Establishment of *in vitro* regeneration protocol of *Gerbera jamesonii* from different explants

B.Sc. THESIS

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Submitted by

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Declaration

This is to certify that the research work embodying in the result reported in this thesis entitled **"Establishment of *in vitro* regeneration protocol of *Gerbera jamesonii* from different explants"** submitted by MD MINHAZUR RAHMAN, has been carried out under the supervision and able guidance of **Assistant Professor Jebunnesa Chowdhury, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University** in partial fulfilment of the degree of Bachelor of Science in Biotechnology, BRACU University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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Abstract

Gerbera jamesonii is one of the most popular ornamental plant in the world. During this study three attempts were taken in three different phases. These phases were establishment of a reliable sterilization protocol, selection of suitable explants, and selection of suitable media along with hormonal composition for *in vitro* shoot regeneration protocol. To maintain the sterility of explants, all explants were washed with tap water for 15 minutes with 0.2% HgCl2 and 70% ethanol for 20 and in the wet season best result was obtained by washing the explants in tap water for 30 minutes followed by 30 seconds washing with 0.1% HgCl2, 70% ethanol and 20% fungicide respectively. In this study two varieties of Gerbera having red and yellow petal were used. Leaf with mid rid, flower bud and flower stalks were used as explants in this experiment. Regarding the position and age of the explants, 3rd position leaf of the plant showed the best responses and 6-7 days old flower bud and flower stalks gave better responses in the *in vitro* regeneration. All these three types of explants were cultured in the MS medium with different hormonal composition and concentration. Among all the composition 5 mg/l BAP with 1mg/l NAA gave the best response by forming multiple shoots from all explants. Shoot proliferation was started within 6-7 weeks after inoculation of explants in the suitable medium. The best mean no. of shoot was found from the leaf with mid rib and the no. was 7.75 per explant. In future, attempts can be made to find out the suitable rooting medium for both Gerbera varieties to complete regeneration protocol.

List of Abbreviations

BRAC- Bangladesh Rural Advancement Committee

BAP- 6-Benzylaminopurine

NAA- Napthalene Acetic Acid

IBA- Indole-3 Butyric Acid

Kn- 6-Furfurylaminopurine

MS- Murashing and Skoog medium (1962)

LS- Linsmaier and Skoog medium (1965)

HgCl2- Mercuric Chloride

NaoCl- Sodium Hypochloride

Ph- Power of Hydrogen

ml- Milliliter

mg- MIligram

RM- Regenaretion Medium

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Introduction:

Gerbera jamesonii is one of the most famous flowering plant in the world. In recent years, it became very important ornamental crops in the world along with other flowering plants, like- rose, chrysanthemum and tulip. Gerbera is an exclusive item in the global floral trade, ranking about fifth in cut flower use**.** ("The Secret of Long-Lasting Gerbera Daisies" 2008). The production of Gerbera was approximately US$ 220 million in 2001 representing 70 million stems sold in US alone (Broek*et al.,* 2004). Horticultural and floriculture greenhouse production has increased 92.4% during the last 10 years (Tzouramani *et al*., 1995). Gerbera is famous to the farmers for its significant economic importance. Due to the increasing demand of flower, flower farming is rapidly flourishing in Bangladesh as well.

The origin of this flower is south eastern Africa. This flower is commonly known as a 'Barberton Daisy'. In 1889, it was first introduced by Robert Jameson in the Barberton area of South Africa. The first official description of "*Gerbera jamesonii"* which *is* also known as Barberton Daisy, was made by J. D. Hooker in 1889 in the Curtis Botanical Magazine. (Codd,1979). The genus Gerbera was named inhonor of a German naturalist Traugott Gerber, who traveled in Russia during 1743. There are about 30 to 40 species of Gerbera. Commercial breeding started in the 19th century when the *Gerbera jamesonii* species from Natal in South Africa was crossed with *Gerbera viridifolia* from the Cape Province. It was cultivated first in Europe and later planted in the Himalayan foothills in Kashmir at heights between 1,300 and 3,200 meters. (Hind, 1 992).

