IN VITRO REGENERATION OF GERBERA (*Gerbera jamesonii)* FROM DIFFERENT EXPLANTS

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CERTIFICATE

This is to certify that thesis entitled **"*IN VITRO* REGENERATION OF GERBERA (*Gerbera jamesonii)* FROM DIFFERENT EXPLANTS"** submitted to the Department of Horticulture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE in HORTICULTURE**, embodies the result of a piece of *bona fide* research work carried out by **SAIDA PARVIN,** Registration No. **06-02692** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

Dated: June, 2015
Dhaka, Bangladesh

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The greatest gift from Allah I ever had

“I had come to the world through my parents”

Dedicated to –

Prof. Dr. Jamal Sir

(My Honorable supervisor)

Without his guideline it is not possible for me to come this far.

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The Author

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ABSTRACT

An experiment was accomplished at Advanced Seed Research and Biotech Center, ACI Dhaka, Bangladesh during the period from September, 2014 to September, 2015 to find out suitable explant for culture establishment to develop a standard protocol. Five explants (E_1, Flower bud; E_2, Capitulum; E_3, Leaves; E_4, Peduncle; and E_5, Petiole) and six different growth regulators combination were used for culture establishment. The flower bud is the best explants, due to higher survival rate (75.6%), early for culture establishment (16.4 days) and lowest contamination rate (7.1%). MS medium supplemented with 3 mg/l BAP + 0.01 mg/l NAA; (85.8%) is best medium showed longest shoot (25.4 mm) and higher response for shoot regeneration in shortest time. The lowest performance found by the control treatment. Various levels of auxin were tried for root initiation. MS medium of half strength supplemented with 0.3 mg /l NAA; rooted early (12.0 days) was found to be the best medium for rooting of *in vitro* shoots because it showed highest root number (9.30). Media consists of sand, coco dust, vermicompost (1:1:1, v/v) resulting as the best hardening materials with maximum survival rate with better plant vigour. The overall micropropagation technique is effective and promising for regeneration of gerbera.

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ABBREVIATIONS AND ACRONYMS

SAU	---	Sher-e-Bangla Agricultural University
SAURES	---	Sher-e-Bangla Agricultural University Research System
BARI	---	Bangladesh Agricultural Research Institute
Intl.	---	International
Biol	---	Biological
Agril	---	Agricultural
PGRs	---	Plant Growth regulators
BAP	---	6-Benzyl Amino Purin
BA	---	Benzyl Adenin
NAA	---	Naphthalene Acetic acid
IAA	---	Indole Acetic Acid
IBA	---	Indole Butyric Acid
2,4-D	---	2,4-Dichlorophenoxy acitic acid
2-ip	---	2-isopentyladenine
TDZ	---	Thidiazuron
mm	---	Milimeter
ppm	---	parts per million
pH	---	Negative logarithm of hydrogen ions concentration
gl^{-1}	---	Gram per litre
mgl^{-1}	---	Miligram per litre
Mol	---	Molecular
µm	---	Micro mol
DMRT	---	Duncan's Multiple Range Tesr
CRD	---	Complete Randomized Design
ANOVA	---	Analysis of Variance
df	---	Degrees of freedom
CV%	---	Percentage of Coefficient of Variation
LSD	---	Least Significant Difference
J	---	Journal
Sci.	---	Science
ASRBC	---	Advanced Seed Research and Biotech Centre
Res.	---	Research
et al.	---	And others (at elli)

CHAPTER I

INTRODUCTION

Gerbera (*Gerbera jamesonii* Bolus), which is also known as Transvaal Daisy, is an important cut flower both in the domestic and the international markets. The genus *Gerbera* was named in the honor of a German naturalist, Traugott Gerber. Variety in color has made this flowering plant attractive for use garden decoration, such as herbaceous border, bedding and pots and for cut flowers as it has a long vase life (Chung *et al*., 2005; Chauhan, 2005). The flowers are of various colours suit very well in different floral arrangements. The cut blooms also have a long vase life of about 7 to 8 days. Gerbera is ideal for beds, borders, pots and rock gardens. *Gerbera* is one of the leading cut flowers and ranks fifth among the top ten cut-flowers of the world (Parthasarathy and Nagaraju, 1999). The genus *Gerbera* consists of about 40 species comprising half-hardy and perennial flowering plants. Among the different species, *Gerbera jamesonii* is the only species under cultivation. Gerbera belongs to the family *Asteraceae*. Plants are stem less, tender and perennial herbs. Leaves are radical, petiolate, lanceolate, deeply lobed, sometimes leathery, narrower at the base and wider at the top and are arranged in a rosette at the base. Flower heads are solitary, many flowered, with conspicuous ray florets in one or two rows, florets of inner disc when present are very short, sub-tubular and two lipped. Based on flower heads, they may be grouped into single, semi-double and double cultivars. The flowers occur in a wide range of colors including yellow, orange, cream-white, pink, brick red, scarlet, salmon, maroon, terracotta and various other intermediate shades. The flower stalks are long, thin and leafless.

It is considered to be native to South African and Asiatic regions. is an important commercial flower grown throughout the world in a wide range of climatic conditions. Gerbera is growing best at altitudes ranging between 1300 and 3200

meters above mean sea level. In our country it is grown as a commercial crop in Jessore from past few years. For its adopting nature it has been popular in farmers within short times.

A well drained, rich, light, neutral or slightly alkaline soil is most suitable for Gerbera production. Day temperature of 22 - 25^0C and night temperature of 12 - 16^0C are ideal for cultivation. Gerbera is generally propagated by division of suckers or clumps. Propagation through seeds is not preferred as the plants exhibit heterozygosity and non-uniformity. Also, the improved semi-double and double cultivars do not set seeds. Propagation by division of suckers or clumps gives true to type plants, but the multiplication rate is very low. It is very costly due to gerbera plant imported from India for commercial cultivation. It is not only costly; sometimes plants are of low quality. Farmers multiply the plants by dividing clumps every year, which is result poor quality flower. It causes loss in commercial business..

Clonal propagation is one of the potent solution others countries has been used for rapid multiplication. This could be accomplished through micropropagation technique. Tissue culture finds its tremendous application in ornamental crops, especially in areas of propagation and crop improvement. Plant propagation either through meristem or non-meristem culture enables production of large number of plants within a short span of time in limited space. Propagation through meristem has an added advantage of virus elimination, while regeneration through non meristematic tissue like leaf helps in production of transgenic plants by introducing new genes with desirable characters like novel colours, shapes, resistance to pests and diseases, and enhanced vase life. Plants raised through non meristematic tissue like leaf are also amenable for somaclonal variations. Micropropagation work in Gerbera was initiated by shoot tip culture for rapid multiplication of elite varieties as early as 1974 by Murashige *et al.* and in 1985 by Huang and Chu. Recently, regeneration through leaf has been utilized to create

somaclonal variations and to introduce genes of desirable characters (Elomaa *et al.*, 1993). Over the years, gerbera has been propagated by direct or indirect organogenesis using various explants, including stem tips, floral buds, leaf, capitulum etc. (Kanwar and Kumar, 2008).

To decrease the cost of production for commercial cultivation it is necessary to produce seedling in our country. There is an attempt made by BRAC of producing seedling by micropropagation. But as per farmers review the flowers produce by that tissue culture plantlet producing flower was not uniform in size and color. In Bangladesh different study is done on gerbera micropropagation. That causes this study on hybrid *Gerbera jamesonii* Bolus. Hence keeping above points in view, present investigation has been undertaken with following objectives.

- To standardize the protocol for *in vitro* clonal propagation of gerbera from best explants
- To study the response of different explants on medium supplemented with different concentrations of growth regulators
- To standardize the optimum concentration of plant growth regulators

CHAPTER II

REVIEW OF LITERATURE

The present experiment was conducted to find out the best explant, growth regulators and hardening medium for *in vitro* multiplication of gerbera. Some of important and informative works have so far been done in home and abroad related to this experimentation have been presented (Year wise) in this chapter. The relevant literature about these aspects have been reviewed and presented below.

The concept of totipotency which is an inherent part of the cell theory of Schleiden (1838) and Schwann (1839) is the basis for plant tissue culture. Major breakthroughs in plant tissue culture were achieved with the discovery of auxins and cytokinin.

2.1 Concept of gerbera tissue culture:

An experiment conducted by Chung *et al*, (2005) on misty red gerbera (*Gerbera hybrida*) developed from crossing of Ximena and florense, performance trial at the Flower Breeding Research Institute, Gyeongnam Agricultural Research and Extension Services, Korea, in 2000. Study showed that Misty red gerbera is suitable for cultivation in greenhouse conditions in Korea. It stated that different color variety of gerbera is responsible for attractive garden as herbaceous border, bedding and pot flower and as cut flower it has long vase life.

Seed propagation in gerbera is not always satisfactory since impurity of strain produces a great deal of variations (Schiva 1975). Moreover, traditional seed production technique is too slow for their exploitation in commercial purposes (Murashige et al. 1974). It also requires longer time to produce flower. Vegetative

propagation overcomes the problem of unpredictable characters and plants obtained by this method perform better than those developed from seeds. In vitro techniques of propagation have been recognized as the preferred method for multiplication of selected elite clones (Reynoird *et al*. 1993, Aswath and Choudhary 2002, Xi and Shi 2003, Prasanth and Sekar 2004, Kumar *et al*. 2004, Chakrabarty and Datta 2007 and http://www.bari.gov.bd). A survey of literature indicates that tissue culture of Gerbera has been studied by various scientists using different types of explants (Barbosa *et al*. 1994, Parthasarathy *et al*. 1997, Le *et al*. 1999, Posada *et al*. 1999, Zhang *et al*. 2002, Modh *et al*. 2002, Tyagi and Kothari 2004, Aswath and Wanzeen 2004, Thakur *et al*. 2004, Kumar and Kanwar 2005, 2006, Sharma and Srivastava 2005 and Ray *et al*. 2005).

Murashige *et al.* (1974) showed that gerbera can be propagated vegetatively by shoot tip culture. A comparison of the two propagation methods, shoot tip culture and capitulum explant culture showed that each method has advantages and disadvantages. They reported that shoot tip system is much more rapid, but the initial number of shoot tips required is very high due to the high infection rate (80%) and in turn require a great number of mother plants. In case of capitulum explants, the number of shoots per flower was much smaller, but loss by infection was only 10 percent. The use of capitulum explants had the advantage of leaving the mother plant intact. They proposed that for practical purposes both methods can be used, but the capitulum system in the initial phase and shoot tip system in the later phase are better for mass propagation.

2.2 Establishment of *Gerbera jamesonii* Bolus plantlets

The success of plant tissue culture as a means of plant propagation depends on multiplication and establishment of plantlets which in turn are greatly influenced by number of factors.

2.2.1 Explant

The type of explant, its size, position, age, physiological state and the manner in which it is cultured can all determine, whether the cultures could be successfully maintained and the morphogenesis could be induced. Shoot tip, bud, flower, leaf, peduncle, petiole are the commonly used explants for *in vitro* gerbera multiplication.

Leffring (1971) concluded that the most efficient *in vitro* method for the vegetative propagation of *Gerbera jamesonii* was by rhizome cuttings. Dormant buds on excised rhizome can be forced to form shoots which can root. This method was rather slow, particularly for plant breeders, as an average of only 20 plants could be produced per year from a single plant.

Pierik and Segers (1973) reported that when inflorescence was cut into six instead of four explants, the total number of shoots per flower was the same, but it decreased when the inflorescence was cut into eight, ten or twelve explants. A slightly higher number of shoots per explant was obtained from the inflorescence collected in the month of June and July compared to those in other months. They concluded that there was a very small probability that these shoots were of adventitious origin. They insisted that shoot primordia in a quiescent state existed in the axils of the involucral bracts. Murashige *et al.* (1974) showed that Gerbera can be propagated vegetatively by shoot tip culture. A comparison of the two propagation methods, shoot tip culture and capitulum explant culture showed that each method has advantages and disadvantages.

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capitulum system in the initial phase and shoot tip system in the later phase are better for mass propagation. Pierik *et al.* (1975) propagated Gerbera using fully developed inflorescence. They found no differences in shoot formation between wide open and older flowers. However, they found closed flowers and very young flowers were inferior and had more infection than other flowers. Gregorini *et al.* (1976) opined that i*n vitro* culture of vegetative apices was a valid method for clonal multiplication of Gerbera while, Pierik *et al.* (1979) reported that shoot tips are more suitable for mass propagation than capitulum. Shoot formation from leaves was noticed during *in vitro* propagation of Gerbera cultivar 'Vulkan' (Hedtrich, 1979).

Mother plants with leaves removed were planted in green houses and tissues taken from the new shoots or the rhizomes were grown on nutrient base for propagation of gerbera through shoot tip culture (Raalte-D-Van, 1978).

Huang and Chu (1985) cultured ex vitro derived shoot tips of cultivars 'Arendsoog' and 'Super Giant Yellow' and observed that ten shoots developed per explant after four weeks of culture. Schum and Busold (1985) observed in vitro shoot production to be higher and quicker from one to two cm long, flower bud explants. They further noticed that shoot development in the axils of involucral bracts was most common in buds, while shoots from undifferentiated callus occurred more frequently in fully developed inflorescence.

The culture of portions of young capitulum at the bud stage of inflorescence was proved more productive than that using fully developed inflorescence (Laliberte et al., 1985). They used immature inflorescence of 0.5 to 0.7 cm in diameter and after sterilization, involucral bracts were removed. The hypothesis of Pierik and Segers (1973) and Pierik et al. (1975), that the dormancy of pre-existing buds in the axils of the involucral bracts could be broken in the culture conditions, but this was excluded by Laliberte et al. (1985), since involucral bracts had been removed from the explants. According to them the buds resulted from the reorientation of

meristematic tissues in the explant. Kafarski and Hauzinska (1974) also used young capitulum as explants for the multiplication of *Gerbera jamesonii*.

Arello et al. (1991) used capitulum explants to produce a large translucent callus. Sitbon (1981), Meynet and Sibi (1984), Ahim and Vieth (1986) and Tosca et al. (1990) showed the feasibility of producing haploid plants through in vitro ovule culture. Adventitious shoot formation from leaf explants occurred at the base of leaf petiole (Jezry and Lubomskii, 1991). Leaf, petiole and apex as explants of Gerbera were used to establish tissue and cell cultures by Ruffoni and Massabo (1991). Leaf explants produced numerous roots and apex explants produced more shoots and calli.

Effect of the age of plant material on in vitro regeneration was tested in Dianthus caryophyllus by Altorst et al. (1992). The leaves of different ages (3, 5, 8, 10 and 12 weeks after subculture of donor cuttings) were taken from donor plants and explants were examined for regeneration. The youngest leaves gave the best regeneration results with seven shoots per regenerated explant. Regeneration percentage decreased with increase in the age of leaf.

