In vitro multiplication of strawberry (*Fragaria* x *ananassa* Duch.), cultivars Chandler and Ofra

BY

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CERTIFICATE – I

This is to certify that this thesis entitled "***In vitro* multiplication of strawberry (*Fragaria* x *ananassa* Duch.), cultivars Chandler and Ofra**", submitted for the degree of Master of Science in the subject of **Horticulture** of the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Mr. Krishna Madhav Rai, Adm. No. 2008A28M** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE – II

This is to certify that this thesis entitled **"*In vitro* multiplication of strawberry (*Fragaria* x *ananassa* Duch.) cultivars Chandler and Ofra",** submitted by **Mr. Krishna Madhav Rai Adm. No. 2008A28M** to the Chaudhary Charan Singh Haryana Agricultural University, in partial fulfilment of the requirements for the degree of **Master of Science** in the subject of **Horticulture**, has been approved by the Student's Advisory Committee after an oral examination on the same.

HEAD OF THE DEPARTMENT **MAJOR ADVISOR**

DEAN, POST-GRADUATE STUDIES

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LIST OF ABBREVIATION

2,4-D	=	2,4-Dichlorophenoxy acetic acid
BAP	=	6 Benzyl aminopurine
CH	=	Casein hydrolysate
IAA	=	Indole acetic acid
Kn	=	Kinetin
MS	=	Murashige and Skoog (1962) basal medium
µM	=	Micromolar
NAA	=	α-Napthalene acetic acid
EDTA	=	Ethylene diamine acetic acid
In vivo	=	In nature
In vitro	=	Under aspectic conditions
mg/l	=	Milligram per litre
gm/l	=	Gram per litre
µg	=	Microgram
V/V	=	Volume/Volume
W/V	=	Weight/Volume
IBA	=	Indole 3 butyric acid
GA_3	=	Gibberellic acid
%	=	Per cent
NaOH	=	Sodium hydroxide
HCl	=	Hydrochloric acid
pH	=	Negative logarithm of hydrogen ion concentration
Ppm	=	Parts per million
$HgCl_2$	=	Mercuric chloride
^oC	=	Degree Celsius
et al.	=	et alia = and other

Chapter-1

Introduction

Strawberry belongs to genus Fragaria of family Rosaceae. The cultivated strawberry (*Fragaria* × *ananassa* Duch.) is a hybrid between *Fragaria chiloensis* × *Fragaria virginiana*. It is an attractive luscious tasty, nutritious fruit with pleasant aroma and delicate flavour. It has unique place among cultivated berry fruits and is consumed by millions of people. In India, it is commercially cultivated in Maharashtra, Himachal Pradesh, Haryana, Uttar Pradesh, West Bengal, North Eastern state etc. During the last two decades strawberry cultivation has gained momentum in different parts of India because it is amongst the fewer crops which give quicker and very high returns per unite area on the capital investment, as the crop is ready for harvesting within six month of planting.

The strawberry plant is a low- creeping, perennial herb in which stem is composed in to a rosette crown (Galletta and Bringhurst, 1990) with 2 mm long internodes (Guttridge, 1985). The axiliary buds in the leaf nodes of the crown either remain dormant or developed in to branched crown or stolon (runners) depending on the prevailing environment. Stolon of most species consists of two nodes. A daughter plant is produced at the second nodes, whereas the first node remains dormant or develops another stolon. Leaves are arranged spirally on the central stem or crown and are generally pinnate and trifoliate. It has two types of roots primary one large, which originate from crown and the small secondary lateral roots which arise from the primary roots.

Propagation of strawberry by seed is not used commercially as the plants do not come true-to-type. Strawberry is conventionally propagated by runners. Although propagation by runner perpetuates all the characters of the parent, viral diseases are quite often transmitted through runner especially when the parent plants are already infected. Many experiments have shown that viral disease infection reduce vigor and yield to considerable extent, even no obvious symptoms are developed. Healthy plants get viral disease from infected plant through runners. Some of the most commonly occurring viral diseases are strawberry crinkle virus (SCV), strawberry vein bending virus (SVB), strawberry mild yellow edge (SMYE) and strawberry

latent C disease (Hancock, 1999). Thus, development and production of virus free planting material is of prime importance to the strawberry industry.

Micropropagation can be a potential tool in this direction to produce true-to-type, disease free and quality plants throughout the year. The possibilities of obtaining large numbers of strawberry plants from meristems or *in vitro* cultured strawberry plants and from meristem callus have been explored by several workers (Mullin *et al*., 1974 and Sobczykiewicz, 1979). This technique could be applied industrially. Since then, interest in strawberry micropropagation has increased and the method is used worldwide by institutes and private nurseries for the multiplication of clean stock.

The choice of donor plant and the time of explanting the meristem are of immense importance. There is no difference in using meristem excised from apical or lateral bud (Boxus *et al*., 1977). Strawberry meristem explants showed a remarkable adaptability to growing in different salt formulations including those of Knops (McGrew, 1965 and Mullin *et al*., 1974). Although virus-free plantlets could be obtained from meristems grown without growth regulators (Miller and Bellkengren, 1963), the supplements of plant hormones and mainly of benzyl adenine in the medium associated with an auxin improved the percentage of meristems that gave plantlets.

Large-scale commercial propagation by tissue culture is now being used widely in the strawberry industry. A million of plants can be produced in a year from a single meristem tip (Boxus, 1974 and Damiano, 1980). The effectiveness of meristem culture in producing virus-free plant material is reported to vary among cultivars (Beloshapkina, 1962). The wilt resistant cultivars Zinet and Kulon have high regenerative capacity from meristem producing high quality planting material (Popova *et al*., 1985). Strawberry plant have been regenerated through the shoot tip of runner is well established as described by number of researchers (Paredes and Lavin, 2005; Kaur and Chopra, 2003). Beside shoot tip of runner other organs are also serving as tissue sources like leaf (Liu and Sanford, 1988), apical bud (Negi *et al*., 2008) and nodal segments (Sakila *et al.,* 2007).

Presently availability of good quality plant material of a required cultivar in a large quantity is a major limitation in expansion of strawberry cultivation in northern India. No micropropagation oriented research work has been done in the newly introduced cultivars Chandler and Ofra which are performing well under North Indian conditions. Taking these problems in mind, the present study was planned with the following objective:

To optimize the culture conditions for high frequency shoot multiplication, rooting and transplantation in strawberry (*Fragaria x ananassa* Duch.) cultivars Chandler and Ofra.

Chapter-2

Review of Literature

Micropropagation is the rapid multiplication method of plants under environmentally controlled and aseptic conditions. It is an attractive technique because large number of uniform propagules can be generated in a relatively short period of time with predictable dates of harvest. Plant can be produced at any given time using this technology as it is season independent whereby planting material can be produced round the year, variability encountered in size and propagule density can also be minimized since plants are grown under controlled conditions *in vitro*.

Since the last three decades *in vitro* studies on strawberry (*Fragaria x ananassa* Duch.) have been conducted. Boxus (1974) determined the proper concentration of BAP necessary to obtain a large number of shoots from meristem tip explants in strawberry. Since then it became possible to multiply strawberry plants on an industrial scale. Earlier investigations aimed at the formation of fruits by culturing pollinated flowers and the induction of healthy virus-free plants from meristem (Nitsch, 1950; Bajaj and Collins, 1968).

Cryopreservation and cold storage of plantlets or meristems are valuable tools which help in preservation of genetic resources. Micropropagation techniques are now established and commercially exploited for propagation of strawberry in many countries including India. Protocols for protoplast isolation and regeneration have been developed for different strawberry cultivars. Tissue culture techniques such as protoplast fusion, *in vitro* cell selection and somatic embryogenesis have also been used with different objectives in strawberry (Joshi *et al*., 2003).

In this chapter, an attempt has been made to review the available literature on micropropagation studies relevant to strawberry *in vitro* culture under the following sub-headings:

2.1 Selection of explant

Selection of healthy explant is important in micropropagation (William and Maheshwaran, 1986). The suitability of which depends upon:

2.1.1 The organ that is to serve as a tissue source

In vitro propagation of strawberry cultivar through the shoot tip of runner is well established as described by a number of researchers (Wang *et al.,* 1984; Paredes and Lavin, 2005). Beside shoot tip of runner other organs are also serving as tissue sources like axillary buds of crown, leaf petioles, leaf disk, nodal segments and fruit receptacles. Boxus (1974) found that strawberry plants growing readily *in vitro* from axillary buds and the presence of cytokinin (6-BAP) in the medium stimulates growth. The soft, friable and non morphogenic callus was induced on medium without 2, 4-D but containing NAA, while compact and organized structure was formed when explants receptacles were cultured on a medium containing 2, 4-D and BA (Wang *et al*., 1984).