Out of the recorded species, *Gerbera jamesonii* is one which is under cultivation. Few important cultivars of Gerberas are Cream Clementine, Maron Clementine, Delphi, Vesta, Uranus, Terraqueen, Dusty, Valentine, Diablo, Mariso and Pascal. Based on the flower heads, they may be grouped into single, semi-double and double cultivars. (Loeser, 1986).

Scientific Classification of *Gerbera jamesonii*

Kingdom: Plantae – Plants

Division: Magnoliophyte – Flowering plants

Class: Magnoliopsida – Dicotyledons

Order: Asterales

Family: Asteraceae/Compositae – Aster family

Genus: Gerbera J.F. Gmel. – Transvaal daisy

Species: Gerbera jamesonii Bolus ex Hook. f. – Barberton daisy

Gerbera became very popular for its various vibrant colors. It is specially founded in red, yellow, white, purple and many solo as well as bicolor. It is widely popular as a decorative garden plant. The vase life of this flower is longer than others flowers in the market. Vase life of Gerbera dependent upon the verity of it but it is almost 5 to 7 days. (Ranwala, 2010). *Gerbera jamesonii* has been hybridized with other Gerbera species to produce the variation and long-lasting cut flower. Literally, hundreds of new varieties are tested in Dutch greenhouses each year to discover new shape and color combinations that ship well and last for a long time in the vase. ("The Secret of Long-Lasting Gerbera Daisies" 2008).

Gerbera is an herb or forbs for its physical condition and for the growth habit. The stem of the Gerbera plants is cylindrical and unbranched also. The full steam contains green pigments all over the body. The height of the stem is different for different varieties but it is in between 40-70cm. The leaves of Gerberas are deep green and those deeply lobed leaves covered with silky hairs arising from a crown. Leaves occur in basal rosettes, petiole, oblong-spatulate and deeply lobed to 25 cm (10 inch) long by half as wide.

Gerbera has a large capitulum with striking, 2-lipped ray florets. The capitulum on the Gerberas has the appearance of a single flower, but is actually a cluster of hundreds of individual flowers. The morphology of the flowers varies depending on their position in the capitulum of the Gerbera. The flower head of the flower is 3 to 4 inches wide. The multiple soft petals come from the centerpiece

of stamens, anthers and carpels. The flowers from the hybrid plant produce two or more single stemmed stalks with a single flower. (Grimsby, 2011).

Gerbera need cool well ventilated condition with good light in the green house. The suitable temperature for the cultivation of Gerbera is 10-20°C. Temperature below 7°C hinders the normal growth of this plant. Gerbera usually grow well in a soil mix consisting of 2 parts peat moss to 2-parts sand to 1-part loam. Organically rich, medium moisture and well-drained soil is favorable condition for the average growth of Gerbera. Gerbera is a perennial plant contains dicotyledons. Though Gerbera founds round the year, the growth rate of this plant is higher in winter and the flower production rate is high in the spring.

Diseases of Gerbera

Gerbera can be attack by various insects that causes physical damage of the plants and those insects are one of the main obstruct for good flower production. Like White Fly *(T. vaporariorum*)- It is a sucking insect and most of the Gerbera plants in the greenhouse attack by those Whitefly. Leaf Miner (*Liriomyzatrifolii*)- it is a leaf mining fly and a severe pest of Gerbera. This pest born into the leaf and make irregularly shaped tunnels or blotches which are generally light yellowish tan to brown in color. Mites (*hemitarsonemus latus* and *Steneotarsonemus pallidus*) - Plants are affected by this pest often which hamper the development of leaves and flower bud. Flowers are malformed as well as unsaleable for this pest. Aphids- This insect infests young leaves and buds and causes injury by sucking the sap which results in distortion of tissues. And Nematodes- Gerbera plants occasionally affected by this pest. Root-knot nematode is very dangerous for Gerbera. (Moorman, 2016).