Efficient bud regeneration was obtained from leaf explants of a clone of Gerbera hybrida cultured on modified MS medium. Morphogenic potential varied with the development stage of the leaves. Up to 90 percent of excised developing leaves formed three to five shoots per plant. Bud regeneration was not obtained on same medium with fully expanded leaves (Reynoird et al., 1993). They noticed the effect of developmental stage of leaf on morphogenic potential in clones 10 and 11 of *Gerbera hybrida*. Further, they observed that younger leaves located in the centre of the rosette were quite difficult to excise, but displayed the greatest ability to regenerate. The regeneration capacities progressively decreased with the stage of leaf development and fully expanded leaves were non-regenerative. A similar observation was made by Orlikowska et al. (1995) where, the production of

adventitious shoots was best from the young leaf explants compared to the explants from petiole bases of old leaves.

Sreelatha et al. (1998) compared the effect of basal and apical portions of leaf explants on callus initiation in Anthurium species. They noticed that callus initiation was better in the basal portions of leaf explants. The difference in response between the basal and apical portions was attributed to the difference in the physiological state as well as the number of cells undergoing de-differentiation. Physiological changes may account for the changes in the contents of endogenous hormones, nutrients and metabolites.

Gerbera plants were regenerated and propagated in vitro from floral peduncle explants by Le-CL *et al*. (1999). De novo regeneration of adventitious buds was possible by cutting peduncle tissues on a basal salt Murashige and Skoog medium, which led to the production of axillary buds at the rate of 10 newly, formed shoots per initial explant and per subculture.

Zhao et al. (2002) reported that the contamination rate could be lowered greatly if the receptacle was used as explant for Gerbera. Maximum per cent contamination was observed in shoot tip and flower (fully developed) explants.

Modh *et al*. (2002) successfully used young capitulum having a diameter of 0.5 to 1.0 cm as an explant from Gerbera cultivar 'Atella' plants for in vitro culturing. Shailaja (2002) studied the suitable explant for multiplication of Gerbera at Department of Horticulture, UAS, Dharwad and found bud and flower as suitable explants for the establishment of *in vitro* Gerbera culture.

The shoot tips of 0.5 - 1.0 cm length and leaf discs and petiole of 1 - 2 cm length were used as explants for *in vitro* culturing by Mohanty *et al*. (2005). The shoot tip was widely used as explant for Gerbera tissue culture studies by many workers (Murashige *et al.*, 1974; Gregorini *et al.*, 1976; Raalte-D-Van, 1978; Petru and Matous, 1984; Huang and Chu, 1985; Parthasarathy and Nagaraju, 1995).

The technique of using immature flower buds (1 - 1.5 cm long) as explants was followed in carnation by Karami et al. (2007). Tui *et al.* (2005) also used young capitulum as explants for in vitro regeneration of *Gerbera jamesonii.*

2.3 Surface sterilization of explants

Sterilization of explants is an essential requirement in order to improve the success of micropropagation.

Gerbera plants of cultivars 'Arendsoog' and 'Super Gaint Yellow' were stripped of roots and divided into portions from each of which all but the last unexpanded leaf, were removed. The periderm of the rhizome was peeled off and the shoots were sterilized in 1% NaOCl (sodium hypochlorite) solution for 10 minutes, rinsed three times with sterilized water and reduced to 2 - 3 mm explants (Huang and Chu, 1985).

Immature inflorescences, 0.5 to 0.7 cm in diameter of *Gerbera jamesonii* cultivars 'Pastourelle' and 'Mardi Gras' were collected and sterilized for 2 minutes in 70 percent ethanol, for 9 minutes in sodium hypochlorite (1.5%) with Tween-20 (0.01%), and washed in sterilized distilled water for 45 minutes. After sterilization, involucral bracts were removed, and the capitula were cut into 20 to 25 pieces. Each explant, containing 25 to 40 sessile flowers was inoculated separately on solid medium (Pierik *et al*., 1975; Laliberte et al., 1985).

Pre-treatment of mother plants by Ruffoni et al. (1987) comprised of defoliation, a short immersion in a solution containing 100 g/1 Streptomycin + 1 g/1 Benlate + 1 g/1 Ridomil and transplanting into sterile 1:1 (peat : perlite) in sterile pots. The best among the six explant treatments was provided sequential treatment with 3% NaOCl for 20 minutes, 70% ethanol for 30 minutes followed by two changes of sterile water for 10 minutes each.

Capitula collected at the moment of the expansion of ray florets, were surface disinfected in 2 per cent sodium hypochlorite for 10 minutes and then rinsed three times in sterile water (Tosca *et al.*, 1990). Terminal buds of *Chrysanthemum morifolium* cv. 'Pennine Reel' were excised from vigorously growing shoots and immersed for 20 minutes in a solution of sodium hypochlorite containing 1.0-1.5 per cent (v/v) available chlorine. Buds were immersed in two changes of sterile distilled water, each for 20 minutes and inoculated onto multiplication medium (Roberts and Smith, 1990). Capitula (1 - 3 per genotype and 0.7 - 1.0 cm diameter) were collected from August to November and sterilized in commercial bleach (2% sodium hypochlorite) for 30 minutes and then rinsed in sterile distilled water (Conti *et al.*, 1991).

Shoot tip explants were obtained from axillary shoots of greenhouse grown plants of Gerbera jamesonii Bolus. A few outer leaves were removed, the blades of the remaining leaves were cut off and the shoots were rinsed free of soil in autoclave sterilized water. Subsequently, the shoots were disinfected for 20 minutes in commercial bleach solution (1.3% NaOCl) containing 0.1 per cent Tween-20, and rinsed in disinfectant solution with autoclave sterilized water. These explants of about 1 - 3 mm in length were transferred to nutrient medium, one explant per culture tube (Jerzy and Lubomski, 1991).

The shoot tips of carnation were washed under running tap water for 1 - 1 ½ hours and dipped in 70 per cent alcohol for 45 seconds, they were then disinfected with 0.2 per cent $HgCl_2$ along with two drops of Tween-20 for ten minutes and thoroughly rinsed with sterile distilled water for 3 - 4 times (Jagannatha, 1997). Shoot tips, nodes and flower buds of carnation were immersed in a solution of carbendazim (0.1%) each for 3 minutes and then washed thoroughly thrice with distilled water, then they were immersed in 0.1 per cent $HgCl_2$ for 3 minutes and washed with sterile distilled water for 3 - 4 times in the laminar air flow cabinet (Hiremath, 2000).

The ray florets and young leaves of chrysanthemum were surface sterilized with 0.1 per cent sodium hypochlorite and then rinsed 4 to 5 times with sterile distilled water (Tanaka *et al*., 2000). Explants from young capitula of Gerbera were surface sterilized in 70 per cent ethanol for 25 - 30 seconds, followed by 0.1 per cent $HgCl_2$ solution for 5 minutes (Modh *et al.,* 2002).

Solutions of carbendazim (0.1%) and streptomycin (0.1%) each for 3 minutes were used for immersion of explants, which were later washed thoroughly thrice with distilled water and were again immersed in 0.1 percent $HgCl_2$ for 3 minutes before finally washing with sterile distilled water for 3 - 4 times in the laminar air flow cabinet (Shailaja, 2002).

The explants size of 5 mm x 5 mm cut transversely to the midrib from mature leaves were washed thoroughly with water for 5 minutes, disinfected with 70 percent ethanol for 30 seconds and surface sterilized with filtered sodium hypochlorite (0.1%). After 10 minutes, they were rinsed five times with sterilized distilled water, blotted on sterile filter paper and 15 explants were plated with adoxial surface in contact with the medium (25 ml) in 100 mm petri dishes (Aswath and Choudhary, 2002b).

Inflorescences were detached at developmental stage 2 for in vitro culture. They were sterilized with 1% NaOCl for 10 minutes and rinsed with sterile distilled water for three times. They were then incubated on the medium (8 g/1 agar with 3% sucrose) under 14 hours light/10 hours dark cycles at 23 - 25°C (Meng and Wang, 2004).

Mohanty et al. (2005) treated the segments of mature and immature leaves as well as petioles with Bavistin (0.2%) for 30 minutes and washed under running tap water. The explants were washed with Teepol for 10 minutes followed by tap water. Then they were treated with 0.1 per cent $HgCl_2$ solution for different durations under laminar air flow cabinet. Further they were dipped in 1 per cent

KCl solution for one minute to remove toxic Hg_2+ ions from the surface of the explants. The sterilized explants were cut with a scalpel and surgical blade and washed thrice with autoclaved distilled water before inoculation.

Ranjan and Gaurav (2005) in their experiment took leaf segments and pieces of petiole of 1 - 2 cm size as explants. They were washed under running tap water for 15 minutes and treated with carbendazim (0.2%) for 30 minutes and surface sterilized with $HgCl_2$ (0.1%) for 10 minutes, followed by washing 3 - 4 times by sterile distilled water. Mature explants were collected from greenhouse-grown plants and washed thoroughly with tap water followed by distilled water containing a few drops of Teepol. The explants were dipped in 10% calcium hypochlorite solution for 5 minutes, followed by a dipping in 0.1% HgCl2 solution for 10 minutes. Again they were rinsed with sterile distilled water for 4 - 5 times and blotted on a sterile tissue paper (Negi et al., 2006).

Nhut et al. (2007) reported that receptacles (1.5 - 2.0 cm in diameter) of 10 inter-specific crosses of young Gerbera flowers (7 - 14 days old) were washed thoroughly under tap water for 20 minutes, soaked in My Hao detergent (Vietnam) for 15 minutes, then washed thoroughly under tap water again for 2 hours, rinsed six times with distilled water and then submerged in a 7% (w/v) solution of $Ca(ClO_2)$ for 25 minutes followed by rinsing six times in sterile distilled water in the laminar air flow cabinet.

2.4 Culture media

The success of plant tissue culture is greatly influenced by the nature of the culture medium used. Plant tissue culture media provides major and minor nutrient elements and carbohydrates. A wide variety of media have been reported. The choice depends on the plant species and the intended use of the culture.

Murashige and Skoog (1962) medium characterized by high concentration of mineral salts has been widely used for general plant tissue culture. No other factor

has received as much attention as media, since the success in plant cell culture is largely determined by the quality of nutrient media (Vasil and Thorpe, 1994).

Improved results were obtained by providing trace amounts of organic compounds notably vitamins and amino acids (George and Sherrington, 1984). A nutrient media comprising of MS macro elements at half strength, Heller's micro elements, Na_2Fe-EDTA at 21.4 mg/1, MS organic constituents and 10 g/1sucrose with pH adjusted to 5.6 was used for excised scape culture (Huang and Chu, 1983).

Growth and morphogenesis in in vitro are regulated by the interaction and balance between the growth regulators supplied in the medium and the growth substances produced endogenously by the cultured cells. Apart from the direct effect on cellular mechanisms, many synthetic growth regulators may in fact modify the level of endogenous growth substances (George and Sherrington, 1984).

Schum and Bichler (1986) cultured Gerbera cultivars 'Hildegard' and 'Th-102' in ten different media and then potted in a greenhouse. Subsequent growth was best in plants cultured on media of Pierik et al. (1975) and poorest on that of Murashige *et al*. (1974), which had the highest salt concentration of macro elements.

Villalobos (1986) reported that when carnation apical meristems with 2 leaf primordia were cultured on various concentrations of MS salts (100, 75, 50 or 25 per cent), explant growth and differentiation were best at the full strength or the highest concentration.

Shoots of Gerbera cultivars 'Clementine', 'Saskia' and 'Terravisa' were multiplied on media without or with the addition of thiamin, pyridoxine, nicotinic acid, myo-inositol, tyrosine and adenine sulphate, in the original strength or in concentration reduced to one-half or one quarter. In three subsequent passages, the multiplication rate of the shoots did not depend on the concentration of thiamin, pyridoxine, myo-inositol and adenine sulphate (Soczek and Hempel, 1988).

Tosca et al. (1990) reported that naked mature unfertilized ovules, collected from April to October, were cultured successfully on modified MS basal medium plus 0.88 µM BA (6-benzyl adenine) and 0.57 µM IAA (3-indole acetic acid). Culture initiation using shoot tip explants was carried out on medium containing MS salts, 100 mg/1myo-inositol, 0.4 mg/1thiamine-HCl, 10 g/1sucrose, 8 g/1Bacto agar with the addition of 0.5 mg/1IAA (Jerzy and Lubomskii, 1991).

Shoots were regenerated on Murashige and Skoog's (1962) basal medium (3% sucrose, 0.8% Difco Bacto agar) supplemented with three concentrations (0.5, 1, 2 mg/1) of 6-benzyl amino purine (BAP) in combination with four concentrations (0.2, 0.5, 1, 2 mg/1) of α-naphthalene acetic acid (NAA) (Chin-Yi et al., 1990). MS media with 3 mg/1BA, 0.5 mg/1IBA, 100 mg mg/1 myo-inositol, 0.4 mg/1thiamine-HCl, 10 g l^{-1} sucrose and 8 g/1Bacto agar was used for regeneration studies using in vitro derived leaf explants of Gerbera (Jerzy and Lubomskii, 1992).

Barbosa *et al*. (1993) cultured three-leaf bud explants of Gerbera cultivar "Applebloesen" on MS medium supplemented with 3 percent sucrose, 80 mg adenine sulfate, 100 mg tyrosine, 7 g Difco Bacto agar and growth regulators.

The initial explants of three to four of the youngest leaves with petioles in Gerbera, were obtained from 3-week-old shoot cultures multiplying on a medium comprised of modified MS mineral salts with the addition of 85 mg/1NaH_2PO_4 and vitamins, 9.3 µM kinetin, 0.6 µM 3-indole acetic acid, 20 mg l^{-1} adenine sulfate, 20 g/1sucrose and 7 g/1Bacto agar (Orlikowska *et al*., 1999).

Topoonyanont et al. (1999) reported that Gerbera jamesonii cultivars 'Rosabella' (bushy) and 'Sunset' (normal or non-bushy) were multiplied in vitro on a basal medium containing Murashige and Skoog (1962) macro elements, 40 mg l^{-1} NaFe-EDTA, 100 mg l^{-1} myo-inositol, 40 g l^{-1} sucrose, 8 g l^{-1} BDH agar, 0.86 mg l^{-1} kinetin and 0.5 mg l^{-1} IAA.

The effect of MS, half MS, one-fourth MS and one-eight MS medium in Gerbera was evaluated by Aswath and Choudhary (2002). The number of shoots was highest on half MS medium and lowest in one-eighth medium. This result was similar to that of Huang and Chu (1985). Shoot proliferation from leaf segment of Gerbera was found to be the best on half MS medium and poor when diluted further. However, Jerzy and Lubomskii (1991) reported that half or full strength MS salts did not influence the adventitious shoot formation from petioles.

Modh et al. (2002) used MS medium supplemented with various levels of BAP (1.0 to 10.0 mgl^{-1}) and IAA (0.1 mg l^{-1}) for culturing the explants cut from capitula, whereas Ranjan and Gaurav (2005) used the modified Murashige and Skoog medium supplemented with various combinations and concentrations of plant growth regulators such as IAA, IBA, NAA, 2,4-D, kinetin and BAP with sucrose (3%) and agar (0.8%).