Bhatt and Dhar (2000) developed an efficient method of micropropagation with increased percent survival of explants and reduced phenol induced browning in wild strawberry. Nodal segments, when cultured on MS medium supplemented with 4.0 µM BAP and 0.1 µM NAA showed 94.4% explant establishment. Shoots showed 70% survival rates, when transferred to half strength MS medium supplemented with 1.0 µM NAA.

Elmana *et al.* (2003) reported that strawberry cultivar Selva was micropropagated by using shoot tip explant. Using MS salt mixture at full strength containing 1.0 mg/l IBA and 1.0 mg/l BAP, gave the best results for mass propagation. Devy (1997) reported rapid *in vitro* multiplication procedure for strawberry using runner tip as explants. Shoot runner tip of local (Bali) and California cultivar cultured on initiation medium developed into leafy shoot successfully. Similarly, Kaur and Chopra (2003) reported that experimental plant material of cultivar Chandler was raised through tissue culture MS nutrient medium using runner tips as explants.

2.1.2 Size of explant and overall quality of parent plant (Murashige, 1974)

Many workers have confined that the quality of explants primarily determines the establishment of *in vitro* cultures (John and Murrey, 1981; Kim *et al.,* 1981 and Keathely, 1983).

The larger explants regenerated earlier but more chances of contamination while using too small shoot explants chances of contamination is less but growth is slow.

2.2 Methods of explant disinfection

Successful disinfection of explants is a pre-requisite for *in vitro* culture and often involves a standard set of treatment, which vary with the type and species of explant (Thorpe and Patel, 1984). Contamination in tissue culture can originate from two sources: either through carryover of microorganisms on the surface of explant or in the tissue itself (endophytic microbes). The contamination usually decreases the propagation rate in the *in vitro* cultures, causing reduced chlorophyll formation and the rooting is seriously diminished. The physiological behavior of true axenic or contaminated cultures might be completely different because some added or released constituents of the nutrient medium are metabolized more or less by bacterial or fungal contaminations and the cultures can be hampered by substances which are released from microbes.

Disinfection of the isolated plant material to some extent depends on the physiological state of the mother plants. Some investigators prefer the practice of removing the explant from the plant in winter, when more stringent disinfection techniques can be applied (Antonelli, 1979). On the other hand, tips taken from vertically exposed runners do not need the same vigorous disinfection treatment before the isolation of meristems. Plant material to be used for culture is generally treated with an appropriate sterilizing agent to inactivate the microbes present on the surface. The sterilizing agents used were 9-10% calcium hypochlorite for 5-13 minutes, 2% sodium hypochlorite for 5-30 minutes, 0.1-1% mercuric chloride for 2-10 minutes, 1% silver nitrate for 30 minutes, 1-2% bromine water for 2-10 minutes, 10-12% hydrogen peroxide for 5-15 minutes, 70-95% ethyl alcohol for few seconds and antibiotics (4-50 mg/l) (Singh, 2005).

Yeoman (1977) recommended that disinfection by surface sterilizing agents must be undertaken according to concentration and time of treatment in explants taken from actively growing strawberry plants. Material from actively growing plants should be treated for 10 seconds to 10 minutes, depending on the number of cell layers which cover the meristem, and taking into account the likelihood of whether or not spores have to be removed. Wang *et al*. (1984) reported that disinfection with 1% sodium hypochlorite for 4 minutes is highly effective for strawberry receptacles. Surface sterilization of runner tips to control fungal contamination can be done by immersing them into 70% alcohol, use of an antioxidant and 25% sodium hypochlorite (Paredes and Lavin, 2005). The runner tips are easier to decontaminate then the

crowns. Runner tips should be collected in the spring or summer, as bacterial and fungal contamination is less than in the fall and winter (Adam *et al*., 2008).

Jungnickel and Gliemeroth (1986) reported that extremely sensitive or rare explants were short-term disinfected twice with an interval of 24-48 hours. Oxidizing agents (calcium hypochlorite: 3-10%, sodium hypochlorite: 1-7% or hydrogen peroxide: 3-12%) are characterized by having a very good effect when they are fresh. During storage they lose their oxidation power, and this can be controlled by titration. In the case of hypochlorites, however, ageing is accompanied by the formation of chlorates, which are well known herbicides. Ko *et al*. (2009) reported an effective disinfection protocol for regeneration from shoot tip culture strawberry. Explants were surface sterilized in sodium hypochlorite (0.5%) containing a few drops of Tween 20 for 7 min and rinsed 4-5 times with sterile water and subsequently outer leaves were separated and again explants were treated in 0.25% sodium hypochlorite containing Tween 20 for 1 min. The survival rate of genotypes was between 89.2 to 100%.

2.3 Culture media, its composition and growth regulation

Composition of basal medium used for culturing is an important factor to achieve the objective of targeted crop in tissue culture. Conditions favoring callus growth may not be suitable for organ differentiation. Goutheret (1955) emphasized the importance of nutrition in plant tissue culture. Several media have been developed by various workers to suit particular requirement of a culture tissue. Depending upon the overall salt concentration divergent nutrient media have been formulated (Murashige and Skoog, 1962; Gamborg *et al.,* 1968 and McCrown and Lloyed, 1981).

The basal medium is a means to induce rapid shoot multiplication (Vuylsteke and De Langhe, 1985; Boxus *et al.,* 1991 and Hamill *et al.,* 1993), which results into several clones of the original plant, over a short period of time. The growth medium provides all the requirements for growth, inorganic and organic salts (macronutrients, micronutrients and vitamins), moisture, a support matrix, and sugar. Growth regulators have been used for callus induction shoot regeneration and rooting in tissue culture technology. They affect growth and differentiation. The effect of different plant growth regulators, such as auxin, cytokinin, gibberellins etc., varies with the type and the quantity applied.

Lee and Fossard (1975) reported rapid multiplication of strawberry using MS medium supplemented with 1.56 µM IAA and 50µM kinetin. The concentration of cytokinin ranging from 0.25-2.5 µM/l coupled with auxin contraction ranging from 0.25-1.0µM/l, were most suitable, ensuring rapid proliferation of adventitious buds (James and Newton, 1977). James and Newtan

(1977) studied the effect of growth regulators on *in vitro* shoots of strawberry and reported that cytokinin are more necessary for organ differentiation and their data suggested that the best concentration of BAP is spread over the range of approximately 0.05 to 0.5 mg/l.

Jones *et al*. (1977) reported shoots regeneration from the callus cultures of strawberry cultivars Bogatta, Brighton, Cambridge Favourite, Hapil, Ostara, Rapella and Redcontlet and the promising selection JILA 33. Callus was initiated from explants of petiole and lamina of *in vitro* micropropagated shoots and greenhouse grown plants. There was more shoot regeneration with calli derived from lamina than from petiole. Out of 7 cultivars used in this study, in six cultivars shoot regeneration occurred on culture media supplemented with 6-BAP and 2,4-D, while in cultivar Cambridge favourite, it occurred only with medium which contained IAA β-alanine conjugate in place of 2, 4-D. Regenerated shoots rooted readily.

Liu and Sanford (1988) reported various methods for shoot regeneration from leaf and runner tissues of strawberry cultivars Allstar and Honeoye. Allstar leaf tissue derived from *in vitro* cultures regenerated shoots most efficiently in a Linsmaier and Skoog (LS) medium containing 2.5 mg/l BA and 0.5 mg/l IBA. Cultivar 'Allstar' leaf tissues derived from green house plant regenerated shoots best in LS medium containing 3.0 mg/l BA and 0.1 mg/l IBA. Additions of casein hydrolysate (CH) at either 400 or 600 mg/l stimulated the shoot production. A supplement of KNO_3 at 2000 mg/l also enhanced shoot regeneration efficiency from greenhouse grown leaf tissue explants. Lower regeneration potential was recorded in most treatments in Honeoye cultivar than Allstar. In Honeoye cultivar, shoots were only produced in LS medium containing 5.0 mg/l BA, 0.5 mg/l IBA and 400 mg/l casein hydrolysate (CH). In both the cultivars, shoots from runner tissues were best obtained using LS medium containing 10.0 mg/l BA, 2.0 mg/l IAA and 500 mg/l CH.