Gerbera plants are often infected by various bacteria and virus. The rate of viral infection in Gerbera plants is much higher than any other plant. Some of the diseases by the viral and bacterial infections are:

Alternaria Leaf Spot- this disease caused by *Alternaria*. Brown dots form on the florets and the leaves. Centers on the leaf spots become white. **Bacterial Leaf Spot**- *Pseudomonas cichorii* is responsible for that. Circular small and large spots are form in the leaves and then become irregular. The leaves color turninto black from dark brown. **Botrytis Blight**- The petioles have a long brown spots. The leaves become yellow then die. Petals have tan spots. Stems at soil level are killed and

the infected tissues become covered with gray fungal growth. The agent of this disease is *Botrytis cinerea*. **Fusarium Stem Rot**- *Fusarium solani* is the cause of this disease. Petiole of leaves blacken at the base as the plant collapses. **Phytophthora Crown Rot**- *Phytophthoracryptogea* is responsible for this disease. The symptoms are plants wilt suddenly and the color of leaves become brown. Roots are rotted and a crown rot develops. **Powdery Mildew**- This is a fungal infection cause by *Golovinomyces cichoracearum***.** A white fungal growth develops on the surface of leaves. **Pythium Root Rot**- Agent of this disease is *Pythium*. The plants wilt and die just like roots rot. **Rhizoctonia Crown Rot**- Stems at the soil level have a brown lesion. Plants wilt and die. And the agent is *Rhizoctonia. Thielaviopsis.* **Root Rot**- Plants become yellow, wilt, and die. Color of the Roots becomes dark brown to black. This is cause by *Thielaviopsisbasicola*. **Tobacco rattle, Gerbera mosaic, impatiens necrotic spot,** and **cucumber mosaic viruses** are also attack on the Gerbera plants but the rate is low but those has the potential to damage in a large amount to the plants and flower. They can cause color breaking of the flower, Line or ring spots to the leaves and sometime can cause for the mottled leaves. Yellow spotting and dead flecks even the plants may be distorted by those viruses. (Moorman, 2016).

Gerbera is a commercially grown flower plant all over the world. This plant can be propagated by seed but cultivated Gerbera plants are extremely heterozygous. Those plants also take long time to flower. Conventional propagation and breeding are facing with problems that require the application of modern methods of biotechnology. Gerbera can propagate asexually also. In case of asexual propagation, the multiplication rate is low. Among the various methods division clumps is the most common method. Though it is not suitable for a large number of production. On the other side, multiplication through tissue culture can propagate millions of plants in a year and this method is also very effective. (Murashige *et al*., 1974 and Aswath *et al*., 2002, 2003). In tissue culture studied so far, plant regeneration was uniformly achieved with different explants as the source material (Murashige, 1977).

In vitro regeneration of Gerbera has been successful through different explants including stem tips, floral buds, leaf, capitulum etc. Some of the examples are, Plants produce from explants of capitulum (Pierik *et al.,* 1973, 1975), leaves (Hedtrich, 1979) and (Barbosa *et al.,* 1994), floral buds (Posada *et*

al., 1999), floral bracts (Maia *et al*., 1983), torus (Zhang, 2002) and inflorescence (Schum and Busold, 1985) can be mentioned.

The nutrient medium is important for successful tissue culture and information is still scanty on the various medium used for Gerbera explants. MS medium (Murashige and Skoog, 1962) was successfully used by many workers for callus formation as well as shoot regeneration (Pierik *et al.,* 1982; Le *et al*., 1999; Modh *et al.,* 2002; Aswath and Wazneen, 2004; Kumar and Kanwar, 2005, 2006). (Parthasarathy *et al*., 1996) and (Verma and Anand, 2006) used N6 (Chu, 1978) and B5 (Gamborg *et al.,* 1968) medium for culture establishment in Gerbera. (Mandal and Datta, 2002) used LS (Linsmaier and Skoog, 1965) medium for establishing organogenic callus cultures from immature flower buds. (Chen *et al.* 2006) used DKW (Driver and Kuniyuki, 1984) medium for tissue culture studies of Gerbera stem nodes with buds. (Kanwar and Kumar, 2008).

Bangladesh is a land of agriculture.Due to the increasing demands of flower in local and international arena; floriculture became very popular in recent years especially in the urban areas. To meet up the local demand, many nurseries started to cultivate this plant from cutting imported from India. (Rahman *et al.,* 2014). Along with nursery cultivation, biotechnological approach might be a prospective alternative for mass propagation of Gerbera. As the economy of Bangladesh is largely dependent on agriculture, this flowering plant can have a great contribution to the economic growth as well as for opening a new dimension in the field of agriculture in Bangladesh.