Mohanty et al. (2005) concluded that MS medium supplemented with BAP (1 ppm) was the best for in vitro proliferation of Gerbera cultivar 'Alsmeera' from shoot tip explants. Maximum callus induction was achieved through amendment of 2,4-D (5 ppm) on MS medium from leaf disc during summer season, keeping in diffused light condition.

2.5 Growth regulator

Hasbullah et al. (2008) also reported that addition of auxins along with cytokinins was essential for shoot induction in gerbera. When BAP was supplemented with IAA callus was induced but no shoot proliferated. On the other hand when BAP was supplemented with NAA, in some combinations callus induction as well as shoot proliferation occurred. Pierik et al. (1973) also reported that addition of strong auxin (NAA) with BAP promoted better shoot formation compared to weak auxin (IAA).

Chakrabarty and Datta (2008) obtained best shoot multiplication on MS containing only BAP, Kn and TDZ. Kanwar and Kumar (2006) also reported that IBA was the best medium for root induction in Gerbera.

Murashige *et al*. (1974) rapidly multiplied shoot tips and a satisfactory rate of increase in divisions was obtained by simply lowering the IAA level and in the basal medium. Favorable effect of cytokinins (especially zeatin riboside) on formation of new buds on vegetative apices was noticed by Gregorini et al. (1976).

Pierik et al. (1975) reported that no shoots developed from excised capitulum explants on cytokinin free media. The addition of BA or BAP in higher concentration was very essential, the optimum being 10 and 5 mg l^{-1} respectively. BAP was found to be slightly more effective than BA. They felt auxin was not essential for shoot formation, although the addition of a low auxin concentration (IAA at 0.1 mg l^{-1} or IBA at 0.05 mg l^{-1}) was slightly more stimulative in comparison to no auxin. Shoots were successfully regenerated from excised scape explants on MS medium supplemented with 10 mg l^{-1} BA (Huang and Chu, 1983).

The regeneration of adventitious shoots from leaf blades was observed by Hedtrich (1979) during his study on Gerbera jamesonii cultivar 'Vulkan' on modified Murashige and Skoog medium supplemented with 1 mg l^{-1} BAP and 0.1 mg l^{-1} gibberellic acid.

Petru and Matous (1984) propagated shoot tips of cultivar 'Lada' on MS medium containing 7 mg l^{-1} kinetin and 0.2 mg l^{-1} IAA. The shoot tips were successfully cultured on a medium containing 5 mg l^{-1} BA and 0.1 mg l^{-1} IAA (Huang and Chu, 1985). Mariska *et al*. (1989) obtained greatest number of shoots (4.9) from a lateral bud with kinetin at 5 mg l^{-1} + IAA at 0.5 mg l^{-1}.

Laliberte et al. (1985) reported that 'Mardi Grass' capitulum explants responded best to the MS medium with 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} IAA. However, the vigour and number of shoots decreased when the concentration of BAP was raised

to 2.0 - 3.0 mg l^{-1}. Pierik's medium enriched with IAA and kinetin or BAP was found suitable for inflorescence buds (Pawlowska, 1977), while that of MS nutrients + 5 mg l^{-1} BAP + 0.1 mg l^{-1} IAA + 1% Bacto- agar was suitable for shoot tips (Huang and Chu, 1985).

Hempel et al. (1985) in their study on the effect of kinetin, BA or 2 iP, each at 1.25 to 20 mg l^{-1} on shoot multiplication of cultivar 'Marleen', found that kinetin at 5.0 mg l^{-1} was the best for shoot multiplication. Zakharova (1987) found BA at 1 mg l^{-1} was the most beneficial cytokinin for proliferation of cultivars 'Auriga Karmezin' and 'Sonia'. Roberts and Smith (1990) used the multiplication medium consisting of MS salts and vitamins, 30 g l^{-1} sucrose, 0.1 mg l^{-1} benzyl amino purine (BAP) and 0.01 mg l^{-1} α-naphthalene acetic acid (NAA), which was adjusted to pH 5.6. Agar at the rate of 8 g l^{-1} was added and the medium was autoclaved at 121°C for 15 min. Plantlets were subcultured from nodal sections every six weeks.

Arello et al. (1991) cultured capitulum explants of the varieties 'Applebloesem', 'Marleen', 'Clementine' and 'Pimpernel' on MS medium supplemented with benzyl adenine (BA), indole acetic acid (IAA) or kinetin. The best results were obtained with 2 mg BA and 0.5 mg IAA per litre. Jerzy and Lubomskii (1991) observed that the addition of BAP to the nutrient medium was more effective than the addition of kinetin. The highest number of shoots of 8 to 11 was obtained on medium with 10 mg l^{-1} BAP, but the shoots were frail, concise and showed vitrification symptoms. The optimum BAP concentrations were 3 and 5 mg l^{-1} and caused formation of 4 to 6 shoots with insignificant vitrification symptoms.

Reynoird et al. (1993) obtained efficient bud regeneration from a clone of Gerbera hybrida from leaf explants cultured on modified MS medium supplemented with 10 µM BA and 2.5 µM NAA. Addition of 0.05 µM or 0.5 µM TDZ to the above medium significantly promoted regeneration from mature leaves.

Barbosa et al. (1993) in their study with Gerbera cultivar 'Applebloesem', obtained the highest multiplication (9.5 - 11.2) rates on modified MS medium supplemented with 1.0 mg l^{-1} BAP. The supplementation of MS medium with 0.75 mg l^{-1} BAP was found to give highest number of shoots in cv. 'Gold Dust' of Gerbera (Partasarathy and Nagaraju, 1995).

Parthasarathy and Nagaraju (1995) and Parthasarathy et al. (1996) confirmed that low concentration of BAP (0.75 - 1.0 mg l^{-1}) is sufficient for obtaining more number of better sized shoots in 'Gold Dust' (17.83) and 'Popular' (14.6) cultivars of Gerbera. Though maximum number of shoots was recorded at 2.0 mg l^{-1} BAP, it was not significantly different from that obtained with 1.0 mg l^{-1} BAP.

Leaf disc explants (5 x 5 mm) excised from young and mature leaves of 6 - 8 week old in vitro grown plants of garland chrysanthemum were grown on MS medium supplemented with: (1) different combinations of cytokinin (benzyl adenine, kinetin and zeatin) and auxin (IAA, IBA, NAA or 2,4-D) each at 2.5 µM; and (2) 0.5, 2.5, 5, 10 and 25 µM BA in combinations with 0, 0.25, 0.5, 1, 2.5 and 5 µM NAA. The media were solidified with either 0.8% Bacto agar or 0.4% Agarose (Lee et al., 1997).

Deepaja (1999) in her in vitro studies with Gerbera at University of Agricultural Sciences, Bangalore obtained two distinct type of calli from leaf explant, viz. creamy callus which was frothy in GJ-1 and GJ-2 and friable in GJ-3 with 2,4-D and BAP combination while green/greenish white granular callus was produced by IAA and BAP.

Aswath and Choudhary (2002a) observed that cytokinins (kinetin or benzyl amino purine) in half MS medium at the concentration of 0.5 - 5.0 mg l^{-1}, strongly suppressed shoot proliferation. The suppression of shoot proliferation was more in BAP supplemented media than in kinetin.

Shailaja (2002) in her in vitro studies with Gerbera at University of Agricultural Sciences, Dharwad reported that MS medium with 2.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA gave more number of better sized shoots.

2.6 Cultivar

Pierik et al. (1982) observed significant differences among cultivars and levels of kinetin (1, 5 or 10 mg l-1) in the medium with respect to axillary branching of subcultured shoots. The optimum level for the highest quality shoots (highest leaf weight/shoot, no leaf malformation and no callus formation) was not always the same as the level producing the highest number of axillary shoots.

Schiva et al. (1982) conducted a study on micropropagation of six cultivars of Gerbera, with varied auxin and cytokinin concentrations. The multiplication of cultivar 'Peter' was found to be the best with kinetin concentration of 2 x 10^{-6} g l^{-1}, while that of 'Tunisia' was best with 10 x 10-6 g l^{-1} kinetin.

In a trial on the effect of cytokinin and cultivar (28 cultivars were used) on shoot formation using capitulum explants, shoot formation was found to be dependent on both cultivar and cytokinin level in the medium. With capitulum explants, shoot formation was very low for some cultivars regardless of the level of BA (5, 10 or 20 mg l^{-1}); whereas other cultivars had individual optimum BA levels (Pierik et al., 1982).

Petru and Matous (1984) propagated shoot tips of cultivar 'Lada' on MS medium containing 7 mg l-1 kinetin and 0.2 mg l^{-1} IAA. Topoonyanont and Dillen (1988) in their study on capitulum culture of 'Red', 'Pink', 'Orange' and 'Yellow' cultivars, concluded that 'Orange' cultivar which produced up to 8 shoots per explant with BA at 5 or 7.5 mg l^{-1}. The response of 'Yellow' cultivar was best with 10 - 15 mg l^{-1}, while that of the 'Red' cultivar was limited to 25 mg l^{-1}. Zakharova (1987) found BA at 1 mg l^{-1} to be the most beneficial cytokinin for proliferation of Gerbera cultivars 'Auriga Karmezin' and 'Sonia'. Capitulum

explants of the varieties ‘Applebloesem’, ‘Marleen’, ‘Clementine’ and ‘Pimpernel’ were cultured on modified MS medium supplemented with BA, IAA or kinetin. The best results were obtained with 2 mg l^{-1} BA and 0.5 mg l^{-1} IAA (Arello et al., 1991).

Harel *et al.* (1993) observed that the highest rate of shoot production (8 shoots per inoculum) in a period of 4 weeks was showed by the cultivars ‘Ansofie’, ‘Terracerise’ and ‘Lablinel’. The cultivars ‘Maria’, ‘Shangha’ and ‘Fresultane’ showed moderate rates (6 - 7 shoots), while ‘Croduro’, ‘Raisa’, ‘Fredigor’, ‘Terramaxima’ and ‘Fredibel’ recorded the lowest rates (4 - 5 shoots)

Reynoird *et al.* (1993) conducted a leaf regeneration study on two clones 10 and 11 of Gerbera hybrida (G. jamesonii x G. viridifolia) and two wild types of Gerbera virifolia and Gerbera piloselloids. Response of clone 10 was better than that of clone 11 for regeneration. Weak responses were observed from the leaves of clone 11 when placed in optimum conditions defined for clone 10. While Gerbera viridifolia leaves exhibited similar response to those of G. hybrida under optimum conditions, Gerbera piloselloids leaves were more recalcitrant to bud regeneration.

Gerbera jamesonii cultivars ‘Monoco’, ‘King’ and ‘Marleen’ were cultured on MS medium containing auxins alone or in combination with activated charcoal. Activated charcoal markedly inhibited root formation in the cultivars ‘Monoco’ and ‘King’. The number of roots was found to be higher in ‘Monoco’ cultivar with increasing IBA concentrations (0 - 25 mg l^{-1}) in combination with IAA (0.2 mgl^{-1}) and it was found slightly lower in ‘King’ cultivar and least the number was recorded in ‘Marleen’ cultivar. The combination of IBA + NAA found to inhibit root growth in cultivars ‘Monoco’ and ‘King’ but stimulated root growth in ‘Marleen’ (Szule and Rogozinska, 1994).

Aswath et al. (2003) observed that GJ-1 produced higher number of shoots when cultured on MS medium supplemented with 0.2 mgl^{-1} IAA plus 1 mg l^{-1} BAP. GJ-2 produced higher number of shoots when cultured on MS medium supplemented with 0.1mgl^{-1} IAA plus 1 mgl^{-1} BAP. Whereas, GJ-3 produced maximum shoots on MS medium supplemented with 0.2mgl^{-1}IAA plus 1 mgl^{-1} BAP. They concluded that effect of growth regulators was different in all three varieties, since varieties did not perform uniformly in one common nutrient medium combination.

2.3 Establishment of *Gerbera jamesonii* Bolus in rooting media

2.3.1 Explant

Pierik and Segers (1973) studied the factors affecting adventitious root production in isolated explants of a red-flowering Gerbera jamesonii clone. Root formation was optimal in the segments taken from young leaves, 6 - 14 cm long, which had been cultivated in continuous darkness at 25 - 29ºC. It was reduced markedly by placing the explants with their basal ends downwards instead of inverted position in the medium.

Huang and Chu (1985) used shoots measuring more than 2 cm to obtain 90 per cent rooting in sand bed under mist condition. Laliberte et al. (1985) obtained rooting from all shoots over the size of 0.5 cm, irrespective of the IAA level in the medium.

Ruffoni and Massabo (1991), in a study on tissue culture in *Gerbera jamesonii*, used leaf, petiole and apex explants of *Gerbera jamesonii* to establish tissue and cell cultures. Leaf explants produced more roots and apex explants produced more shoots and callus.

2.3.2 Media

In vitro plantlets should have sufficient number of roots for absorption of nutrients and to provide anchorage for establishment of tissue culture plants in open conditions. Auxins play a crucial role in the formation of adventitious roots in the micropropagated plantlets. Pierik and Segers (1973) observed that rooting was influenced only by sugar and auxin in the medium, but not by the presence or absence of macro elements. A decrease in root growth was noticed by addition of organic compounds and increase in the concentrations of macroelements, whereas manganese and saccharose were found to increase rooting. Schum and Bichler (1986) reported that, increase in saccharose and Mn improved rooting. IAA stimulated branching and growth of roots better than NAA, while NAA improved shoots growth.

2.3.3 Growth regulator

Rooting and shoot growth were found to be better at low light intensity (800 lux) when compared to high light intensively (2100 lux), Optimum rooting was obtained by IAA or IBA at a concentration of 10 mg l^{-1}; IAA induced a smaller number of long roots, whereas IBA larger number of short roots (Pierik *et al*., 1975).

Growth and morphogenesis in vitro are regulated by the interaction and balance between the growth regulators in the medium and the growth substances produced endogenously by the cultured cells. Apart from the direct effect on cellular mechanisms, many synthetic growth regulators may in fact modify the level of indigenous growth substances (George and Sherrington, 1984).

Shoot tips of the shy-rooting Gerbera cultivars ‘Fleur’ and ‘Florence’ were cultured on amended MS medium containing either IAA at 3 - 10 mg l^{-1}or NAA at

1 - 3 mg l^{-1}. The medium containing NAA was found to give 100 per cent rooting and more number of roots produced per culture was achieved in both the cultivars (Pierik and Sprenkels, 1984).

Huang and Chu (1985) obtained 90 per cent rooting within a period of two weeks from the explants of shoots of cultivars 'Arendsoog' and 'Super Giant Yellow' by treating with 0.1 per cent IBA solution for 30 seconds and planting into a sand bed. Meyer and VanStaden (1988) reported that the shoots rooted in vitro on MS medium supplemented with 5 to 10 µM indole-butyric acid (IBA) or 5 to 10 µM α-naphthalene acetic acid (NAA).

Zakharova (1987) reported that cultivars 'Auriga-Karmezin' and 'Sonia' produced best roots on MS medium supplemented with IAA at 1 mg l^{-1}. Mariska *et al*. (1989) obtained best rooting of shoots with IAA at 0.1 mg l^{-1}, while the use of NAA gave short swollen roots.