Green *et al*. (1990) reported shoot regeneration from leaf tissue explants in *Fragaria vesca* species of strawberry using Murashige and Skoog (1962) basal medium containing Gamberg's B_5 vitamins, 3% sucrose and 8 g/l agar supplemented with the various concentrations of growth hormones. Media containing 5µM BA with 5µM 2,4-D or NAA gives best callus growth from both lamina and petiole explants. Petrovic and Jacimovic (1990) studied that apical meristems excised from stolon buds were culture on basal media supplemented with growth regulator. The best supplements were 1.0 mg/l BA and 1.0mg/l IBA and o.1 mg/l GA_3 for leaf rosette development whereas, 1.0 mg/l BA and 1.0 mg/l IBA for shoot multiplication; and 0.5 mg/l IBA for rooting. MS basal medium was preferred over other media.

Kour *et al*. (2000) developed a low cost medium for *in vitro* propagation of strawberry (*Fragaria* × *ananassa* Duch) cultivar Chandler. Vegetative buds were cultured on Murashige and Skoog (MS) medium supplemented with low cost medium components. The major medium manipulations were made by replacing agar-agar, sucrose and distilled water with tapioca granules, table sugar and tap water, respectively. Maximum *in vitro* multiplication of shoots was obtained on MS medium supplemented with kinetin 0.5 mg/l, BAP 1.0 mg/l and GA_3 2.0 mg/l and table sugar in place of sucrose and maximum *in vitro* rooting was induced on one-fourth MS medium supplemented with IBA 1.0 mg/l, charcoal 200 mg/l and table sugar 20.0 g/l in place of sucrose. Hundred per cent survival of micropropagated plants was recorded in the field.

Mereti *et al*. (2002) reported micropropagation of the strawberry tree. The optimum shoot proliferation was achieved on a basal WPM containing MS vitamins, sucrose agar and 22.2µM BA. Microshoots rooted successfully in basal *in vitro* medium containing 10µm IBA or IAA.

Tahmatsidou and Voysatzi's (2002) worked to examine the possibility of a rooting medium for strawberry cultivars Elvira and Selva. Experiments were conducted on four medium (a) MRA= MS medium + 30g/l sucrose + Plant Growth Regulators (b) MRXA= MS medium + 15 g/l sucrose + Plant Growth Regulators, (c) MSYA= MS medium + 15g/l sucrose + No Plant Growth Regulators, (d) ENSHI= Enshi liquid medium with inorganic salts only. Rooting was not significantly different in all four media but plantlets rooted on ENSHI medium produced significantly higher numbers of daughter plants. Rooting on MSYA and ENSHI media, also showed that there was no need of PGR for root development in cultivars Elvira and Selva.

Zebrowska *et al*.(2003) reported plant regeneration from petiole and leaf blades of cultivar Kama and clone B-302m of strawberry on MS medium supplemented with various concentration of 6-BAP. Leaf explants showed shoot regeneration in media containing BAP only at concentration of 3.2 mg/l and 6.4 mg/l with clone B-302 displaying better shoot formation than cultivar Kama. In both genotypes regeneration from petioles was better than from leaf blades but number of plantlets per leaf blade was higher than obtained from petioles. Lal *et al*. (2003a) studied on *in vitro* propagation in strawberry for *ex situ* conservation and reported that the maximum number of shoots on MS medium with BAP (0.5 mg/l) and IBA (0.1mg/l) with 100% regeneration was observed in strawberry cultivar Ofra.

Lal *et al*. (2003b) developed an efficient protocol for plant regeneration in strawberry cultivars Ofra, Chandler and Oso Grande using runner tips as explants. Hundred percent shoot proliferation was observed on MS medium containing 4.0 mg/l BAP by cultivar Ofra followed by

91.7% in Oso Grande and 91.0% in Chandler. In case of Knops based media, 70% shoot regeneration frequency was recorded on 4.0 mg/l IBA + 0.4 mg/l BAP + 0.4 mg/l GA_3 by cultivar Chandler followed by 60%, 60% and 50% shoot regeneration in cultivars chandler, Oso Grande and Ofra respectively in Knops medium containing 3.0 mg/l IBA, 0.3 mg/l BAP and 0.3 mg/l GA_3. Solitary as well as multiple number of shoots were obtained in MS based media, while only solitary shoot per explant were observed on various combinations of Knops media. After shoot formation, plants were transferred to rooting media (MS media containing IBA). All the transferred plants showed 100% survival rate.

Kaushal *et al*. (2004) standardized protocol for regeneration of complete plantlets from callus culture using leaf and petiole explants of strawberry cultivars Chandler and Ferns. MS medium supplemented with 0.50 mg/l BAP and 0.75 mg/l NAA was found to be best for the induction and multiplication of callus. The response of leaf explant was significantly higher than petiole explant in both the cultivars. Khan and Spoor (2004) reported shoot regeneration in strawberry *cv.* Tango using leaf disc explants. It was found that callus induced from *in vitro* grown leaf disc explants exhibited higher shoot regeneration then those induced from greenhouse grown plants. MS medium supplemented with 2.5 mg/l BA + 0.18 mg/l NAA or 1.0 mg/l IBA recorded maximum shoot regeneration. Media containing 2, 4-D and BA in 1:1 ratio resulted in callus production while higher 2, 4-D and BA ratio produced large number of shoots from greenhouse grown leaf disc explants.

Paredes and Lavin (2005) reported clonal propagation in strawberry through axillary bud proliferation. Axillary buds aseptically isolated from runner tips and crowns, were cultured on agar-solidified MS nutrient medium supplemented with 1.0 mg/l IBA, 0.1 mg/l BA and 0.1 mg/l GA_3. Differentiated shoots were transferred to MS-agar medium supplemented with 0.1 mg/l IBA, 0.5 mg/l BA and 0.1 mg/l GA_3 in order to induce mass proliferation of shoots. Plantlets were then transferred to MS-agar medium with BAP (0.1 mg/l) for shoot elongation and rooting. Biswash *et al.* (2007) virus free strawberry plantlets production was investigated through meristem culture of strawberry clones. Meristem from runner tip cultured on different concentration and combination of growth regulator in MS liquid medium. After four weeks establishment meristems were transferred to growth regulator IBA which effective for root development and GA_3 for shoot elongation.

Sakila *et al*. (2007) studied Nodal segments of strawberry gave rise to multiple shoots when cultured on MS medium supplemented with different concentration of BA with kinetin or

GA_3. The highest response of shoot multiplication was obtained on MS medium containing 1.5 mg /l BA + 0.5-0.1 mg/l kinetin. The regenerated shoot lets were rooted on MS basal medium with different concentrations IBA and IAA. The maximum frequency of rooting and highest number of roots was produced on medium containing 1.0 mg/l IBA. The plantlets, thus developed were hardened and successfully established in soil.

Negi *et al*. (2008) standardized a protocol for production of strawberry plants, used axillary bud as explants with MS medium supplemented with BAP (0.5 mg/l) and kinetin (0.5 mg/l) and it was found best for culture survival (88.92%), earlier days to shoot emergence (9.18 days), shoot elongation (11.19 days) and number of shoots per explants (14.50). Debnath (2009) reported Reproducible protocol for regeneration of complete plantlets from 'Bounty' strawberry (*Fragaria* x *ananassa* Duch.), using a combination of gelled medium and bioreactor system. *In vitro* culture on TDZ and zeatin containing nutrient media apparently induced the juvenile branching characteristics that enhanced vegetative growth with more crown, runners, leaf, and berry production.

2.4 Rooting of regenerated shoots

After the regenerated shoot, the next stage is to induce roots, which are adventitious in origin (Hancock, 1999), arising from shoot base when inoculated in suitable medium. No general technique can be used for *in vitro* rooting because of variation in responses obtained from plantlets (William *et al*., 1985). Auxin (IBA, IAA, NAA etc.) growth regulator has been most suitable and used individually or in combination for rooting (Mante *et al*., 1989). Hunter *et al*. (1984) studied on micropropagation of strawberry and reported that IBA was most desirable additive for root formation, the optimum concentration being in range of 0.75 to 1.0 mg/l. Pereira *et al*. (1998) reported that ¾ to ½ strength salts of MS medium were better than full concentration for *in vitro* rooting of plantlets of Hofla and Tangi cultivars of strawberry. Maximum rooting of 100% on both full and half strength MS media supplemented with IBA at 1.0 mg/l reported by

Lal *et al*. (2003b). On the other hand Knops medium supplemented with 4.0 mg/l IBA + 0.4 mg/l BAP + 0.4 mg/l GA_3 when used maximum rooting of 60% was found.