This research was conducted with a view to fulfill the following objects: -

01. Establishment of sterilization procedure for the selected explants.
02. Selection of suitable explants of Gerbera for shoot regeneration.
03. Selection of suitable medium combination for the shoot regeneration.

2 Method and Materials

2.1 Plant materials

In the present experiment two varieties of Gerbera (*Gerbera Jamesonii*) (figure 2.1) were used as following.

- Red
- Yellow

2.2 Sources

All the plantlet of Gerbera ware collected from the BRAC Research and Development Center, joydebpur, Gazipur. During the research time those plants ware maintained in the roof top garden of the BRAC University under an expert gardener.

2.2 Medium used during the experiments

2.2.1. Medium used for tissue culture

For the different segments of experiment in the tissue culture Murashige and Skoog (MS) medium (1962) was used with different hormonal combination.

2.2.1.1. Callus induction medium

For the callus induction from different explant MS medium containing BAP hormone with different other hormones (NAA, KN, IBA) were used with different combination.

2.2.1.2. Shoot regeneration medium

For the initiation and development of the shoot direct from the explant MS medium with high amount of different combination of hormones were used. On the other side MS medium with different hormones and hormonal combinations were used for the shoot development from the callus of different explants.

2.2.1.3 Root induction medium

Half strength of MS medium (half strength of macro and micro nutrients) with different concentration of auxin named (IBA) was used for the induction of the roots from the base of *in vitro* grown shoots. For the solidification of the medium 0.6 % (w/v) phytagel of Sigma company was used.

2.3 Preparation of stock solutions for medium

2.3.1. Preparation of different stocks solutions for MS medium

The MS medium contain macro and micro nutrients with iron EDTA as well as organic compounds and also different kind of hormonal combination. These are very essential for the in vitro plant regeneration. These elements are needed in different concentration for the medium (Table 2.3). So their stock preparation is the first steps for the medium preparation.

2.3.1.1. Stock solution (Macro nutrients) preparation

All the components of the macro nutrients (mentioned in the table 2.1) were added one after dissolving the previous one in half of the total volume of the distilled water in a volumetric flask. Magnetic stirrer was used to mix them well. The total volume (1000 ml) was made by adding distilled water. The solution was then poured into a clean flask and tagged with the date. The solution was then stored in the refrigerator at 4°C. The concentration of the stock solution is 10 times more than the final strength of the medium.

2.3.1.2. Stock solution (Micro nutrients) preparation

The components of micro nutrients (mentioned in the table 2.1) were added serially in the half of the total volume of the solution. Components were mixed well. Total volume (1000 ml) was made by adding distilled water. The solution was stored in refrigerator at 4°C in a clean flask. The strength of the solution is 100 times of the medium strength.

2.3.1.3. Stock solution (Iron EDTA) preparation

The strength of the solution was 100 times. (27.8mg/l) FeSO4.7H2O was added in distilled water and stirred in hot plate till dissolved. After that Na2EDTA.2H2O (37.3 mg/l) was added and stirred in hot plate till dissolved. The solution is light sensitive so it was stored in the amber bottle at 4°C in the refrigerator.

2.3.1.4. Stock solution (Organic nutrients) preparation

All the components (mentioned in the table 2.1) were added to the distilled water one after dissolving another. The final volume of the solution is 100ml and the strength is 100 times more than the final strength of the medium. The solution was then stored in 4°C in the refrigerator.

2.3.1.5. stocks solutions (Different hormone) preparation

In this experiment different types of Auxin and Cytokinin hormones were used. For the regeneration and development of plant tissues these hormones are essential. These hormones have different molecular weight (mentioned in table 2.3). All the hormones are dissolved in 1N NaOH.