Highest root multiplication rates (9.5 - 11.2) were obtained on modified MS medium supplemented with 1 mg l^{-1}BAP, independently of the IAA concentration used in a study on in vitro propagation of Gerbera cultivar 'Applebloesem' using three-leaf-bud explants (Barbosa et al., 1993). Szule and Rogozinska (1994) reported that more number of roots was obtained in cultivar "Monaco" with IBA at a concentration of 0.25 mg l^{-1}. They also reported that activated charcoal inhibited root formation and reduced number of roots.

Liqing et al. (1996) concluded that the rooting media supplemented with ABT (amino benzo triozole) at a concentration of 0.5 mg l^{-1} resulted in 100 per cent rooting and 98.9 per cent survival of transplanted plantlets.

Deepaja (1999) reported that the rooting response was better with IBA compared to NAA. MS basal media was found to be the best for in vitro rooting in GJ-1 and GJ-2. The cultivar GJ-3 rooted well at 0.5 mg l^{-1}IBA. The medium containing NAA induced swelling of roots in GJ-2 and callus production in GJ-3.

Shailaja (2002) obtained 100 per cent rooting, improved shoot and root growth on MS medium supplemented with 1 mg l^{-1}NAA. Whereas, Hitmi *et al*. (1999) observed that addition of NAA in propagation of in vitro was completely inhibitory to root initiation. The effect of auxins (NAA or 2, 4-D, each at 0.5, 1, 2 and 5 mg l^{-1}) on root formation was studied by Aswath and Choudhary (2002a). The number and length of roots were best in half MS medium supplemented with 0.5 mg l^{-1}2, 4-D and 0.5 mg l^{-1}NAA and the root formation rate was 100 per cent.

Nga et al. (2005) reported that 100 per cent of micro-shoots rooted on MS medium supplemented with NAA (0.1 - 1.0 mg l^{-1}) after 2 weeks of inoculation, while 0.1 mg l^{-1}NAA gave higher number of roots and good plant vigour.

2.4 Hardening medium

The micropropagated plantlets should be hardened before transferring them to open conditions. The medium for hardening should have good water holding capacity, drainage and aeration.

Martyr (1981) observed that cuttings could absorb more water from the medium peat: perlite (1:1 ratio, v/v) than from either peat : grit (1:1, v/v) or from peat alone. Water uptake by cuttings was not directly related to the water content of the medium per unit volume, which was greatest in the peat. This higher rate of uptake was reflected in the quicker rooting of cuttings in the peat: perlite mixture.

Evapotranspiration (ET) measurements were estimated for Gerbera subcultured in perlite, coco peat, sand, zeolite and rock wool substrate (Maloupa et al., 1993). Actual evapotranspiration was higher with coco peat, perlite and rock wool substrate than with sand and zeolite because of the lower hydraulic conductivity of the later. Eapen and Rao (1985) reported 75 percent survival by transferring the regenerated anthurium plants to plastic cups with vermiculite and irrigating with a nutrient solution containing MS salts at half strength. Plants were covered with a bell jar to maintain humidity. Rooted plantlets were first transferred to Jiffy-7 peat

pellets in a glass covered acclimatization chamber and later to a mixture of perlite: sphagnum moss (1:1, v/v) in greenhouse, losses at this stage were less than 5 per cent (Laliberte *et al*., 1985). The regenerated plants with well-established root systems were carefully washed free of agar, prior to transfer to pots (10 cm) containing indoor growing mixture (bark and sand) They were repotted to 15 cm pots, when attained 12 cm height. Potted plants were kept under photoperiod (49 µmol m^{-2} s^{-1}) of 16 hours and at a temperature of 19 ± 10C for vegetative growth and a photoperiod (19 ± 10C, 49 µmol m^{-2} s^{-1}) of 10 hours for flowering (Chin-Yi et al., 1990). Conti et al. (1991) reported that greenhouse acclimatization of plantlets was achieved in 30 days in a peat : perlite (1:1 ratio, v/v) substrate. Qianzhong et al. (1998) obtained better root growth and plant survival (93.8%) in rice chaff as hardening medium. The use of jars as containers was found to be better than pots because high humidity was maintained around the plants and reduced desiccation effect (Parthasarathy and Nagaraju, 1995). Shailaja (2002) reported maximum survival percentage (60) with better plant vigour on perlite as hardening medium. Agar was carefully washed from the regenerated plantlets with well-established root system before transfer to pots (15 cm) filled with a mixture of coco peat and compost (1:1, v/v). Plantlets were maintained under high relative humidity (90%) for 3 weeks. Acclimatized plants were kept under a natural photoperiod condition at a temperature of 25 ± 2 ºC. Survival rate of plantlets was almost 100 per cent when plantlets after root development were transferred to plastic pots filled with coco peat, red soil, and sand in a 3:1:1 ratio (Aswath and Choudhary, 2002b). The regenerated plantlets were transferred to poly-bags containing soil : sand : FYM (1:1:1) mixture and kept under high humidity for 3 days. The humidity was gradually reduced to normal by using polythene covers up to 7 days. They were further allowed to grow for 4 weeks and transferred to field (Modh et al., 2002). Nga et al. (2005) investigated the effect of hardening media, viz. soil, sand, rice husk, humus, soil + humus (1:1, v/v), sand + rice husk (1:1, v/v) and humus + rice husk (1:1, v/v) on survival percentage and root growth of

Gerbera plantlets. The best plant vigour and maximum survival of plants (93.25%) was observed in humus + rice husk (1:1, v/v) mixture followed by sand medium. Agar-based medium was removed from the roots of plantlets (approximately 25 mm in length) by washing them under running tap water. The plantlets were transferred to plastic pots containing a mixture of sand and compost (2:1 ratio) and maintained in a growth room (18 ± 20C, 16 hours photoperiod with 25 µmol m^{-2} s^{-1} illumination). Well-established plants were transferred into pots containing garden soil after 4 weeks for further acclimatization in greenhouse where they were kept for 2 weeks (Karami *et al*., 2006). Negi *et al*. (2006) reported that the rooted plants were taken out from the containers and washed thoroughly with tap water to remove adhering agar. These plantlets were dipped in 0.1% carbendazim solution for two minutes and transplanted in tray pots containing sterilized peat moss. The potted plants were kept inside a small glasshouse and watered regularly with sprinkler for a week. Further, they were watered twice a day with the help of a water cane. A temperature of 26 ± 2ºC and humidity of 85 - 90% were maintained inside the glasshouse. These plants were hardened well within a period of 12 - 14 days. Germinated secondary somatic embryos were transferred into plastic pots containing autoclaved mixture of soil, sand, and compost (1:1:1, v/v) and kept for 2 weeks, they were transplanted into plastic pots containing garden soil and allowed to be grown in the growth room (18 ± 2ºC, 16 hours photoperiod under 25 µmol m^{-2} s^{-1} irradiation). They were further acclimatized for 3 weeks in a greenhouse at temperature of 28°C and transferred to another greenhouse without temperature control (Karami *et al*., 2007).

CHAPTER III
MATERIALS AND METHODS

3.1 Time and Location of the experiment:

The study was carried out at Advanced Seed Research and Biotech Center (ASRBC), ACI during the period from September, 2014 to September, 2015 to obtain *in vitro* regeneration of gerbera from different explants. Materials and methods followed to conduct the present investigation have been presented in this chapter.

3.2 Experimental materials:

3.2.1 Plant material

One promising cultivar of gerbera was used for the present study. Varietal characteristics of this cultivar are presented below:

Type of flower: Double

Length of stalk: 40.24 - 43.52 **(cm)**

Girth of stalk: 0.44 - 0.50 **(cm)**

Diameter of flower: 9.54 - 10.72 **(cm)**

Flower color:

-**Ray florets:** Magenta or hot pink

- **Disc florets:** black and yellow

3.2.2 Source of materials:

The explants are collected from ASRBC rooftop net house.

3.2.3 Types of explants:

The plant parts uses for this experiment are (Plate 2)

a) Flower bud
b) Capitulum
c) Leaf
d) Peduncle
e) Petiole

3.2.3.1 Flower bud

The young inflorescence buds were used. Immature inflorescences of 0.5 to 0.7 cm in diameter were collected from the plants. After sterilization, the buds were cut into two halves. Each explant was inoculated separately on solid medium (Plate 2a).

3.2.3.2 Capitulum

Fully developed inflorescences were collected; both disc florets and ligulate ray florets were removed before sterilization. After sterilization, the capitulam were quartered. Explants were subsequently placed on solid medium with the bracts above the medium (Plate 2b).

3.2.3.3 Leaf

The mid portion of the leaf was carefully separated and made into 4 - 5 mm square pieces along with the portion of midrib. They were inoculated such that the basal portion of the leaf was in contact with the medium (Plate 2c).

3.2.3.4 Peduncle

An immature part o f flower stalk from flower bud was cut to around 0.5 - 1.0 mm pieces. Each was cultured separately on solid medium (Plate 2d).

3.2.3.5 Petiole

The part between leaf blade and petiole was cut and made into 4 - 5 square pieces. They were inoculated such that the basal portion of the leaf was in contact with the medium (Plate 2e).

3.3 Culture media

The degree of success in any technology employing cell, tissue and organ culture is related to relatively some major factors. A significant factor is the choice of nutritional components and growth regulators. Both for shoot regeneration and rooting of multiplied shoots, MS medium (Murashige and skoog, 1962) was used with different vitamins and hormonal supplementation. Hormones were added separately in different media according to the requirements. For the preparation of media, readymade MS media packet of 1 liter with and without sugar, and stock solutions was used. Packets and stock solutions were stored at 4 ± 1^{0}C temperature. The respective media were prepared from the readymade MS media packet and stock solutions as well.

3.3.1 Preparation of hormonal stock solution:

The first step in the preparation of the medium was the preparation of hormone stock solutions. To accelerate the preparation of the medium separate stock solutions for growth regulators were prepared and used.

The following growth regulators were used in this present investigation:

Auxins

NAA - 0.01, 0.02, 0.03 mg/l

IAA - 0.01, 0.02, 0.03 mg/l

Cytokinin

BAP - 1.0, 2.0, 3.0, 4.0, 5.0 mg/l

These hormonal supplements were dissolved in proper solvent as shown against each of them

Hormones	Solvent
NAA	70% Ethyl alcohol
IAA	70% Ethyl alcohol
BAP	0.1 N NaOH

Separate stock solutions of hormones were prepared by dissolving desired quantity of ingredients to the appropriate solvent and made the final volume with distilled water and stored in a refrigerator at 4ºC for later use. To prepare the stock solutions of 3-Indole Acetic acid (IAA), Indole Butyric Acid (IBA) and α-Naphthalene acetic Acid (NAA) (1mg/ml), 100 mg of solid hormone was placed in a small beaker and then dissolved in 10 ml of 70 % ethyl alcohol and Stock solution of 6-Benzyl Amino Purine (BAP) (1mg/ml), was prepared by dissolving it first in few drops of 1N NaOH solvent. Finally the volume was made up to 100 ml by the addition of sterile distilled water using micro pipette (10 ml). The labeled hormone solution stored at 4 ± 1^{0}C for use up to two months

3.3.2 MS media preparation:

While preparing individual medium using stocks, required quantity of stock solutions were added along with sucrose and the volume was made up by adding distilled water. The pH was adjusted between 5.6 and 5.8 with the help of 0.1N NaOH or 0.1N HCl. The volume was finally made up and required quantity of agar was added into the medium. Agar in the medium was completely melted by in a microware. Then 15 - 20 ml of hot medium was poured into 25 x 150 mm pre-sterilized glass culture vessels and plugged with non-absorbent cotton. These tubes were then autoclaved.

After the preparation of stock solution, the later step was the preparation of culture media. To prepare 1 liter of above mentioned media the following steps were followed:

i. 500 ml of sterile distilled water was added into 2 liter buckets.
ii. 4405.19 mg/l of Duchefa Biochemic MS media including vitamin was added to beaker.
iii. Then thirty gram of sucrose was added and gently agitated to dissolve completely
iv. Different concentration of hormonal supplements were added to the solution either in single or in combination as required and mixed well
v. Since each hormonal stock contained 10 mg o the chemical in 100 ml of solution to make one liter of medium addition of 10 ml of stock solution of any of the hormones , resulted in 1 mg/L concentration of that hormonal supplement.
vi. The volume was made up to 1000 ml with addition of sterile distilled water.
vii. The pH of the medium was adjusted to 5.8 with a digital pH meter with the help of 0.1N HCL or 0.1N NaOH as necessary.
viii. After adjusting the pH, 8 g/L agar was added to solidify the medium.
ix. As additives, 1% of Activated charcoal was directly added to the medium.
x. The mixture was then heated for 10 minutes in an electric oven for melting of agar.\
xi. Required volume of hot medium was dispensed into culture vessels viz., vial. After dispensing the medium, the culture vessels were plugged with its cover and marked with different codes with the help of a glass marker to indicate specific media.

3.3.3 Autoclaving

The test tubes with nutrient media were autoclaved at a pressure of 1.06 kg/cm² (121ºC) for 20 minutes. The tubes were then removed from the autoclave and allowed to cool. They were kept in the culture room at temperature of 25 ± 2ºC. The inoculation was done after 4 - 5 days ensuring that the tubes were free from contaminations.

3.4 Sterilization:

For in vitro techniques, aseptic condition is pre requisite. So, all instruments, glass wares and culture media were sterilized.

3.4.1 Sterilization of culture media:

The culture vessel containing the medium were autoclaved with 1.06 kg/cm² (15 PSI) of pressure at 121ºC for 20 minutes .After autoclaving the culture vessels (vials) containing the medium were allowed to cool in culture racks.

3.4.2 Sterilization of glassware and instruments:

Beakers, test tubes, conical flasks, pipettes, metal instruments viz., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121ºC for 20 minutes at 1.06 kg/cm² (15 PSI) pressure .

3.4.3 Sterilization of culture room and transfer area:

The culture room was initially cleaned by gently washing all over the floors and walls with detergent or Lysol (germicide) followed by wiping with 70% ethyl alcohol. The process of sterilization was repeated at regular intervals. Generally, switching on the laminar airflow cabinet and sterilized the cabinet by wiping the working surface with 70% ethyl alcohol and then UV light was on for 30 minutes so that the working area of the cabinet is sterilized. After in the cabinet was delayed for at least 5 minutes to ensure safe environment.

3.4.4 Maintenance of aseptic conditions

All the aseptic manipulations such as surface disinfection of the explants, preparation and inoculation of explants and subsequent sub-culturing were carried out in the laminar air flow cabinet. The working table of laminar air flow cabinet and spirit lamp were sterilized by swabbing with absolute alcohol. All the required material like media, spirit lamp, lighter, glass wares, etc. were transferred on to the clean laminar air flow. The UV light was switched on for half an hour to achieve aseptic environment inside the cabinet before working in the lamina air flow cabinet.

3.5 Culture establishment

3.5.1 Surface sterilization of explants

The explants were washed under running tap water with few drops of Tween twenty for 15 – 20 minutes. Then they were immersed in 70% ethanol for 1 minute and washed thoroughly with distilled water. They were later immersed in 0.1 per cent $HgCl_2$ for 7 minutes and finally washed with double glass distilled water for 3-4 times in laminar air flow cabinet to remove any traces of $HgCl_2$.