2.5 Hardening of tissue culture produced plant

Plantlets produced *in vitro* must be acclimatized gradually to withstand the harsh natural environment. Misting, spraying or covering the pots with polythene bags may serve to fulfill the above objectives. Different types of substrates have been used during the acclimatization period

such as coco-peat, vermiculite perlite mixture (Goyal and Arya, 1981), sterilized sand (Bhansali *et al.,* 1988; Thakur *et al*., 2001) and soil (Kurtesn *et al.,* 1990; Sunaina and Goyal, 2000). During acclimatization, the temperature of root zone is important for better root growth. The medium should be warmer than the air for good root activity and to increase the humidity around the plantlets (Dunstan and Turner, 1984; McCown, 1986).

Moore *et al*. (1991) reported establishment of tissue culture grown plants in the green house environment. The tissue culture process is expensive with a large portion of costs associated with labor. The elimination of a laboratory rooting phase makes costs more reasonable. Specific environmental requirements must be recognized for the critical greenhouse acclimatization phase. Greenhouse technique reducing costs through the elimination of a laboratory rooting stagehand discusses the specific greenhouse requirements of selected tissue culture grown plants.

Zhou-Huan *et al*. (2003) stated that to increase the survival of transplanted *in vitro* cultured banana plantlets, the best method involved transplanting the plantlets in to plastic bags. Before transplanting, plantlets were soaked in a 600 time dilution of carbendazim for 1 minute for sterilization. When the first new leaf appeared, after planting in bags, 0.1% urea solution was added. When transplanted plantlets grew to a height of 20-30 cm after 45-60 days, the plant material was suitable for planting in the field.

Kaur and Chopra (2003) studied on hardening and field survival of micropropagated plants of strawberry (*Fragaria* x *ananassa* Duch.) under field condition in Punjab and reported that micropropagated strawberry plants were hardened in polythene bag and plastic trays. The maximum survival during hardening was observed in polythene bags were filled with soil and farmyard manure (1:1).

Chapter-3

Material and Methods

The present investigation entitled, "*In vitro* multiplication of strawberry (*Fragaria* x *ananassa* Duch.) cultivars Chandler and Ofra" was conducted during the year 2009-2010 in the Department of Biotechnology and Molecular Biology, CCS Haryana Agricultural University, Hisar. The present study was conducted with an objective to optimize the culture conditions for

high frequency shoot multiplication, rooting and transplantation in strawberry cultivars Chandler and Ofra.

3.1 Materials

3.1.1 Plant materials

The explants of Ofra collected from the PFDC, Department of Horticulture, CCS Haryana Agricultural University, Hisar and the plant of Chandler cultivar were procured from Dr. YSPUHF, Solan. The explant shoot tip 4-6 mm in length was used. The explant were collected and cultured from May to November, 2009 in Ofra and March to May, 2010 in Chandler.

3.1.2 Chemicals

Throughout the course of investigation, only chemicals of high purity were used. The chemicals such as inorganic salts, vitamins, sucrose, agar, myo-inositol, chelating agent etc. were procured from Sigma Co. (USA) and Hi-Media Co. (India). Antibiotics like streptomycin of Duchefa Co. (Netherlands) were used for the experiments.

3.1.2 Glassware

Glassware used during the course of investigation includes the flasks, pipettes, measuring cylinders etc. were of borosilicate quality. Flasks (150 ml) and glass bottles were used as culture vessels in all the experiments.

3.2 Methods

3.2.1 Sterilization of Equipment and glassware

The laminar air flow chamber was first wiped clean with spirit and then UV light was switched on for 45 minutes before use. The spirit lamp was also clean properly with spirit dipped cotton plug before its use. All the apparatus *i.e*. forceps, scalpels, needles and scissor were washed with spirit and were kept dipped in spirit inside the laminar air flow chamber and frequently sterilized on the flame while working.

All the glassware used during the investigation was washed with mild detergent followed by running tap water. The glassware was dried in oven before use. The glass apparatus were sterilized in oven at 180±2ºC for 2-3 hours. Pre-sterilized disposable Petri dishes (Tarson Pvt. Ltd) were also used during present investigation.

Table 3.1: Chemical composition of MS (Murashige and Skoog, 1962) medium

Constituents	Sr. No.	Composition	Quantity (mg/l)

Major salts	1.	KNO_3	1900
	2.	NH_4NO_3	1650
	3.	$MgSO_4.7\ H_2O$	370
	4.	KH_2PO_4	170
	5.	$CaCl_2.2H_2O$	440
Minor salts	1.	$MnSO_4.4\ H_2O$	22.3
	2.	KI	0.83
	3.	$Na_2MoO_4.2H_2O$	0.25
	4.	$ZnSO_4.\ 7\ H_2O$	8.6
	5.	H_3BO_3	6.2
	6.	$CuSO_4.5\ H_2O$	0.025
	7.	$CoCl_2.6H_2O$	0.025
Vitamins	1.	Thiamine HCl	0.1
	2.	Pyridoxine HCl	0.5
	3.	Nicotinic acid	0.5
Chelating agents	1.	Na_2EDTA	37.3
	2.	$FeSO_4.7\ H_2O$	27.8
Others	1.	Glycine	2
	2.	Sucrose	30,000
	3.	Agar	8,000

3.2.2. Preparation of Stock Solutions and their Storage

The stock solution required for preparing the media were prepared by dissolving the required chemicals in double distilled water. Salts were dissolved by adding one compound at a time and precipitation was usually avoided by dissolving the inorganic nitrogen source first. Stock solutions were prepared as 10 or 100 fold concentration. Stock solutions of growth hormones such as auxin, cytokinins were prepared dissolving small volume of NaOH and HCl and then finally by dissolving water in 1:1 ratio. All stock solutions were stored in glass containers with a lid kept at 4±1ºC temperature in refrigerator. Stock solution of growth regulator

and chelating agent were kept in amber coloured bottles for protection against light. All the stocks were consumed within a month of their preparation.

3.2.3 Culture media

In the present investigation, Murashige and Skoog (1962) basal medium was used. MS basal media supplemented with different concentrations of auxins and cytokinins alone and in combination were used for shoot induction, shoot multiplication and root induction.

3.2.4 Media preparation, sterilization and storage

The MS basal medium was prepared by mixing the required quantity of each stock solution in 500 ml of distilled water by continuous stirring. Myo-inositol (100 mg/l) and sucrose (30 g/l) were added to this solution. The growth regulator added in measured quantity, whenever required. The final volume was made to desire quantity by adding distilled water. The pH of medium was adjusted to 5.8 with 1N NaOH and 1N HCl using pH meter (Elico-digital). Agar was added (8.0 g/l) to this medium followed by melting of the medium in oven. The medium was stirred regularly to avoid agar clumps till boiled thoroughly allowed to cool for few minutes at room temperature and poured in flasks/glass, bottled with plastic screw caps (approximately 50 ml/vessel). The media were sterilized in autoclave at 121^0C for 20 minutes at 15 lb per square inch and were stored at room temperature for further use. The flasks/bottles containing culture media were used within a week.

3.2.5 Sterilization of plant material

Young shoot tip of healthy plants were collected from the polyhouse and brought to the laboratory in polythene bags. The explants of appropriate size (4-6 mm) were excised with the help of scalpel. Excised explants were first washed with detergent (Teepol), followed by washing under in running tap water. Then the shoot tip explants of both the cultivars were treated with different concentrations of antifungal and antibacterial agents for different period of time to avoid contamination. The explants were then surface sterilized with mercuric chloride (various concentration for different time) followed by washings with sterilized double distilled water in the laminar air flow chamber for further use.

Table 3.2: Disinfection/sterilization methods used for surface sterilization of tissue explants in cultivars Chandler and Ofra

Code	Disinfection Procedure
S1	Shoot tip explants were washed with double distilled water containing two drops of Teepol for 3 minutes and rinsed with double distilled water.