- BAP: 10 mg of the BAP (Sigma) was dissolved in 1-2 ml of 1 N NaOH. Distilled water was added to make the final volume (100ml). The strength of the stock solution was 1mg/10ml. The solution was then filtered and stored in a clean flask at 4°C in the refrigerator.
- NAA: 10 mg of the NAA (Sigma) was dissolved in 1-2 ml of 1 N NaOH. Distilled water was added to made the final volume (100ml). The strength of the stock solution was 1mg/10ml. The solution was then filtered and stored in a clean flask at 4°C in the refrigerator.
- KN: 10 mg of the KN (Sigma) was dissolved in 1-2 ml of 1 N NaOH. Distilled water was added to make the final volume (100ml). The strength of the stock solution was 1mg/10ml. The solution was then filtered and stored in a clean flask at 4°C in the refrigerator.
- IBA: 10 mg of the IBA (Sigma) was dissolved in 1-2 ml of 1 N NaOH. Distilled water was added to make the final volume (100ml). The strength of the stock solution was 1mg/10ml. The solution was then filtered and stored in a clean flask at 4°C in the refrigerator.

2.4 MS medium preparation

Murashige and Skoog (1962) medium (MS) was widely used medium for the regeneration of the plant in plant tissue culture.

- All components of this medium (mentioned in the table 2.3) were added to a volumetric flask. Distilled water was added to make the final volume (1000ml).
- The pH of the medium was adjusted to 5.8±2 with help of 1 N NaOH or HCl.
- For the solidification of the medium 0.8% (w/v) ager was used in the medium.
- The mixture was heated 8-10 minutes to the micro oven (Model: MH6548SR, LG, China)
- The medium was divided into conical flasks in 100ml and sealed with aluminum foil then tagged with a permanent marker.

2.5 Medium sterilization

Conical flashes were prewashed before pouring the medium. And after pouring the medium in a 100ml the flasks were sealed with the aluminum foil paper. Then the conical flasks were autoclaved (ALP-32, Japan) at 15lb/sq. inch at 121°C temperature for 20 minutes. Medium containing conical flasks were stored at 25°C in the culture room.

2.6 Maintenance of Aseptic condition

All the inoculation was carried out in a laminar airflow cabinet (SCV-AI, Singapore). The UV light was on for 30 minutes before work in the cabinet and also cleaned the surface with 70% of ethanol to avoid the contamination. Instruments like petri dishes, filter paper, forceps, scalp etc. were pre autoclaved (ALP-32, Japan) and stored in the incubator. Before the work those instruments were cleaned with 70% of ethanol and also sterilized by flaming method. To maintain the aseptic condition other things like the biker, explant carrier etc. was cleaned by 70% of ethanol. Before work the hands were sterilized with the soap and cleaned with 70% of ethanol to maintain the aseptic condition in the work. Every time after opening and before sealing the conical flasks the covers were flamed.

After inoculation or subculture the flasks are covered with the Para film paper for maintaining the aseptic condition.

2.7 Applied culture technique

In this experiment for the different purposes and for the different segments of the experiment these following technique were used.

- Axenic culture
- Explant culture
- Subculture of the explants.
- Rooting

2.7.1 Axenic culture

Fungal contamination is one of the main barrier for tissue culture. The rate of fungal contamination changes with the season. So the sterilization process of the explants was different. In the time of rainy season more precaution was needed then the other times.

2.7.1.1 Axenic culture (Rainy season)

The explants were collected in a jar from the garden to the lab. The surface of the explants was washed with the running tap water for 30 minutes. Then the explants were deepened in the fungicide Bavistin DF (1mg/500ml) for 5 minutes and washed with the distilled water. The explants were then deepened in 0.1% HgCl2 for 5 minutes. After that those were washed with the autoclave distilled water in the laminar flow cabinet for 5 times. Then those were washed by 70% of ethanol for 20 seconds and again washed with autoclaved distilled water for 7 times.

2.7.1.2 Axenic culture (Other seasons)

The collected explants from the garden were washed with the running tap water for 15 minutes. Then the explants were washed with the distilled water for five times. In the laminar flow cabinet, the explants were deepened in the HgCl2 for 7 minutes and washed with the autoclaved distilled water

for 5 times. After that the explants were deepened in the 70% of the ethanol for 20 seconds. And finally the explants were again washed with the autoclaved distilled water.