3.5.2 Inoculation

The sterilized explants were inoculated in slant tubes containing the media (15 – 20 ml). The cut ends of explants were kept in such a way that they have maximum contact with medium.

3.5.3 Incubation:

The culture vials transferred to culture racks and allowed to grow in controlled environment. The temperature of the culture room was maintained within 25± 1ºC by an air conditioner and 16 hour photoperiod was maintained along with light intensity of 3000 lux for proper growth and development of culture .

3.6 Maintenance of proliferating shoots:

Initial sub culturing was done after 30 days when the explants had produced some shoots. For sub culturing, the entire samples of in vitro shoot were cut into small pieces so that each piece would contain about one shoot. Leaf and blackish or brownish basal tissues were removed. Each piece was inoculated into a similar fresh medium .It was practiced at the interval of 20-25 days.

3.6.1 Subculture

Micro shoots formed in the vessels were taken out after four weeks of inoculation. The shoots were separated by dissecting them in the sterile environment of laminar air flow cabinet with sterile dissecting needle and forceps. They were again placed in the slant tubes containing fresh media.

3.7 Rooting

The micro shoots of more than 1.0 cm in height were taken out and placed in the vessels containing media with different concentrations of NAA, IAA, IBA and half strength of MS media for rooting.

3.8 Experimental condition

The experiments were conducted under well defined conditions of the culture room maintained at temperature of 25 ± 2 ºC. Uniform light was provided by using fluorescent tubes (7200 lux) over a light/dark photoperiodic cycle of 16 hours light/8 hours' dark. All inoculation works were carried out in a laminar air flow chamber. For all the experiments, analytical grade chemicals were used.

3.9 Hardening

Young rooted plants were taken out of the test tubes, washed with distilled water and planted in net pots containing different hardening media. These plants were maintained in a prototype poly tunnel. The plants were watered once in two days

initially, then once in a day after eight to ten days. Then they were transferred to greenhouse after two weeks for further acclimatization.

3.9.1 Hardening media

3.9.1.1 Coco peat

Coco peat is coir fiber pith that is having coconut husk as its base. It is a soil conditioner and growing medium. Its uniqueness is that it can hold 8 times of water of its own weight. It releases nutrients in solution over long intervals. It is best for commercial and home gardening application. It provides breathing space, *i.e.* letting in and letting out of air for roots which help better growth. Added to above mentioned characters, it encourages favorable micro organisms around the root zone. Since it is having slow degradation level it is universally accepted. It will re-wet easily without the use of chemical wetting as it is hydrophilic by nature. It can easily be mixed with other growing media.

3.9.1.2 Vermicompost

Vermicompost is the product or process of composting using various worms, usually red wigglers, white worms, and other earthworms to create a heterogeneous mixture of decomposing vegetable or food waste, bedding materials, and vermicast. Vermicast, also called worm castings, worm humus or worm manure, is the end-product of the breakdown of organic matter by an earthworm. Containing water-soluble nutrients, vermicompost is an excellent, nutrient-rich organic fertilizer and soil conditioner. Vermicompost has been shown to be richer in many nutrients than compost produced by other composting methods. It has also outperformed a commercial plant medium with nutrients added. It is rich in microbial life which converts nutrients already present in the soil into plant-available forms. Unlike other compost, worm castings also contain worm mucus which helps prevent nutrients from washing away with the first watering and holds moisture better than plain soil.

3.9.1.3 Sand

It consists of small rock grains of size ranging from 0.05 - 2.0 mm in diameter, formed as a result of weathering of various rocks. Its mineral composition depends upon the type of parent rock. Quartz sand, generally used for propagation purposes, consists chiefly of silica complex. Sand used in plastering is satisfactory for rooting of cuttings. Sand is the most widely acceptable rooting medium for cuttings; relatively inexpensive and readily available. It does not retain moisture unlike other media, hence needs frequent watering. It is enough to retain some moisture around the cutting, yet coarse enough to allow water to drain through it. It is used mostly in combination with organic material.

3.10 Details of experiment

3.10.1 Experiment 1: Effect of different treatments and explants on culture establishment

Design: CRD (Completely randomized design)

Replications: 3

Number of factor: 2 factorial

Number of treatment combinations: 30

Number of vessels cultured per treatment: 25

3.10.1.1 Treatment combinations:

1	E_1-T_0	7	E_2-T_0	13	E_3-T_0	19	E_4-T_0	25	E_5-T_0
2	E_1-T_1	8	E_2-T_1	14	E_3-T_1	20	E_4-T_1	26	E_5-T_1
3	E_1-T_2	9	E_2-T_2	15	E_3-T_2	21	E_4-T_2	27	E_5-T_2
4	E_1-T_3	10	E_2-T_3	16	E_3-T_3	22	E_4-T_3	28	E_5-T_3
5	E_1-T_4	11	E_2-T_4	17	E_3-T_4	23	E_4-T_4	29	E_5-T_4
6	E_1-T_5	12	E_2-T_5	18	E_3-T_5	24	E_4-T_5	30	E_5-T_5

1. Explants	**2. Growth regulators**
E_1 : Flower bud	T_0: 0 mg/l BAP + 0 mg /l NAA
E_2 : Capitulum	T_1: 1 mg/l BAP + 0.01 mg /l NAA
E_3 : Leaves	T_2: 2 mg/l BAP + 0.01 mg /l NAA
E_4 : Peduncle	T_3: 3 mg/l BAP + 0.01 mg /l NAA
E_5 : Petiole	T_4: 4 mg/l BAP + 0.01 mg /l NAA
	T_5: 5 mg/l BAP + 0.01 mg /l NAA

3.10.1.2 Observations:

1. Per cent contamination
2. Per cent death
3. Per cent survival
4. Number of days taken for callus induction

3.10.2 Experiment 2: Effect of concentrations of BAP on shoot multiplication

Design: CRD

Number of factor: Single factor

Number of treatment combinations: 6

Replications: 3

Number of tubes cultured per treatment: 40

3.10.2.1 Treatment combinations:

	Growth regulators
1	T_0: 0 mg/l BAP + 0 mg /l NAA
2	T_1: 1 mg/l BAP + 0.01 mg /l NAA
3	T_2: 2 mg/l BAP + 0.01 mg /l NAA
4	T_3: 3 mg/l BAP + 0.01 mg /l NAA
5	T_4: 4 mg/l BAP + 0.01 mg /l NAA
6	T_5: 5 mg/l BAP + 0.01 mg /l NAA

3.10.2.2 Observations:

1. Number of responsive explants
2. Percent of responsive explants
3. Number of days taken for shoots initiation
4. Number of shoots per clump
5. Number of leaves per shoot
6. Shoot length (mm)

3.10.3 Experiment 3: Effect of concentrations of Auxins on rooting of *in vitro* shoots

Design: Factorial - CRD

Number of factor: Single factor

Number of treatment combinations: 7

Replications: 3

Number of tubes cultured per treatment: 10

3.10.3.1 Treatment combinations:

Growth regulators

1 T_0: 1/2 MS
2 T_1: 1/2 MS + 0.01 mg /l NAA
3 T_2: 1/2 MS + 0.02 mg /l NAA
4 T_3: 1/2 MS + 0.03 mg /l NAA
5 T_4: 1/2 MS + 0.01 mg /l IAA
6 T_5: 1/2 MS + 0.02 mg /l IAA
7 T_6 1/2 MS + 0.03 mg /l IAA

3.10.3.2 Observations:

1. Number of days taken for root initiation
2. Number of roots per shoot
3. Root length (mm)

3.10.4 Experiment 4: Standardization of hardening medium for regenerated plantlets

Design: Factorial - CRD

Number of factor: Single factor

Number of treatment combinations: 6

Replications: 3

Number of plants per treatment: 5

3.10.4.1 Treatment combinations:

	Hardening materials	
1	T_0:	Sand
2	T_1:	vermiculite
3	T_2:	coco pit
4	T_3:	Sand + vermicompost
5	T_4:	Sand + coco pit
6	T_5:	Sand + vermicompost + coco pit

3.10.4.2 Observations:

1. Survival of plantlets
2. Survival percentage of plantlets
3. Number of leaves per plantlet
4. Plant height (mm)

3.11 Collection of data:

3.11.1 Percent of contamination

Out of total vessels, the numbers of vessels contaminated were counted and it was expressed as percentage.

3.11.2 Percent of death of explants

The numbers of explants that died out of total inoculated were counted and it was expressed in percentage.

3.11.3 Percent of survival of explants

The number of explants survived out of total inoculated was counted and it was expressed in percentage.

Percent of survival = no. of survived plantlet/total no of plantlet X 100

3.11.4 Number of days taken for primordial emergence

The numbers of days taken for emergence of shoot primordium from the date of inoculation of explants was recorded and was expressed as mean number of days.

3.11.5 Number of responsive clumps

The number of explants responded out of total inoculated was counted.

3.11.6 Percent of responsive clumps

The number of explants responded out of total inoculated was counted and were expressed in percentage.

3.11.7 Number of days taken for shoot initiation

The number of days taken for initiation of shoots was recorded and expressed as mean number of days.

3.11.8 Number of shoots per explant

The number of shoots produced per explant was noted after eight weeks of inoculation in the experiment on culture establishment and expressed as mean.

3.11.9 Number of shoots per clump

The number of shoots produced per culture was counted after four weeks of inoculation and expressed as mean.

3.11.10Number of leaves per shoot

The number of leaves produced per shoot was counted after four weeks of inoculation and expressed as mean.

3.11.11 Shoot length

The shoot length was measured from base to the top of the shoot with scale during subculture and average was expressed in terms of millimeters.

3.11.12 Number of days taken for root initiation

The number of days taken for root initiation was counted and was expressed as mean number of days.

3.11.13 Number of roots per shoot

The number of roots produced per shoot was counted after four weeks of inoculation and expressed as mean.

3.11.14 Percent of root

The number of roots produced per shoot was counted after four weeks of inoculation and expressed as percentage.

3.11.15 Root length

The length of the longest root from the collar region to the root tip was measured using a scale and expressed in millimeters.

3.11.16 Survival percentage of plantlets

The number of plantlets survived out of total plantlets subjected to hardening was counted and expressed as percentage.

3.11.17 Number of leaves per plantlet

The number of leaves produced from single plantlet was counted after four weeks of hardening and expressed as mean.

3.11.18 Plant height

The plant height was measured from the base to top of the plantlet using a scale after four weeks of hardening and average was expressed in millimeters.

3.9 Statistical analysis of data:

Collected data were statistically analyzed using MSTAT-C computer package programme. Mean for every treatments were calculated and analysis of variance for each one of characters was performed by F–test (Variance Ratio). Difference between treatments was assessed by Least Significant Difference (LSD) test at 5% level of significance (Gomez and Gomez, 1984).

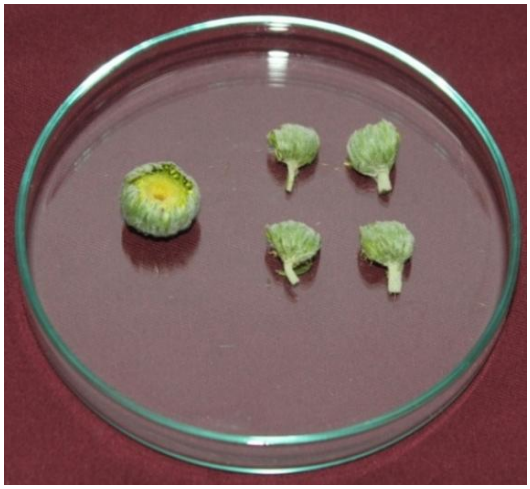
(a)

(b)

(c)

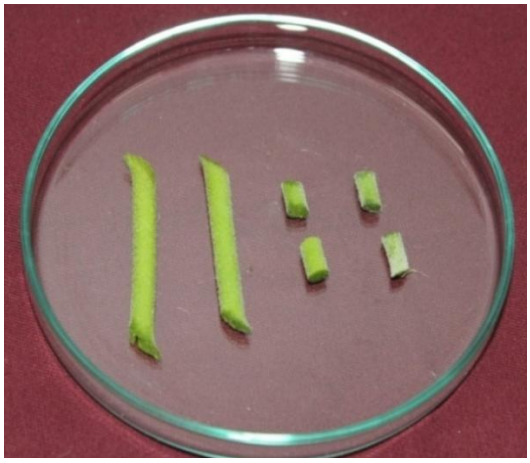
Plate 1: (a) ACI rooftop net house, (b) Gerbera plant, (c) close view of the flower used for the study

(a) Flower bud

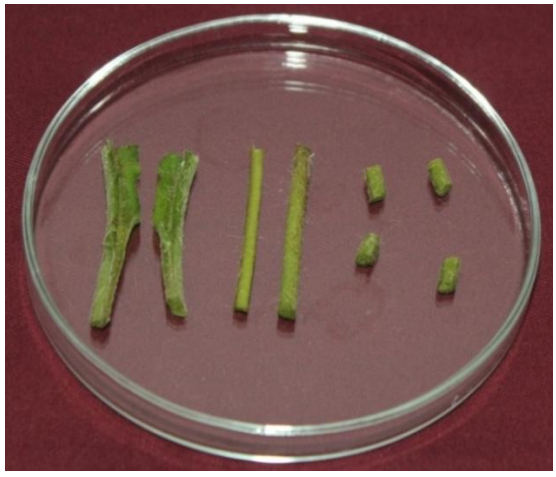


(b) Capitulum

(c) Leaf



(d) Peduncle

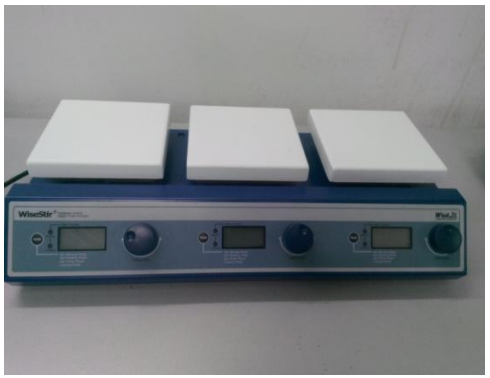


(e) Petiole

Plate 2: Gerbera explants used in the study

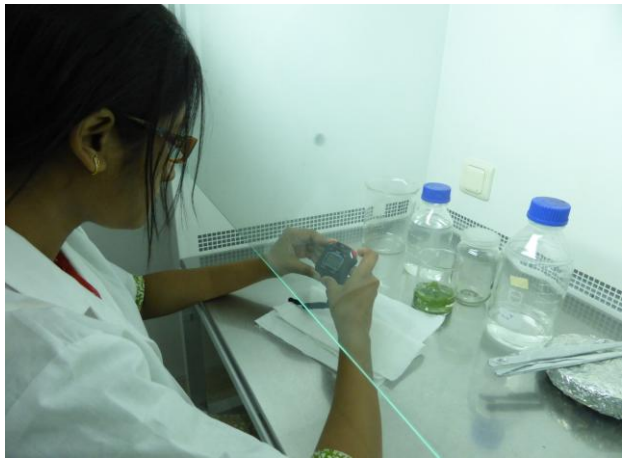
(a)

(b)



(c)

(d)



(e)

(f)

Plate 3: (a) Autoclave machine, (b) Digital weigh machine, (c) Digital stirrer, (d) pH meter, (e) Laminar air flow cabinet, (f) Growth room

CHAPTER IV

RESULTS AND DISCUSSION

The results obtained in the present investigations entitled "*Iin vitro* regeneration of *Gerbera jamesonii* Bolus from different explants" are presented under the following headings in this chapter.