	Treatment of explants with 70% alcohol (v/v) for 2 minutes. Treatment of explants with 1% sodium hypochlorite (w/v) for 2 minutes. Treatment of explants with 0.1% $HgCl_2$ (w/v) for 2 minutes. 7-8 times washing of explants with sterilized double distilled water
S2	Shoot tip explants were washed with double distilled water containing two drops of Teepol for 3 minutes and rinsed with double distilled water. Treatment of explants with 70% alcohol (v/v) for 2 minutes. Treatment of explants with 0.05% $HgCl_2$ (w/v) for 2 minutes. 7-8 times washing of explants with sterilized double distilled water.
S3	Shoot tip explants were washed with double distilled water containing two drops of Teepol for 3 minutes and rinsed with sterilized double distilled water. Treatment of explants with 0.1% bavistin (w/v) for 30 minutes with continuous shaking on a shaker. Treatment of explants with 2% streptomycin (w/v) for 20 minutes. Treatment of explants with 70% alcohol (v/v) for 2 minutes. Treatment of explants with 0.1% $HgCl_2$ (w/v) for 2 minutes. 7-8 times washing of explants with sterilized double distilled water.
S4	Shoot tip explants were washed with double distilled water containing two drops of Teepol for 3 minutes and rinsed with sterilized double distilled water. Treatment of explants with 70% alcohol (v/v) for 2 minutes. Treatment of explants with 0.05% $HgCl_2$ (w/v) for 10 minutes. 7-8 times washing of explants with sterilized double distilled water.
S5	Shoot tip explants were washed with double distilled water containing two drops of Teepol for 3 minutes and rinsed with sterilized double distilled water. Treatment of explants with 0.4% bavistin (w/v) for 20 minutes with continuous shaking on a shaker. Treatment of explants with 70% alcohol (v/v) for 2 minutes. Treatment of explants with 0.1% citric acid (w/v) and 0.1% ascorbic acid (w/v) for 2 minutes.

	Treatment of explants with 0.1% $HgCl_2$ (w/v) for 2 minutes. 7-8 times washing of explants with sterilized double distilled water.
S6	Plants foliage was pretreated with 0.2% bavistin (w/v) two days before culture. Shoot tip explants were washed with double distilled water containing two drops of Teepol for 3 minutes and rinsed with sterilized double distilled water. Treatment of explants with 0.1% $KMnO_4$ (w/v) for 10 min with continuous shaking on a shaker. Treatment of explants with 0.1% bavistin (w/v) for 30 min with continuous shaking on a shaker. Treatment of explants with 70% alcohol (v/v) for 2 minutes. Treatment of explants with 0.1% $HgCl_2$ (w/v) for 2 minutes. 7-8 times washing of explants with sterilized double distilled water
S7	Shoot tip explants were washed with double distilled water containing two drops of Teepol for 3 minutes after that rinsed with sterilized double distilled water. Treatment of explants with 0.1% $KMnO_4$ (w/v) for 8 minutes. Treatment of explants with 0.3% bavistin (w/v) for 15 minutes. Treatment of explants with 70% alcohol (v/v) for 2 minutes. Treatment of explants with 0.1% $HgCl_2$ (w/v) for 2 minutes

3.2.6 Inoculation of explants into culture medium

The surface sterilized and aseptically excised explants were finally placed on media by working in a laminar air flow cabinet. The bottles containing medium prepared as per different treatments, were unplugged by holding them over spirit lamp and inoculations were performed by

placing explants on the surface of the medium with the help of flame sterilized long forceps and again plugged with screw cap of the bottles. During inoculation the explants were properly positioned on the media and were gently pressed with forceps to secure their firm contact with the media.

3.2.7 Incubation of culture

The culture flasks/tubes after inoculation were kept in culture room at 25±2ºC temperature for germination. The explants incubated for shoot induction/ proliferation were maintained at 25±2ºC temperature and photoperiod (2000-3000 lux) of 16 hours light and 8 hours dark in culture room.

3.2.8 Culture conditions

The culture was kept in the incubation room, fitted with photoperiodic controller and sequential timer. Temperature was maintained at 25±2^{o}C and light intensity of 100 μEM^{-2} sec^{-1} (1000 lux) was provided using florescent tubes. Light and dark period of 16 and 8 hours was provided.

Table 3.3: List of different MS based media used for shoot proliferation strawberry cultivars Chandler and Ofra.

Sr. No	Medium code	Basal medium	Growth regulators (mg/l)			
			Auxin	Cytokinin		
			IAA	BAP	Kinetin	TDZ
1	MS0	MS	---	---	---	--
2	MS1	MS	0.5	1.0	--	--
3	MS2	MS	0.5	2.0	--	--
4	MS3	MS	0.5	--	0.5	--
5	MS4	MS	0.5	--	1.0	--
6	MS5	MS	0.5	--	1.5	--
7	MS6	MS	0.5	--	2.0	--
8	MS7	MS	--	0.5	--	0.5
9	MS8	MS	--	1.0	--	0.5
10	MS9	MS	--	1.5	--	0.5
11	MS10	MS	--	2.0	--	0.5
12	MS11	MS	--	--	0.5	0.5
13	MS12	MS	--	--	1.0	0.5
14	MS13	MS	--	--	1.5	0.5

15	MS14	MS	--	--	2.0	0.5

3.3 Observations recorded

3.3.1 Number of days for shoot initiation

The number of days taken for shoot initiation were counted numerically from the date of inoculation for proliferation separately for each treatment and represented as number of days required for shoot initiation.

3.3.2 Numbers of days for completion of proliferation

The number of days taken for complete proliferation was counted numerically from the date of inoculation for proliferation till the proliferation completed for each treatment separately and represented as number of days required for completion.

3.3.3 Per cent shoot proliferation

The number of cultures which responded to shoot proliferation was counted in each treatment medium including the contaminated cultures inoculated and multiplied with 100 were calculated and represented as percent shoot proliferation.

$$\text{Percent shoot proliferation} = \frac{\text{No of culture proliferating}}{\text{Total no of culture inoculated}} \times 100$$

3.3.4 Number of shoots/explant

The number of shoots developed from each explants were numerically counted separately in each treatment after complete proliferation. The numbers of shoots/explant from different medium were recorded.

3.3.5 Shoot multiplication

Regenerated shoots of both the cultivars were subcultured on selected shoot multiplication media having MS basal salts supplemented with various concentration of growth regulator after an interval of 15-20 for their proliferation. Data were recorded for number of shoots obtained after each subculture and effect of subculture on shoot multiplication.

3.3.6 Root induction

Regenerated shoots of both the cultivars (Chandler and Ofra) were excised aseptically along with some of the surrounding basal portion and were placed on media having MS basal salts supplemented with IBA and NAA. Data were recorded for time taken to root initiation, no.

of shoot showing root formation and nature and quality of roots (type of roots, length of roots and density of roots).

Table 3.4: Root induction media for regenerated shoots of strawberry cultivars Chandler and Ofra

Sr. No.	Medium code	Basal medium	Growth regulator (mg/l)
1	R1	MS	1.0
2	R2	MS	1.0
3	Control	MS	--

R1- 1.0 mg/l IBA

R2- 1.0mg/l NAA

Control- without growth regulator

3.3.7 Transplantation of micropropagated plants

The rooted plantlets were separated gently from the medium and washed thoroughly in tap water to remove agar medium. Then the plantlets were planted in sterile mixture of coco-pit, vermiculite and perlite in 3:1:1 ratio with a polythene bag inverted to keep the humidity level high enough for plant to avoid any undesirable desiccation. The plants were kept for 10-15 days in culture room to avoid temperature shock at 25±2 ºC in diffused light conditions and irrigated with Hoagland solution. Initially, 10 ml Hoagland solution per plant was provided twice a day during the growth period. The inverted polythene bags on the plants were removed daily for 20-30 minutes to provide fresh air to the plant.

3.3.8 Plants survived after hardening

$$\text{Plant survived (\%)} = \frac{\text{No of plants survived}}{\text{Total no of plants transferred to pots}} \times 100$$

Table 3.5: Composition of Hoagland solution

(a) Solution A (Macronutrients)

Salt	g/l of stock solution
KNO_3	221.3
$Ca(NO_3)$	364.0
KH_2PO_4	62.1
$MgSO_4.7H_2O$	217.6

The above listed salts were dissolved in one liter distilled water.

