tech)
- Horizontal Gel electrophoresis unit (Geni)
- Ice maker (orumsem)
- Incubator shaker (Pelican)
- Mini centrifuge (Biofuge)
- PCR (Peq Star)
- Spectrophotometer (BL-190)
- Soxhlet apparatus
- Water bath (GFL)
- Water purification system (Borosil)
- Lyophilizer (Christ)
- Rotary shaker (Remi)