4.1. Effect of different explants on culture establishment

4.1.1 Contamination %

Data (Appendix I) shows that there were significant differences between treatments for per cent contamination. Among the explants, the lowest per cent contamination was recorded in E_1, Flower bud; (7.1%) while the highest contamination rate was recorded in E_2, capitulum (61.6%). There were significant differences between growth regulator treatments for per cent contamination. Among the treatments, the lowest per cent contamination was recorded in T_5, 5 mg/l BAP + 0.01 mg /l NAA; (29.1%) while the highest contamination rate was recorded in T_0, 0 mg/l BAP + 0 mg /l NAA; (33.2%). Combined effect of different treatments and different explants also showed a significant variation in terms of percent contamination (Appendix I). Contamination percent showed statistically significant inequality among treatments combinations. the lowest per cent contamination was recorded in T_2E_1 (1.7%), T_4E_1 (1.3%),), T_5E_1 (2.3%) while the maximum contamination rate was recorded in T_1E_2 (79.0%) followed by T_5E_2 (73%) and T_2E_2 (71.3%).

Zhao *et al.* (2002) reported that the contamination rate could be lowered greatly if the receptacle was used as explant in Gerbera. Maximum per cent contamination was observed in capitulum explants. The capitula contain more spores of bacteria and fungus carried from the air. Similar report was made by Murashige *et al.* (1974), wherein they found that though shoot tips are highly regenerative and

rapid; the initial number of shoot tips required is very high due to the high infection rate (80%).

4.1.2 Death rate %

Data (Appendix I) on per cent death differed significantly among the explants. The lowest per cent death was observed in E_4, Peduncle (23.9%). Highest per cent death was observed in E_3, Leaves; (44.8%) followed by E_5, Petiole (38.0%).

Data (Appendix I) on per cent death also differed significantly among the treatments. The lowest per cent death was observed in T_2, 2 mg/l BAP + 0.01 mg /l NAA; (18.7%) and T_3, 3 mg/l BAP + 0.01 mg /l NAA; (17.3%). Highest per cent death was observed in T_0, 0 mg/l BAP + 0 mg /l NAA; (66.8%) followed by T_4, 4 mg/l BAP + 0.01 mg /l NAA; (27.1%). Combined effect of different treatments and different explants also showed a significant variation in terms of percent of death rate (Appendix I). Death percent showed statistically significant inequality among treatments combinations. The lowest per cent death rate was recorded in T_2E_1 (71.3%) and while maximum death rate was recorded in T_0E_5 (91.3%) followed by T_0E_4 (83.3%)

This result is in conformity with that of Nga *et al.* (2005) who also opined that flower bud explant gave best survival rate (40%). Low survival percentage, was due to phytotoxicity of the surface sterilizing chemical. High bleaching activity of chloride might have killed the cells thereby causing the death of the explants (Hiremath, 2000).

4.1.3 Survival %

There were significant differences among explants for per cent survival of explants (Appendix I). The highest survival rate was noticed in E_1, Flower bud; (75.6%) followed by E_4, Peduncle; (66.5%), whereas lowest survival was recorded in E_2, Capitulum; (11.9%). There were also significant differences among

treatments for per cent survival of explants (Appendix I). The highest survival rate was noticed in T_3, 3 mg/l BAP + 0.01 mg /l NAA; (64.5%) followed by T_2, 2 mg/l BAP + 0.01 mg /l NAA; (54.8%), whereas no survival was recorded in T_0, 0 mg/l BAP + 0 mg /l NAA; (0%). Combined effect of different treatments and different explants also showed a significant variation in terms of percent of survival rate (Appendix I). Survival percent showed statistically significant inequality among treatments combinations. Maximum survival rate was recorded in T_2E_1 (97.3%) followed by T_1E_1 (93%) T_3E_1 (89.3%) and T_3E_4 (90%) while no survival rate was recorded in T_0, 0 mg/l BAP + 0 mg /l NAA; with all explants. Shailaja (2002) has also obtained in her studies with Gerbera that flower bud scored better as explants than other explants.

4.1.4 Number of days taken for callus induction

There were significant differences among explants for days to callus induction (Appendix I). The earliest to respond to primordial emergence were noticed in E_1, Flower bud (16.4 days) followed by E_2, Capitulum; (20.1 days).and E_3, Leaves (21.2 days) whereas the most delay for callus induction was recorded in E_5, Petiole (22 days). There were significant differences among treatments for Days to callus induction (Appendix I).

The early response was found in T_3, 3 mg/l BAP + 0.01 mg /l NAA; and longest days were required in T_1, 1mg/l BAP + 0.01 mg /l NAA; (29.3 days) followed by T_2, 2 mg/l BAP + 0.01 mg /l NAA; (24.3 days) whereas no callus induction was recorded in T_0, 0 mg/l BAP + 0 mg /l NAA; (0). The interaction between different treatments and different explants also showed a significant variation in terms on days taken for callus induction (Appendix I).Days to callus induction showed statistically significant inequality among treatments combinations. The lowest days recorded by T_3E_1 (16.3 days). Maximum days to callus induction was recorded in T_1E_3, (89.3 days) T_1E_5 (31.3 days) and T_5E_4 (32 days) followed by T_1E_2

(28.7 days). and T_5E_1(17.3 days) while no callus induction was recorded in T_0, 0 mg/l BAP + 0 mg /l NAA; with all explants. Cytokinins play a crucial role in the morphogenesis of Gerbera. Also, every genotype has a specific range of optimum growth regulator concentration (Deepaja, 1999).

The results of the experiment clearly indicated the suitability of flower bud explants over the others on account of lower contamination, higher survival percentage and lowest days required to callus induction. In the present investigation, regeneration of plantlets from the cultured explants occurred directly from flower bud. Shailaja (2002) has also obtained in her studies with Gerbera that flower bud scored better as explants than other explants. Modh *et al.* (2002) and Nga *et al.* (2005) concluded that the young capitulum is most suitable as explants for *in vitro* culturing. Due to best response of flower bud explants further experiment runs on its performance.

Table 1. Effect of different explants on callus growth of Gerbera[Y]

Explants[X]	% of contamination	% of death rate	% of survival	Days to callus induction
E_1	7.1 d	17.3 d	75.6 a	16.4 a
E_2	61.6 a	26.5 c	11.9 e	20.1 ab
E_3	40.9 b	44.8 a	14.2 d	21.2 b
E_4	9.6 c	23.9 e	66.5 b	22.3 c
E_5	10.8 c	38.0 b	51.2 c	22.0 d
CV (%)	8.3	10.7	5.1	6.4
LSD 0.05	1.4	2.1	1.5	0.9

[X] E_1, Flower bud; E_2, Capitulum; E_3,Leaves ; E_4, Peduncle; and E_5, Petiole

[Y] In a column means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

Table 2. Effect of different growth regulators combinations on callus growth of Gerbera[Y]

Treatments[X]	% of contamination	% of death rate	% of survival	Days to callus induction
T_0	33.2 a	66.8 a	0.0 f	0.0 d
T_1	28.3 b	24.5 c	47.1 d	29.3 a
T_2	25.5 c	18.7 d	54.8 b	24.3 b
T_3	18.1 e	17.3 d	64.5 a	22.5 c
T_4	20.9 d	27.1 b	52.0 c	22.5 c
T_5	29.1 b	26.2 bc	44.7 e	23.7 b
CV (%)	8.3	10.7	5.1	6.4
LSD 0.05	1.6	2.3	1.6	0.9

[X] T_0, 0 mg/l BAP + 0 mg /l NAA; T_1, 1mg/l BAP + 0.01 mg /l NAA ; T_2, 2 mg/l BAP + 0.01 mg /l NAA; T_3, 3 mg/l BAP + 0.01 mg /l NAA ;T_4 ,4 mg/l BAP + 0.01 mg /l NAA; and T_5, 5 mg/l BAP + 0.01 mg /l NAA;

[Y] In a column means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

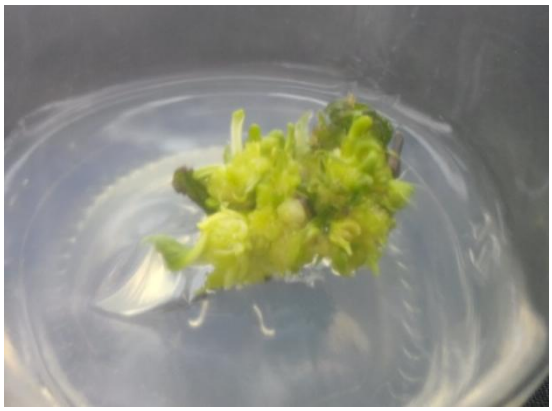
Table 3. Combined effect of different growth regulators combinations and explants on callus growth of Gerbera[Y]

Treatments[X]	% of contamination		% of death rate		% of survival		Days to callus induction	
T_0E_1	30.3	f	69.7	c	0.0	n	0.0	l
T_0E_2	68.7	c	31.3	ghi	0.0	n	0.0	l
T_0E_3	41.7	e	58.3	d	0.0	n	0.0	l
T_0E_4	16.7	g	83.3	b	0.0	n	0.0	l
T_0E_5	8.7	Ij	91.3	a	0.0	n	0.0	l
T_1E_1	33.0	f	56.7	d	10.3	lm	24.0	cdefgh
T_1E_2	79.0	a	18.0	kl	3.0	n	28.7	b
T_1E_3	4.0	klm	3.0	pq	93.0	b	31.3	a
T_1E_4	12.0	hi	16.3	lm	71.7	e	30.3	ab
T_1E_5	13.7	gh	28.7	hij	57.7	f	32.0	a
T_2E_1	1.7	m	1.0	q	97.3	a	20.7	ij
T_2E_2	71.3	bc	19.3	kl	9.3	m	23.3	efgh
T_2E_3	42.3	e	45.7	e	12.0	lm	25.0	cde
T_2E_4	6.3	jkl	11.7	mno	82.0	cd	26.0	cd
T_2E_5	11.0	hi	15.7	lm	73.3	e	26.3	c
T_3E_1	3.0	lm	7.7	op	89.3	b	16.3	k
T_3E_2	32.0	f	33.0	ghi	34.7	i	24.3	cdefg
T_3E_3	45.0	e	27.3	ij	27.7	j	24.0	cdefgh
T_3E_4	3.3	klm	6.7	opq	90.0	b	23.0	efghi
T_3E_5	7.0	jk	12.0	mno	81.0	d	24.7	cdef
T_4E_1	1.3	m	9.0	no	89.7	b	20.0	j
T_4E_2	45.3	e	41.3	ef	13.3	l	21.7	hij
T_4E_3	31.3	f	47.0	e	21.7	k	23.7	defgh
T_4E_4	12.7	h	2.0	pq	85.3	c	22.0	ghij
T_4E_5	13.7	gh	36.3	fg	50.0	g	25.3	cde
T_5E_1	2.3	m	13.7	lmn	84.0	cd	17.3	k
T_5E_2	73.0	b	16.0	lm	11.0	lm	22.3	fghij
T_5E_3	52.3	d	34.0	gh	13.7	l	23.0	efghi
T_5E_4	6.7	jkl	23.3	jk	70.0	e	32.3	a
T_5E_5	11.0	hi	44.0	e	45.0	h	23.7	defgh
CV (%)	8.3		10.7		5.1		6.4	
LSD 0.05	3.5		5.2		3.6		2.1	

[X] T_0, 0 mg/l BAP + 0 mg /l NAA; T_1, 1mg/l BAP + 0.01 mg /l NAA ; T_2, 2 mg/l BAP + 0.01 mg /l NAA; T_3, 3 mg/l BAP + 0.01 mg /l NAA ;T_4 ,4 mg/l BAP + 0.01 mg /l NAA; and T_5, 5 mg/l BAP + 0.01 mg /l NAA; E_1, Flower bud; E_2, Capitulum; E_3,Leaves ; E_4, Peduncle; and E_5, Petiole

[Y] In a column means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

(a)



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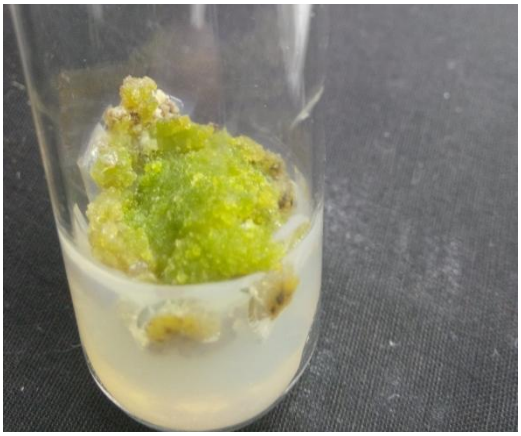

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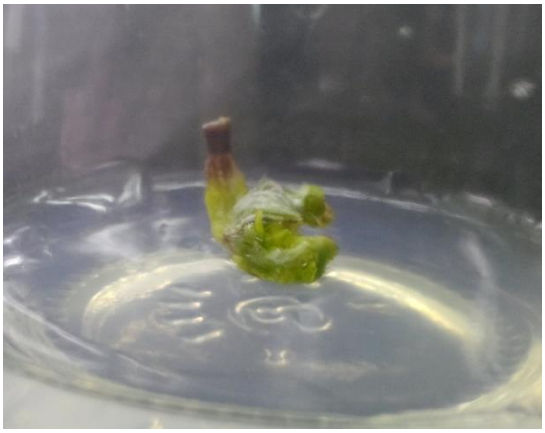

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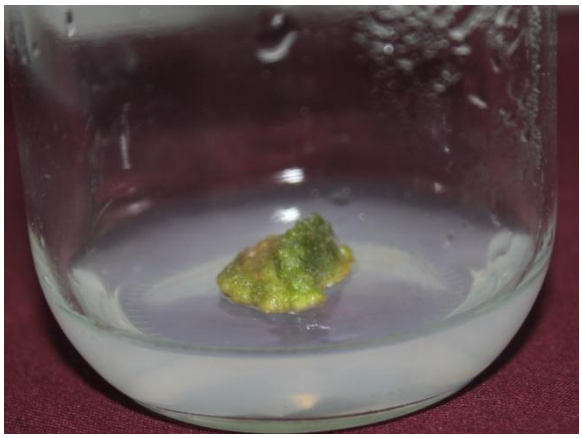


(e)

Plate 4. Gerbera explants response; (a) direct shooting from flower bud (b) direct shooting from Capitulum, (c, d) only callus no direct shoot regeneration from leaves & petiole (e) direct shooting from peduncle

4.2 Effect of concentrations of BAP on shoot multiplication

4.2.1 Number of responsive explants

The number of explants responded recorded after four weeks of inoculation. The data (Table 4) showed that the highest number of explants responded was observed in T_3, 3 mg/l BAP + 0.01 mg /l NAA ;(34.3), followed by T_2, 2 mg/l BAP + 0.01 mg /l NAA; (31.3), while the least number of explants responded in T_1, 1mg/l BAP + 0.01 mg /l NAA and T_5, 5 mg/l BAP + 0.01 mg /l NAA; (29). T_0, 0 mg/l BAP + 0 mg /l NAA; shows no response to shoot initiation.