(b) Solution B (Micronutrients)

Salt	g/l. of stock solution
H_3BO_3	1.26
$MnCl_2.H_2O$	0.60
$CuSO_4.5H_2O$	0.35
$ZnSO_4.7H_2O$	0.097
$NaMoO_4$	0.400

The above listed salts were dissolved in one liter distilled water.

(c) Solution C (Iron)

Salt	g/l of stock solution
$FeSO_4.7H_2O$	5
Tartaric acid	4

The above salts were dissolved in one liter distilled water and used fresh.

62.5 ml each of solution A, B and 15 ml of solution C diluted with 25 liters of distilled water.

Chapter-4

Experimental Results

In present investigation, attempts were made to develop an efficient protocol for micropropagation studies in two strawberry cultivars (Ofra and Chandler). The results obtained are given below:

4.1 Sterilization of explants

Protocol was standardized for surface sterilization of shoot tip explants in two strawberry cultivars *i.e*. Ofra and Chandler. It was quite difficult to get rid of contamination as the mother plants were field grown. Seven disinfection treatments (S1-S7) were used for surface sterilization of explants, which involved teepol, bavistin, sodium hypochlorite, mercuric chloride, alcohol and/or streptomycin. Explants were also treated with antiphenolics like citric acid and ascorbic acid to avoid the release of the phenolic compounds and browning of the cultures.

S1 and S2 disinfection treatments were not able to produce contamination free cultures in strawberry cultivar Chandler. With S4, S5, S6 and S7 disinfection treatments, less than 70% contamination was observed.

Table 4.1: Comparative effectiveness of different disinfection treatments in producing contamination-free shoot tip explants cultures in strawberry cultivars Ofra and Chandler.

Disinfection method	Effectiveness on cultivar	
	Ofra	Chandler
S1	-	-
S2	-	-
S3	++	++
S4	-	+
S5	++	+
S6	+++	+
S7	+++	+

- 100% contamination
+ Less than 75% contamination
++ Less than 50% contamination
+++ Less than 25% contamination

When shoot tips were disinfected with S3 treatment (containing 2% streptomycin) less than 50% contamination was observed. Seven (S1-S7) disinfection treatments were also used in Ofra cultivar for surface sterilization of shoot tip explants. S6 disinfection treatment which involved treatment of foliage with 0.2% bavistin at field and treatment of shoot tip explants with 0.1% bavistin during sterilization was best with 75% contamination free cultures.S7 disinfection treatment also produces less than 25% contamination. S3 and S5 disinfection treatments, which involved bavistin treatment at a concentration of 0.1% and 0.4% for 30 minutes and 20 minutes respectively, produced contamination free culture at a frequency of about 50%. It clearly indicates that use of a fungicide in disinfection procedure is necessary for surface sterilization.

4.2 Shoot induction from explants

Shoot induction/regeneration from shoot tip explants in both cultivars (Ofra and Chandler) was observed on all the media (Table 4.2). Shoot tip explants of 2.0 to 6.0 mm in length were used for shoot regeneration. Small sized (<2.0 mm) explants failed to regenerate and died. Initiation of shoot formation was observed within 12-20 days of culture. The maximum percentage of explants showing shoot formation frequency (100%) was observed on MS6, MS13 and MS14 media in Ofra and on MS13 medium in Chandler. Development of only 1-2 shoots was observed on MS0 medium (without growth regulator). Maximum number of shoot formation (6-10) was observed on MS6 medium in Ofra and in Chandler maximum shoots were obtained (6-12) on MS13 medium.

Table 4.2: Effect of media on shoot proliferation from shoot tip explants in strawberry cultivars, Ofra and Chandler

Sr. No.	Media code	Ofra		Chandler	
		% of explant showing shoot formation	No. of shoots formed/explant	% of explant showing shoot formation	No. of shoots formed/explant
1	MS0	80%	1	80%	1-2
2	MS1	70%	3-5	85%	3-4
3	MS2	60%	3-5	90%	3-4

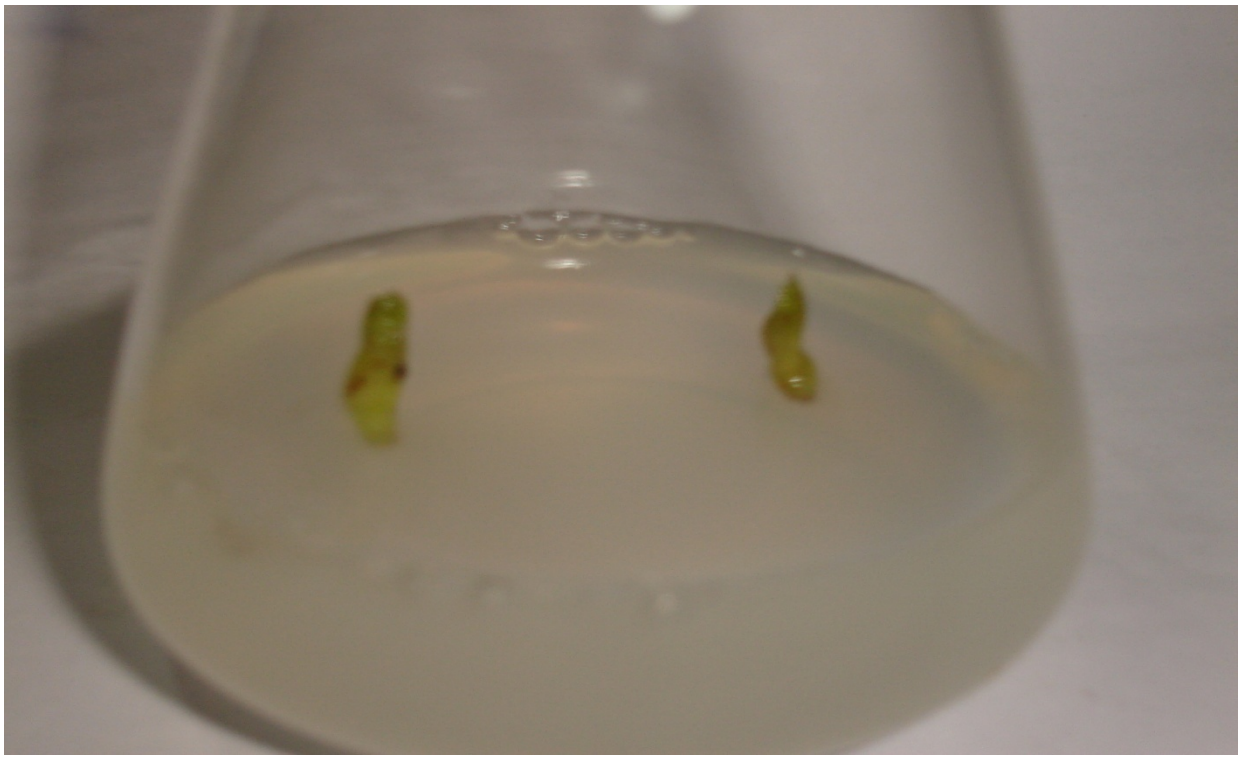

Plate 1. Shoot formation from shoot tip explant in strawberry cultivars Ofra and Chandler

Plate 2. Multiple shoot formation in strawberry cultivars Ofra and Chandler

4	MS3	60%	6-7	85%	3-6
5	MS4	66%	6-7	80%	3-6
6	MS5	60%	6-8	85%	3-6
7	MS6	100%	6-10	90%	3-6
8	MS7	80%	4-6	75%	3-4
9	MS8	90%	4-7	90%	6-8
10	MS9	80%	5-7	90%	5-6
11	MS10	80%	5-7	90%	6-8
12	MS11	90%	4-6	80%	4-6
13	MS12	90%	3-6	90%	6-10
14	MS13	100%	4-6	100%	6-12
15	MS14	100%	5-8	60%	5-7

4.3 Shoot multiplication

Results obtained for shoot multiplication in both the strawberry cultivars (Ofra and Chandler) are shown in Table 4.3. Three MS based media (MS6, MS13 and MS 14) were selected for shoot multiplication in both cultivars. Maximum percent of shoot multiplication (100%) was observed in Ofra on medium MS6 and in Chandler on MS13 medium. The number of shoots in cultivar Ofra and Chandler varied between, 6-10 and 6-12, respectively. Micropropagated shoots took 12-20 days for multiplication in both the cultivars and subculturing was done at an interval of 21 days.