4.2.2 % of responsive explants

The percent of explants responded recorded after four weeks of inoculation. The data (Table 4) showed that the highest number of explants responded was observed in T_3, 3 mg/l BAP + 0.01 mg /l NAA ;(85.8%), followed by T_2, 2 mg/l BAP + 0.01 mg /l NAA;(78.3%) while the least number of explants responded in T_1, 1mg/l BAP + 0.01 mg /l NAA and T_5, 5 mg/l BAP + 0.01 mg /l NAA; (72.5 %). T_0, 0 mg/l BAP + 0 mg /l NAA; shows no response to shoot initiation. Whereas other cultivars had individual optimum BAP levels (Pierik *et al.* 1982).

4.2.3 Number of days taken for shoot initiation

The data on effect of concentrations of BAP and NAA on shoot multiplication recorded after four weeks of culture are present in Table 4. Significant differences were noticed for days taken for initiation of shoots among the treatments (Table 4). T_3, 3 mg/l BAP + 0.01 mg/l NAA; showed the earliest shoot initiation (16 days) followed by T_4 ,4 mg/l BAP + 0.01 mg /l NAA; (17.7 days)and T_1, 1mg/l BAP + 0.01 mg/l NAA; was late (20.7 days). T_0, 0 mg/l BAP + 0 mg /l NAA; shows no shooting. The concentrations of BAP exhibited significant differences

on days taken for initiation of shoot. Various combinations of auxins and cytokinins have been tried to achieve multiple shoot induction in Gerbera (Murashige *et al.,* 1974; Barbosa *et al.,* 1993). This result is contrary to the results of Pierik *et al.* (1975) who reported that addition of high concentration of BA is very essential for regeneration of shoots from capitulum and optimum concentration is 10 mg/l. However, Laliberte *et al.* (1985) reported that 'Mardi Grass' capitulum explants responded best to the MS medium supplemented with 1 mg/l BAP and 0.1 mg/l IAA. The vigour and number of shoots decreased when the concentration of BAP was raised to 2-3 mg/l. Arello *et al.* (1991) also found same with 2 mg/l BA and 0.5 mg/l IAA. Nga *et al.* (2005) also obtained best results in MS medium supplemented with 1 mg/l BA plus 0.3 mg/l kinetin and 0.2 mg/l IAA.

4.2.4 Number of shoots per clump

The number of shoots produced after four weeks of inoculation differed significantly (Table 5). Maximum number of shoots produced was recorded by T_3, 3 mg/l BAP + 0.01 mg/l NAA; (8.0) and minimum by T_5, 5 mg/l BAP + 0.01 mg/l NAA; (2.0). The concentrations of BAP exhibited significant differences with respect to number of shoots production. Laliberte *et al.* (1985) obtained maximum number of shoots per explant on a medium containing 2 mg/l BAP and 0.1 mg/l IAA from 'Pastourelle' variety. The results are in conformity with that of Shailaja (2002) who obtained highest multiplication rates in gerbera with 3 mg/l BAP (14.95) as compared to 1 and 2 mg/l BAP. Maximum number of shoots was recorded at 2 mg/l BAP as compared to 1 mg/l BAP by Parthasarathy *et al.* (1996). Jerzy and Lubomskii (1991) also obtained the optimum BAP concentrations were 3 and 5 mg/l.

4.2.5 Number of leaves per shoot

The flower buds which gave response above (Plate 3) were trimmed and cultured continuously on the same medium for shoot initiation. The number of shoots produced per explant was recorded after eight weeks of inoculation (Table 5). T_3, 3 mg/l BAP + 0.01 mg/l NAA; produced the highest number of shoots (7.70) and T_5, 5 mg/l BAP + 0.01 mg/l NAA; produced the least number of shoots (4.70).

4.2.6 Shoot length (mm)

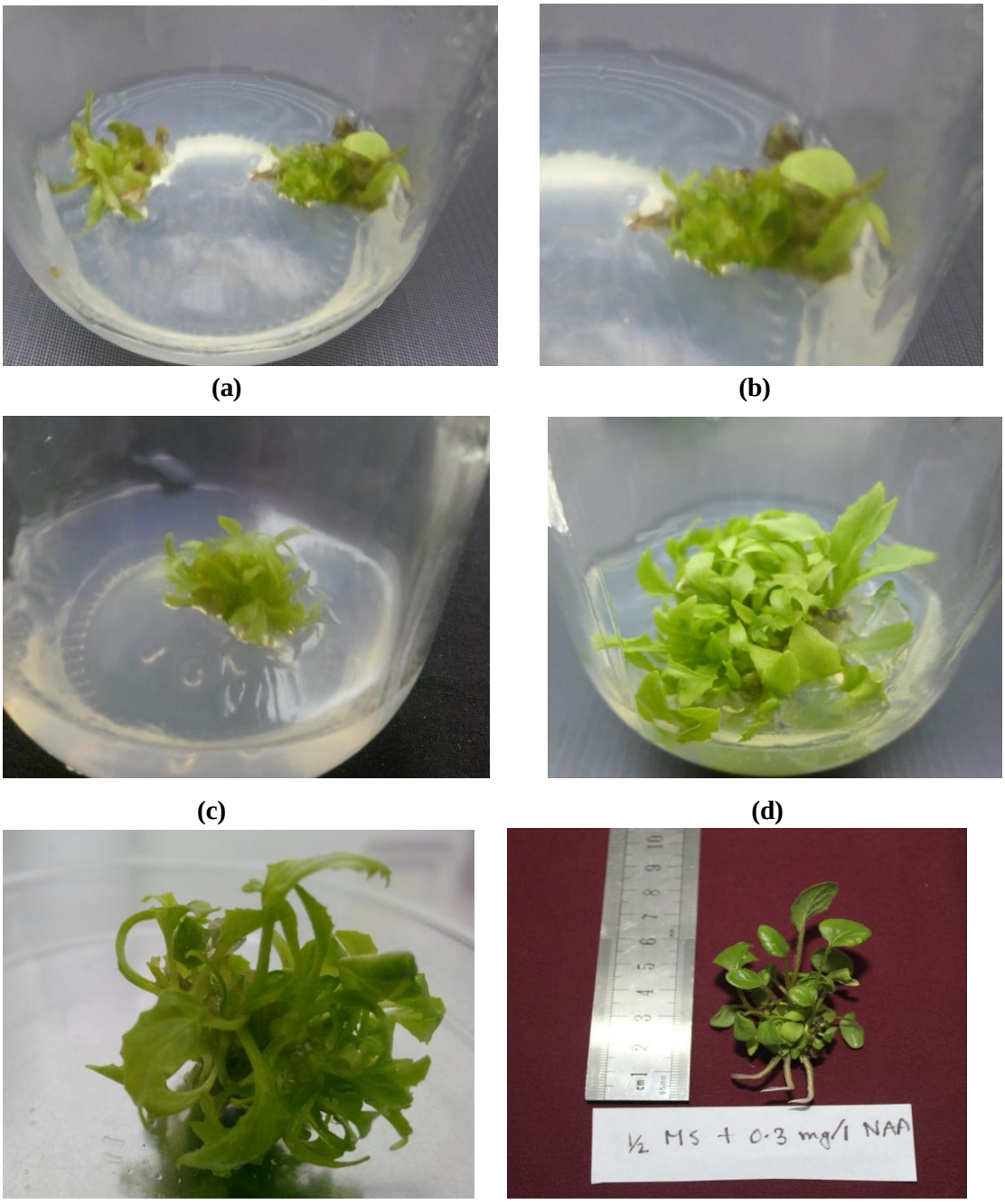
The length of shoot in different treatments after four weeks of inoculation differed significantly (Table 5). The maximum shoot length was observed in T_3, 3 mg/l BAP + 0.01 mg /l NAA; (25.4 mm) followed by and minimum was in Arianna variety (20.84 mm). The concentrations of BAP showed significant differences on shoot length. MS medium with 1 mg l^{-1} BAP and 0.1 mgl^{-1} NAA produced longest shoot (29.24 mm) followed by T_4 ,4 mg/l BAP + 0.01 mg /l NAA; (21.3 mm). Minimum shoot length was noticed on T_1, 1mg/l BAP + 0.01 mg /l NAA; (20.3 mm).

Table 4. Effect of different growth regulators combinations on shooting of Gerbera[Y]

Treatments[X]	No. of responsive clumps	% of responsive clumps	Days taken for shoot induction	No. of shoots/ clumps	No. of leaves/ shoot	Shoot length (mm)
T_0	0.0 d	0.0 d	0.0 d	0.0 e	0.0 c	0.0 d
T_1	29.0 c	72.5 c	20.7 a	2.7 cd	5.3 b	20.3 c
T_2	31.3 b	78.3 b	20.0 a	3.3 c	5.3 b	21.0 bc
T_3	34.3 a	85.8 a	16.0 c	8.0 a	7.7 a	25.4 a
T_4	30.3 bc	75.8 bc	17.7 b	5.3 b	6.0 b	21.3 b
T_5	29.0 c	72.5 c	20.0 a	2.0 d	4.7 b	20.4 c
CV (%)	4.2	4.2	5.5	14.2	17.7	2.6
LSD 0.05	1.9	4.7	1.5	0.9	1.5	0.8

[X] T_0, 0 mg/l BAP + 0 mg /l NAA; T_1, 1mg/l BAP + 0.01 mg /l NAA ; T_2, 2 mg/l BAP + 0.01 mg /l NAA; T_3, 3 mg/l BAP + 0.01 mg /l NAA ;T_4 ,4 mg/l BAP + 0.01 mg /l NAA; and T_5, 5 mg/l BAP + 0.01 mg /l NAA;

[Y] In a column means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability



(a) **(b)**

(c) **(d)**

(e) **(f)**

Plate 5. Gerbera shoot growth; (a, b) shooting from clumps (c, d, e) shoot multiplication, (f) highest shoot growth in number & length

4.3 Effect of concentrations of Auxins on rooting of *in vitro* shoots

4.3.1 Number of days taken for root initiation

The NAA treatments showed significant differences with respect to number of days taken for root initiation. Treatments varied significantly with respect to days taken for root initiation. The data is given in the Table 6. T_3, 1/2 MS + 0.3 mg /l NAA; rooted early (12.0 days) followed by T_2, 1/2 MS + 0.2 mg /l NAA; (14.7 days) and last to root was T_0, 1/2 MS; (31.7 days).

4.3.2 Number of roots per shoot

There were significant differences with regard to number of roots produced among the treatments. The maximum roots were produced with T_2, 1/2 MS + 0.2 mg/l NAA; (9.30) followed by T_1, 1/2 MS + 0.1 mg/l NAA; and T_3, 1/2 MS + 0.3 mg/l NAA; (6.70). The treatment that produced minimum number of roots was observed with T_5, 1/2 MS + 0.2 mg/l IAA; and T_6, 1/2 MS + 0.3 mg/l IAA; (5.0). Pierik and Sprenkels (1984) also obtained rooting in successfully cultured the shoot tips of shy-rooting Gerbera cultivars 'Fleur' and 'Florence' in MS medium containing NAA at 1-3 mg/l. Gregorini et al. (1976) also reported that shoots quickly formed roots on transfer to MS substrate containing NAA

4.3.3 Root length

The auxin treatments showed significant differences in respect of root length. T_2, 1/2 MS + 0.2 mg/l NAA; produced longest root (27.8 mm) followed by T_3, 1/2 MS + 0.3 mg/l NAA ; (25.1 mm), while the treatment produced minimum root length was observed with T_0, 1/2 MS; (19.2 mm). Best quality root type was observed with T_2, 1/2 MS + 0.2 mg/l NAA; long and thick. Short swollen roots

were observed by Mariska *et al.* (1989) with NAA. Deepaja (1999) also reported that NAA induced swelling of roots in GJ-2 and callus production in GJ-3.

Table 5. Effect of different growth regulators combinations on root growth of Gerbera[Y]

Treatments[X]	Days taken for root initiation	Root number/ shoot	Root length/ shoot (mm)	Nature of roots
T_0	31.7 a	4.7 c	19.2 d	long & thin
T_1	20.0 b	6.7 b	23.4 c	long & thick
T_2	14.7 d	9.3 a	27.8 a	long & thick
T_3	12.0 e	6.7 b	25.1 b	long & thick
T_4	21.0 b	6.0 bc	23.3 c	long & thin
T_5	17.0 c	5.0 c	23.4 c	long & thick
T_6	17.3 c	5.0 c	24.2 bc	long & thin
CV (%)	4.6	13.7	2.8	
LSD 0.05	1.6	1.5	1.2	

[X] T_0, 1/2 MS; T_1, 1/2 MS + 0.1 mg /l NAA ; T_2, 1/2 MS + 0.2 mg /l NAA; T_3, 1/2 MS + 0.3 mg /l NAA ;T_4 ,1/2 MS + 0.1 mg /l IAA; and T_5, 1/2 MS + 0.2 mg /l IAA;T_6,1/2 MS + 0.3 mg /l IAA

[Y] In a column means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

(a) **(b)**

(c)

(d)

Plate 6. Gerbera root growth; (a,b) rooting from shoot (c) root performance of different dose of NAA, (d) root performance of different dose of IAA

4.4 Standardization of hardening medium for regenerated plantlets

4.4.1 Survival of plantlets

The survival rate differed significantly among the treatments. The maximum survival rate was noticed on T_5, Sand + vermicompost + coco dust; (1:1:1, v/v) medium with (4.7) followed by T_4, Sand + coco dust; (3.7) after four weeks of transfer to hardening medium. In T_0, Sand; and T_1, Vermicompost, lowest survival rate (2.3) was observed. The water holding capacity of the medium per unit volume was reported to be more in coco peat (Martyr, 1981). On the other hand, evapotranspiration was recorded higher with vermiculite because of higher hydraulic conductivity (Maloupa *et al.*, 1993).

4.4.2 Survival percentage of plantlets

The survival percentage differed significantly among the treatments. The maximum survival rate was noticed on T_5, Sand + vermicompost + coco dust; (1:1:1, v/v) with 93.3 per cent followed by T_4, Sand + coco dust; (1:1, v/v) (73.3%) after four weeks of transfer to hardening medium. In T_0, Sand; and T_1, Vermicompost, lowest survival rate (56.7) was observed.

4.4.3 Number of leaves per plantlet

The treatments were significant differences with respect to the number of leaves production. T_5, Sand + vermicompost + coco dust; (1:1:1, v/v) medium produced maximum number of leaves (23.3) followed by T_4, Sand + coco dust; (16.7) (1:1, v/v) mixture (7.4), while the minimum number of leaves were produced on T_0, Sand; (8).

4.4.4 Plant height

There was found significant differences with regard to plant height among the treatments. The maximum plant height was observed on T_5, Sand + vermicompost + coco dust; (1:1:1, v/v) mixture (23.6 mm) followed by T_4, Sand + coco dust; (1:1, v/v) mixture (21.7 mm), while the minimum plant height was observed on T_1, Vermicompost, medium (16.7 mm).

Table 6. Effect of different hardening medium combinations on hardening of Gerbera[Y]

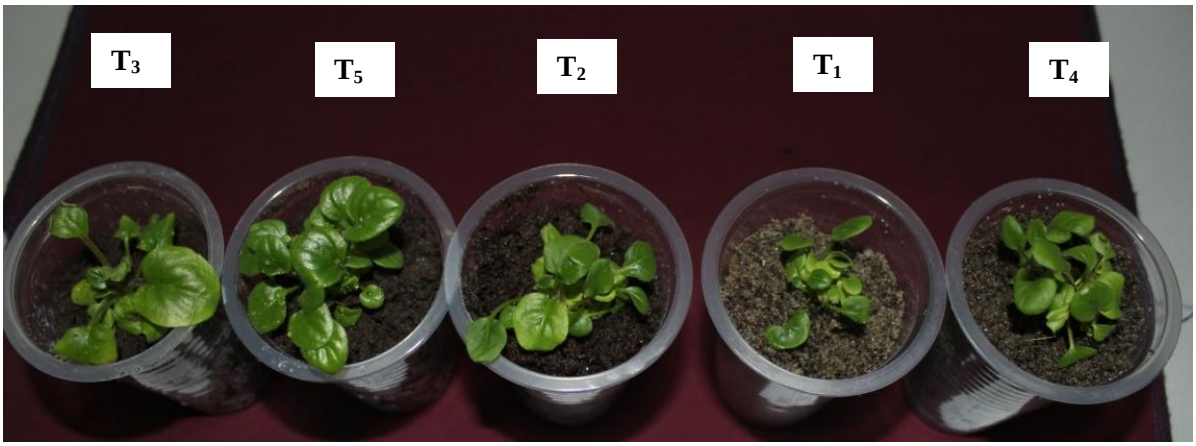
Treatments[X]	Suvival of plantlets	survival percentages	Leaves per plant	Plant height
T_0	2.3 c	46.7 c	8.0 e	18.2 c
T_1	2.3 c	46.7 c	10.0 d	16.7 d
T_2	3.3 bc	66.7 bc	12.7 c	18.1 c
T_3 (1:1, v/v)	3.3 bc	66.7 bc	11.0 d	17.1 cd
T_4 (1:1, v/v)	3.7 ab	73.3 ab	16.7 b	21.7 b
T_5 (1:1:1, v/v)	4.7 a	93.3 a	23.3 a	23.6 a
CV (%)	13.5	13.5	6.2	3.3
LSD 0.05	1.1	21.5	1.5	1.1

[X] T_0, Sand; T_1, Vermicompost ; T_2, Coco dust; T_3, Sand + vermicompost, (1:1, v/v) ;T_4 ,Sand + coco dust, (1:1, v/v); and T_5, Sand + vermicompost + coco dust, (1:1:1, v/v);

[Y] In a column means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

(a)

(b)



(c)

Plate 7. Gerbera plantlet hardening; (a, b) transferred to hardening material, (c) after 4 wks of hardening

(a)

(b)

(c)

(d)

Plate 8. Gerbera plantlet transplanted in rooftop net house of ASRBC, ACI

(a)

(b)

(c)

(d)

Plate 9. Gerbera plant from tissue cultured plantlet; (a) mother plant, (b) clone plant; having true to type flower stalk size, (c) mother plant's flower, (d) cloned plant's flower; with similar diameter and petal arrangement

CHAPTER V

SUMMARY AND CONCLUSION

5.1 Summary

The study was carried out at Advanced Seed Research and Biotech Center (ASRBC), ACI during the period from September, 2014 to September, 2015 to obtain *in vitro* regeneration of gerbera in different explants. Gerbera is one of the important cut flowers having commercial significance. Conventionally propagated Gerbera are heterozygous and their seedlings do not seed. Vegetative propagation is possible through divisions, but rate of multiplication is too slow to be commercially practicable, as divisions can produce only 5 - 10 fold increase per year of selected plant. The plants are also imported legally or illegally from India by the farmers. Division of one plant too many times causes poor quality flower. Each year several new cultivars are introduced in the market. Micropropagation is an important tool for acceleration of plant multiplication and the plants produced through tissue culture are true to type and are free from diseases. Keeping this in view, the present investigation was conducted to find out suitable explant for shoots regeneration, growth regulator for multiplication, rooting and to screen suitable hardening media. Five explants (E_1, Flower bud; E_2, Capitulum; E_3,Leaves ; E_4, Peduncle; and E_5, Petiole), six treatments T_0, 0 mg/l BAP + 0 mg /l NAA; T_1, 1mg/l BAP + 0.01 mg /l NAA ; T_2, 2 mg/l BAP + 0.01 mg /l NAA; T_3, 3 mg/l BAP + 0.01 mg /l NAA ;T_4 ,4 mg/l BAP + 0.01 mg /l NAA; and T_5, 5 mg/l BAP + 0.01 mg /l NAA; .were used for culture establishment. The flower bud is the best explants, due to lowest contamination rate (7.1%) and higher survival rate (75.6%) and early for culture establishment (16.4 days) whereas the lowest performance showed by capitulum and leaves in culture stablishmnt. MS medium supplemented withT_3, 3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA is best medium for culture establishment showed higher response for shoot regeneration. Whereas the lowest performance was by the control treatment withT_0, 0 mg l^{-1} BAP + 0 mg l^{-1} NAA

The best treatment to produce higher shoot number was MS medium supplemented with 3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA and lowest shoot number caused by T_0, 0mg l^{-1} BAP + 0 mg l^{-1} NAA. Six treatments (T_0, 0 mg/l BAP + 0 mg /l NAA; T_1, 1mg/l BAP + 0.01 mg /l NAA ; T_2, 2 mg/l BAP + 0.01 mg /l NAA; T_3, 3 mg/l BAP + 0.01 mg /l NAA ;T_4 ,4 mg/l BAP + 0.01 mg /l NAA; and T_5, 5 mg/l BAP + 0.01 mg /l NAA;) were used to find out best rooting media. T_2, ½ MS + 0.2 mg l^{-1} NAA was the best medium for rooting of *in vitro* shoots. Six different media, *viz.* T_0, Sand; T_1, Vermicompost ; T_2, Coco dust; T_3, Sand + vermicompost, (1:1, v/v) ;T_4 ,Sand + coco dust, (1:1, v/v); and T_5, Sand + vermicompost + coco dust, (1:1:1, v/v);were tried for hardening of plantlets. The T_5, Sand + vermicompost + coco dust, (1:1:1, v/v); gave the maximum survival percentage with better plant vigour resulting as the best medium for hardening. Whereas, the lowest result showed by T_0, Sand; and T_1, Vermicompost.

5.2 Protocol for regeneration of Gerbera:

Based on the results, a protocol for regeneration of Gerbera is given below.

1. Flower bud explants of 0.5 to 0.7 cm diameter should be isolated.
2. The isolated explants should be washed under running tap water with few drops of Tween-twenty for 15 - 20 minutes then immersed in a solution of Bavistin (0.1%) for minutes and then washed thoroughly with distilled water.
3. They are to be treated with 0.1 per cent $HgCl_2$ for 7 minutes and finally washed with double distilled water for 3 - 4 times to remove any traces of $HgCl_2$ in the laminar air flow cabinet.
4. After sterilization flower bud should be cut into two halves and cultured on pre sterilized MS basal medium with 3 mg/1 BAP + 0.1 mg/1 NAA for eight weeks.

5. The shoot may be sub-cultured for shoot multiplication on MS medium supplemented with 3 mg/1 BAP + 0.1 mg/1 NAA after four weeks.
6. The micro shoots of more than 1 cm in length can be transferred to MS medium supplemented with 0.2 mg/1 NAA for four weeks for root initiation.
7. The rooted plantlets are to be washed to remove agar medium, kept in pot with hardening Sand + vermicompost + coco dust; (1:1:1, v/v) medium for four weeks in a in green house.

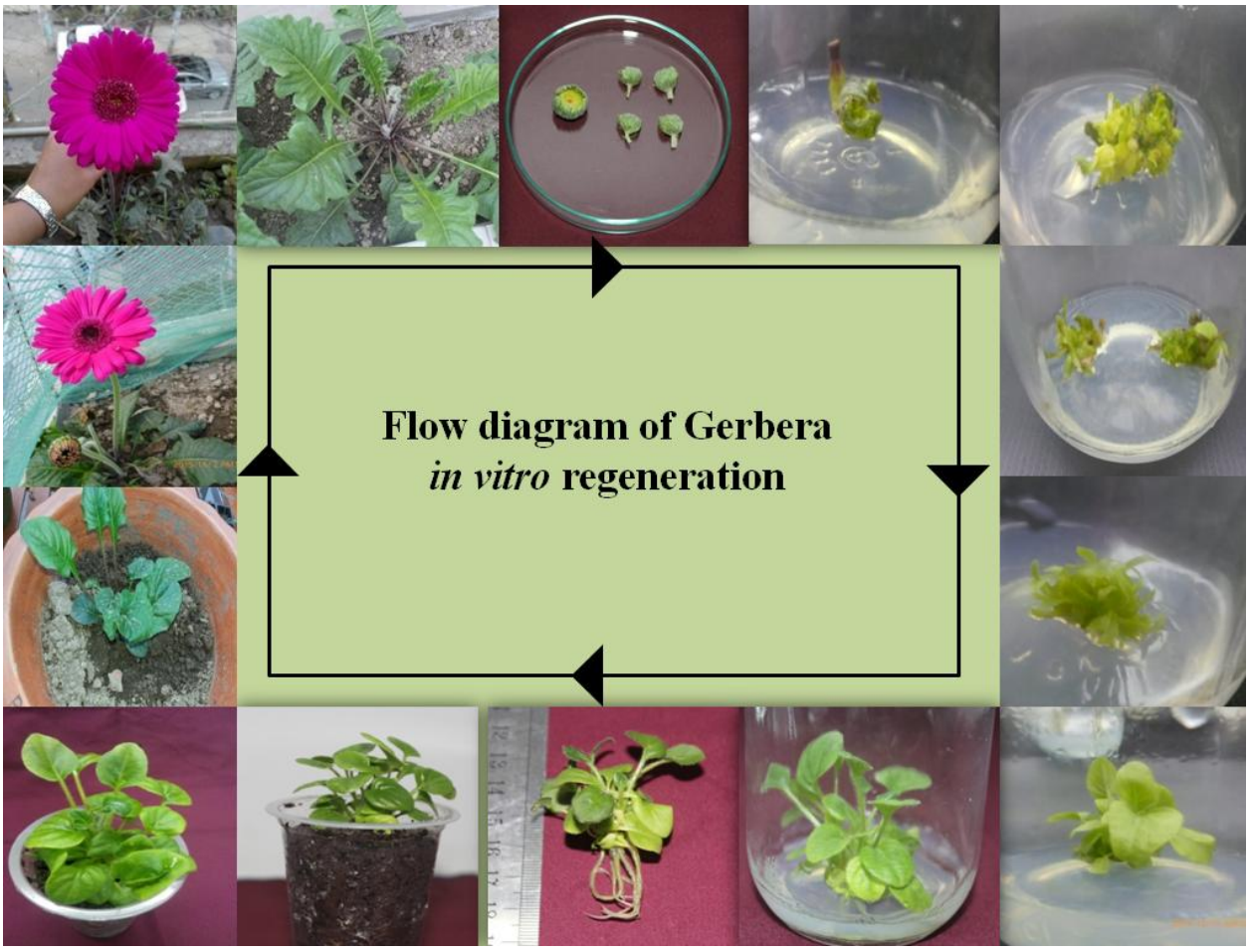


Plate10. Flow diagram of different steps of Gerbera in vitro regeneration

5.3 Conclusion

Following conclusion can be made from the present study:

I. A regeneration protocol has been developed of gerbera in different explants.

II. The flower bud was found to be best explant for culture establishment.

III. MS medium supplemented with 3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA was best medium for culture establishment because it produced more number of shoots per explants.

IV. MS medium supplemented with ½ MS + 2 mg l^{-1} NAA was found to be the best medium for rooting of in vitro shoots

V. Sand + vermicompost + coco dust, (1:1:1, v/v); was the best medium for hardening.

5.4 Recommendation

Based on the findings of this research recommendations are

- ♣ This protocol is useful for *in vitro* plantlet production commercially.

5.5 Suggestions

Further research in the subsequent areas may be suggested:

- ♣ The micro shoots produced *in vitro* may be tried for *ex vitro* rooting to achieve higher establishment rate.
- ♣ As only one variety was evaluated in the current study, protocol needs to be standardized for newly introduced varieties.

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APPENDICES

Appendix I. Analysis of variance of the data on growth of callus of gerbera

Source of Variation	Degrees of freedom (df)	Mean Square for growth of callus			
		% contamination	% death rate	% survival rate	Days to callus induction
Factor A	5	465.6*	5086.8*	7645.5*	1588.1*
Factor B	4	10561.0*	2223.0*	15620.1*	103.0*
Interaction (A x B)	20	305.6*	726.2*	765.3*	16.2*
Error	60	4.6	10.4	4.9	1.7

*: Significant at 0.05 level of probability;

Appendix II. Analysis of variance of the data on growth of shooting of gerbera

Source of Variation	Degrees of freedom (df)	Mean Square for growth of shooting					
		No. of responsive clumps	% of responsive clumps	Days taken for shoot induction	No. of shoots/ clumps	No. of leaves/ shoot	Shoot length (mm)
Replication	2	8.2*	51.1*	2.9*	0.7*	0.7*	0.1*
Factor A	5	486.0*	3037.5*	187.3*	23.3*	20.0*	245.8*
Error	10	1.2	7.3	0.8	0.3	0.7	0.2

*: Significant at 0.05 level of probability;

Appendix III. Analysis of variance of the data on growth of rooting of gerbera

Source of Variation	Degrees of freedom (df)	Mean Square for growth of rooting		
		Days taken for root initiation (days)	Root number/ shoot	Root length/ shoot (mm)
Replication	2	4.7*	0.1*	1.0*
Factor A	6	120.0*	7.8*	19.8*
Error	12	0.8	0.7	0.4

*: Significant at 0.05 level of probability;

Appendix IV. Analysis of variance of the data on hardeningg of gerbera

Source of Variation	Degrees of freedom (df)	Mean Square for hardening			
		Suvival of plantlets	survival percentages	Leaves per plant	Plant height (mm)
Replication	2	0.1*	22.2*	5.8*	1.3*
Factor A	5	2.3*	928.9*	91.0*	23.3*
Error	10	0.4	155.6	0.7	0.4

*: Significant at 0.05 level of probability;