Table 4.3: Percent shoot multiplication response in strawberry cultivars Ofra and Chandler

Medium code	**Ofra**		**Chandler**	
	% shoot multiplication response	**No. of shoots formed/explant**	**% shoot multiplication response**	**No. of shoots formed/explant**
MS6	100	6-10	95	3-6

MS13	80	4-6	100	6-12
MS14	90	5-8	90	5-7

Effect of subculturing on shoot multiplication is shown in Table 4.4. In Ofra cultivar, the maximum shoot multiplication (5-8 shoots) was observed in first subculturing on MS6 medium whereas, in Chandler the maximum shoot multiplication (4-8 shoots) was observed in first subculturing on MS13 medium (Table 4.4).

Table 4.4: Effect of subculturing on shoot multiplication in strawberry cultivars Ofra and Chandler

Cultivars	Media	Sub-culture	Total no. of shoots obtained/explant
Ofra	MS6	1st	5-8
		2nd	6-10
	MS13	1st	4-5
		2nd	4-6
	MS14	1st	4-5
		2nd	5-8
Chandler	MS6	1st	3-4
		2nd	3-6
	MS13	1st	4-8
		2nd	6-12
	MS14	1st	6-8
		2nd	6-8

4.4 Induction of roots:

Shoots were transferred on various rooting media. The roots were visible within 12-15 days after transfer of shoots on rooting media, R1 and R2, based on Murashige and Skoog (1962) medium. Rooting was also observed if explants were kept in shoot proliferation media for 30

Plate 3. Plants with well developed roots in strawberry cultivars Ofra and Chandler


15 4:56AM

Plate 4. Micropropagated plants of strawberry cultivars Ofra and Chandler transplanted into pots

Plate 5. Micropropagated plants produced runners

Plate 6. Micropropagated plants bearing fruit

days. Hundred percent rooting was observed on R1, R2 and control (without growth regulator) in both the cultivars (Table 4.5). R1 (with 1.0 mg/l IBA) proved to be the most suitable for root induction in both the cultivars. Thick and dense root growth was observed on R1 and R2 media but on control MS medium root was comparatively poor in quality.

Table 4.5: Percent shoot induction response in strawberry cultivars, Ofra and Chandler

Medium code	cultivar					
	Ofra			Chandler		
	% of shoots rooted	No. of roots/shoot	Average root length (cm)	% of shoots rooted	No. of roots/shoot	Average root length (cm)
R1	100	6-9	5-8	100	5-7	6-7
R2	100	5-7	4-7	100	5-7	5-7
Control	100	4-5	4-5	100	4-5	3-6

R1- 1.0mg/l IBA

R2- 1.0mg/l NAA

Control- without growth regulator

4.5 Transplantation:

Regenerated plantlets with well developed roots were taken out along with the medium from the culture vessels and rinsed with distilled water to remove trace of sugar and agar medium which might provide a substrate for microorganism. These plants were transferred to pots containing coco-pit, vermiculite and perlite in 3:1:1 ratio. Hoagland solution was used as nutrient supplement (macro and micro nutrients) twice a day at 10 ml/plant. In Ofra percent survival (83%) was higher as compared to percent survival (61%) in Chandler (Table 4.6). Micropropagated plants of cultivar Ofra produced runners after one to one and half months of transplantation and fruiting after three months of transplanting.

Table 4.6: Survival of strawberry plantlets in pots

Cultivars	No. of transplanted plantlets	30 days after transplanting in pots	
		No. of plants survived	% of survival
Ofra	30	25	83

Chandler	21	13	61

Chapter-5

Discussion

Micropropagation is a powerful and well practiced tool for large scale propagation of horticultural crops. It is a rapid technique where mass multiplication of ornamental, timber and fruit tree can be achieved in relatively short period of time with high fidelity index. It is especially important where no other mean of propagation available or conventional method is time consuming, rate of multiplication is very slow or diseases also got transferred with planting material (as in case of strawberry).

Micropropagation has several other advantages over conventional propagation like elimination of pathogen, preservation of breeding stock as juvenile plants and removal of quarantine problems (Maretaki, 1987). The entire sequence of clonal propagation of a species through tissue culture can be divided in to four stage *viz*. stage 1: culture establishment (regeneration of shoots), stage 2: multiplication of propagules (multiplication of shoots), stage 3: induction of roots in regenerated shoots (roots), stage 4: establishment of plantlets in soil (transplantation) (Singh, 2002).

Micropropagation has specific set of requirement and the technique in a particular species is dependent upon fulfilling these conditions. The different conditions and requirements for micropropagation of two cultivars (Ofra and Chandler) of strawberry have been standardized in this investigation and attempts have been made to develop an efficient *in vitro* multiplication of the above cultivars. Various steps involved and their results are discussed below.

5.1 Sterilization of explants

Contamination is a major problem in strawberry *in vitro* multiplication. Thus, successful disinfection of explants is a pre-requisite for *in vitro* culture. Protocol has been standardized for disinfection of shoot tip explants. Shoot tip explants of cultivar Ofra were surface sterilized with S6 and S7 disinfection treatments; we observed less than 25% contamination. In chandler cultivar, sterilization treatment has been standardized for disinfection and less than 50% contamination was observed when explants were surface sterilized with S3 (Table 4.1) treatment. Pierik (1987) also reported that proper surface sterilization of explant is necessary for establishing contamination free axenic culture for micropropagation in plants. Adams *et al*. (2008) reported that sodium hypochlorite and teepol were found to be highly effective to eliminate fungal contamination in strawberry whereas, in present investigation these treatments

with other disinfectants were found not suitable for production of contamination free culture. Similar results were also reported by Bhankhar (2008), who reported 100% contaminated free plants by treating the plant foliage with 0.2% bavistin two days before culture then explants were treated with 0.1% potassium permanganate, 0.1% bavistin, 70% alcohol and 0.1% mercuric chloride. Lal *et al*. (2003b) reported washing of explants with 70% alcohol and 0.1% mercuric chloride produced contamination free culture at reasonably good frequency whereas, in present investigation 70% alcohol and 0.05% mercuric chloride produced less than 50% contamination free culture.

5.2 Shoot induction from explants

Shoot tip explants have been widely used for shoot induction and multiplication in many strawberry cultivars using modified MS and Knops media (McGrew, 1965; Liu and Sanford, 1988; Green and Davis, 1990; Bhatt and Dhar, 2000; Sood *et al*. 2004, Negi *et al*. 2008). Devy (1997) used runner tip a explants and reported that runner tip of local (Bali) and California cultivars cultured on initiation medium containing Murashige-Skoog (MS) developed leafy shoots successfully.

Shoot tip explants exhibited variable response for shoot formation in various media tested (table 3). Shoot tip explants after 45-50 days of culture revealed the maximum (100%) regeneration on MS6 (2.0 mg/l kinetin + 0.5 mg/l TDZ) medium in Ofra cultivar and the maximum (100%) regeneration in cultivar Chandler on MS13 (1.5 mg/l kinetin + 0.5 mg/l TDZ) media. In present investigation different combination of growth regulator on MS based media were suitable for both cultivars and similar results were reported by Boxus (1981) and stated that cultivar differences can be critical in the success of micropropagation. Negi *et al*. (2008) also used MS media supplemented with kinetin (0.5mg/l) in combination with BAP (0.5 mg/l) for shoot induction. They observed earlier shoot elongation (11.19 days) and number of shoots per explants (5.73). They also reported that MS media supplemented with kinetin (0.5 mg/l) with BAP (0.5 mg/l) was found best for culture survival (88.95%) and earliest days to shoot elongation (9.81 days) in Chandler cultivar. Sakila *et.al*. (2007) used nodal segment of strawberry as explants, they reported that nodal segments of strawberry gave rise to multiple shoots when cultured on MS medium supplemented with different concentration of BA with kinetin or GA_3. Elmana *et al*. (2003) used shoot tip as explants and reported that using MS salt mixture at full strength, containing 1.0 mg/l IBA and 1.0 mg/l BAP gave the best result for mass propagation of the strawberry plant. In present investigation we used MS media supplemented

with kinetin, BAP with IAA and TDZ for cultivars Ofra and Chandler of strawberry. For Ofra cultivar best combination was kinetin (2.0 mg/l) + IAA (0.5 mg/l) and for Chandler cultivar kinetin (1.5 mg/l) + TDZ (0.5 mg/l).

5.3 Shoot multiplication

In present investigation, differences in shoot multiplication frequency were observed in two strawberry cultivars, with maximum frequency noticed in cultivar Chandler followed by Ofra. The maximum number of shoots per explant was obtained on MS6 medium kinetin (2.0 mg/l) + IAA (0.5 mg/l) for Ofra and in Chandler on MS13 medium kinetin (1.5 mg/l) + TDZ (0.5 mg/l). Sakila *et al*. (2007) reported that highest response of shoot multiplication was obtained in MS medium containing 1.5 mg/l BA + 0.5 to 0.1 mg/l kinetin. Negi *et al*. (2008) reported that different combinations of growth regulators BAP, IBA, kinetin and GA_3 were tried for further multiplication. In BAP, IBA and GA_3 combination MS medium supplemented with BAP (0.5 mg/l) in IBA (0.5 mg/l) and GA_3 1.0mg/l) resulted maximum shoot length (10.50 cm) while, the maximum number of shoots (14.50) per explants was found on MS medium supplemented with BAP (0.5 mg/l) and IBA (0.5 mg/l). In BAP, kinetin and GA_3 combination MS medium supplemented with BAP (0.5 mg/l) and GA_3 (2.0 mg/l) was resulted maximum shoot length (4.60 cm), number of shoots (4.44), number of leaves (10.3) and number of fragments (3.71) per explant. Kaur and Chopra (2003) reported that used MS medium supplemented with BA (0.5 mg/l) + IBA (0.1 mg/l) for shoot multiplication and observed good results.

5.4 Induction of roots

Hundred percent rooting was observed in R1 (1.0 mg/l IBA), R2 (1.0 mg/l NAA) and control (without growth regulator) media and high rooting intensity was observed on R1 and R2 media. If multiplicated shoot were kept on shoot proliferation media for 30 days rooting was observed. Elmana *et al*. (2003) reported that use of MS basal medium induced the formation of longest and highest number of shoots. In both cultivars have thick and dense root was observed on R1 and R2 rooting media. Hunter *et al*. (1984) reported that IBA was most desirable additive for root formation, the optimum concentration being in the range of 0.75 to 1.0 mg/l. Sakila *et al*. (2007) also reported that out of different concentration of IBA (0.1-1.5 mg/l) and IAA (0.1-1.5mg/l) tested 1.0 mg/l IBA proved to be the most suitable for root induction per explant and the average root length. The rooting was obtained on MS basal medium fortified with IBA (1.0 mg/l) alone also reported by Kaur and Chopra (2003).

5.5 Transplantation

Hundred percent survival of plantlets in the pots containing 3:1:1 ratio of coco-pits, vermiculite and perlite was observed in the plantlets raised from all the media tested, indicating that the survival of plantlets on transfer to the pots depends on the manipulation of external environmental factors like temperature, high humidity and soil moisture, rather than on the composition of culture media from which the plantlets were multiplied. Kaur and Chopra (2003) reported micropropagated plantlets were hardened in polythene bags and plastic trays. The maximum survival during hardening was observed in polythene bags filled with soil and Farmyard manure (1:1). The hardening carried out during the month of February gave maximum survival (71.50%) as compared to March (55.20%). In present investigation, survival micropropagated plantlets were 83% in Ofra as compared to 61% in Chandler cultivar.

Chapter-6

Summary and conclusions

In the present investigation attempts were made to develop an efficient protocol for *in vitro* regeneration in two cultivars (Ofra and Chandler) of strawberry (*Fragaria* x *ananassa* Duch.). The experiments were conducted during May, 2009 to June, 2010. The explants were collected during the period of last week of May to first week of October for Ofra and second week of March to first week of May for Chandler. The results of various experiments conducted to induce shoot multiplication, rooting and transplantation are as given below:

1. Shoot tip was used as explants for *in vitro* propagation of Ofra and Chandler cultivars.
2. In Ofra, maximum survival percentage of explants was obtained with S6, when treatment of foliage with 0.2% bavistin at field and shoot tip explants were surface sterilized with 0.1% bavistin (30 min.), 0.1% $KMno_4$ (8 min.), 70% alcohol (2 min.) and 0.1% $HgCl_2$ (2 min.) and S7, when explants were surface sterilized with 0.1% $KMno_4$ (8 minutes), 0.3% bavistin (15 minutes), 70% alcohol (2 minutes) and 0.1% $HgCl_2$ (2 minutes).
3. In Chandler, maximum survival percentage of explants was obtained when shoot tip explants were surface sterilized with 0.1% bavistin (30 minutes), 2% streptomycin (20 minutes), 70% alcohol (2 minutes) and 0.1% $HgCl_2$ (2 minutes).
4. Per cent shoot formation was observed maximum (100%) on MS6, MS13 and MS14 basal media supplemented with 2.0 mg/l Kinetin + 0.5 mg/l IAA, 1.5 mg/l kinetin + 0.5 mg/l TDZ and 2.0 mg/l kinetin + 0.5 mg/l TDZ in Ofra and 100% in cultivar Chandler on MS13 medium supplemented with 1.5 mg/l Kinetin + 0.5 mg/l TDZ.
5. Maximum number of shoots regenerated per explant (6-12) was obtained in cultivar Chandler as compared to cultivar Ofra (6-10).
6. Both the cultivars showed the development of one to two shoots in MS medium without any growth regulator.

7. Time taken for root initiation was recorded after 12-16 days of culturing on rooting media in both the cultivars.
8. The plantlets, thus developed were hardened and successfully established in 3:1:1 mixture of coco-pit, vermiculite and perlite. Survival rate of regenerated plantlets transferred to soil media was 83% in Ofra followed by 61% in Chandler.
9. Micropropagated plants showed runner production in cultivar Ofra after one to one and half months of transplantation.
10. Micropropagated plants of cultivar Ofra also showed fruiting after three months of transplantation.

In strawberry Scultivar Ofra, MS6 medium supplemented with 2.0 mg/l kinetin and 0.5 mg/l IAA was found best for shoot formation followed by MS13 and MS14. But in case strawberry cultivar Chandler, MS13 medium supplemented with 1.5 mg/l kinetin and 0.5 mg/l TDZ was found best for multiple shoot proliferation. The regenerated shoots were best rooted on MS basal medium containing 1.0 mg/l IBA in both cultivars. The plantlets were hardened and successfully established in 3:1:1 mixture of coco-pit, vermiculite and perlite. Survival rate of micropropagated plant transfer to soil media was 83% in Ofra followed by 61% in Chandler cultivar.

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ABSTRACT

Title of thesis	:	*In vitro* multiplication of strawberry (*Fragaria* x *ananassa* Duch.) cultivars Chandler and Ofra
Full Name of degree holder	:	Krishna Madhav Rai
Title of Degree	:	Master of Science in Horticulture
Name and address of Major Advisor	:	Dr. R.K. Goyal Department of Horticulture, CCS Haryana Agricultural University,Hisar-125004, India
Degree awarding University	:	CCS Haryana Agricultural University, Hisar-125004, Haryana, India
Year of award of degree	:	2010
Major subject	:	Horticulture
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Number of words in the abstract	:	112 (approximately)

Key words: *Fragaria* x *ananassa* Duch., strawberry, shoot tip, regeneration

Shoot tip of strawberry gave rise to multiple shoots when cultured on MS medium supplemented with different concentration of kinetin and BAP with IAA and TDZ. The highest response of shoot multiplication was obtained in MS media containing 2.0 mg/l kinetin + 0.5 mg/l IAA and 1.5mg/l TDZ for Ofra and Chandler cultivar respectively. The regenerated shoots were rooted on MS basal medium with IBA, NAA and control (without growth regulator). The highest root length and number of roots was produced on MS medium containing 1.0 mg/l IBA. The plantlets, thus developed were hardened and successfully established in soil medium. The plants raised through tissue culture exhibited normal growth and fruit setting.

Major Advisor **Head of the Department**

Signature of Student

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"I, **Krishna Madhav Rai Adm. No. 2008A28M** undertakes that I give copy right to the CCS HAU, Hisar of my thesis entitled **"*In vitro* multiplication of strawberry (*Fragaria* x *ananassa* Duch.) cultivars Chandler and Ofra".** I also undertake that patent, if any, arising out of the research work conducted during the programme shall be filled by me only with due permission of the competent authority of CCS HAU, Hisar.

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