2.7.2 Explant culture

Leaf segments with the midrib, flower buds and flower stalks were used as explants. The third number leaf from the top of the plants was selected always for the leaf explants. The flower buds and the flower stalks at the age of 6-7 days was collected as explants. The flower bud was separated from the flower stalk before sterilization process and then dissected 3 to 4 pieces for using as explants. On the other side the flower stalks were divided into 4 pieces. The cutting size of the leaf segments with mid rib were 4 cm. after sterilization and cutting those explants were placed on a filter paper to absorb the extra water of the surface. The leaf segments were placed on the medium in both sides. And some of the flower stalks were placed on the medium without dissection.

2.7.3 Subculture

All of the explants contain a large number of secondary metabolites so subculture is very essential part for Gerbera tissue culture. For the callus formation and also for the direct shoot regeneration explants were transferred into a fresh medium after 10-12 days. During subculture, the death tissues of the explants were cutting out. All the sub cultured explants were under the routine observation. In the time of multiple shoot regeneration all the explants were placed into the individual flask for better regeneration. After the regeneration of multiple shoot those individual explants were dissected into 2 to 3 pieces for the optimum height of the shoots.

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2.7.4 Rooting culture

The 2 to 4 cm size of the shoots were separated and placed on the medium. The medium contained different combination of IBA hormones with half strength of MS supplements. Rooting culture was took placed on the test tubes as well as conical flasks also.

2.8 Incubation condition

The inoculated flasks were incubated in the culture room under fluorescent light of 20,000 lux intensity. The temperature of the culture room was 25±1. The culture room had photoperiod of 16-8 (day-night) hour cycle.

Figure 2.1 Two verities of Gerbera (*Gerbera jamesonii*) used in the experiment

Gerbera (Red)

Gerbera (Yellow)

Figure 2.2 Different Explants (leaf segments with mid rib, Flower buds, Flower stalks)

Leaf with mid rib

Flower bud along with flower stalks

Table 2.1 Composition of stock solution in MS medium.

Macro Nutrients (10x) mg/l	
KNO3	1900
NH4 NO3	1650
MgSO4·2H2O	370
CaCl2·2H2O	440
KH2PO4	170
Micro Nutrients (100x) mg/l	
KI	0.83
H3BO3	6.2
MnSO4·4H2O	22.3
ZnSO4·7H2O	8.6
Na2MoO4 ·2H2O	0.25
CuSO4·5H2O	0.025
CoCl2·6H2O	0.025
Iron EDTA (100x) mg/l	
FeSO4·7H2O	27.8
Na2EDTA.2H2O	37.3
Organic nutrients (100x) mg/l	
Nicotinic acid	0.5
Pyridoxin HCI	0.5
Thaimin HCl	0.1
Glycin	2.0

Table 2.2 Molecular weight of different plant hormones (Sigma Plant Culture Catalogue)

Plant Hormones	Molecular weight (gm/mol)
BAP	225.3
KN	215.2
IBA	203.2
NAA	186.2

Table 2.3 Different components for preparation of MS medium (1000 ml)

Components with stock concentration	Amount (ml)
Macronutrients (10x)	100
Micronutrients (100x)	10
Fe- EDTA (100x)	10
Organic nutrients (100x)	10
Sucrose	30
Myo-inositol	0.1

3 Result

In this study two varieties of gerbera (*Gerbera jamesonii*) having red and yellow flower Patel were used. Three different types of explant (Leaf with mid rib, flower bud, flower stalks) were used in this experiment. The main purposes of this study was to establish an *in vitro* regeneration of Gerbera and also to optimization of different techniques of the regeneration.

3.1 Optimization of sterilization for explants

In this part of experiment, explants were washed with running tap water for several time and treated with fungicide (only in rainy season), HgCl2 and 70% of ethanol eventually and washed with Autoclaved distilled water for several time. Then all the explants were placed to the filter paper to absorb the extra surface moisture of the explants.

To avoid the contamination, explants were treated with different concentration of HgCl2 and different concentration of fungicide (Bavistine). The time duration for washing and time duration for treating with fungicide, HgCl2 and 70% of ethanol had effect the contamination rate. It was found that 0.1 % HgCl2 for 5-7 minutes and 70 % of ethanol for 20 seconds gave the lower number of contamination. The uses of the fungicide also had effect on the rate of responsive explants though the use of 20% of the fungicide for 5 minutes along with the HgCl2 and 70% of the ethanol gave lower rate of contamination in the rainy season. The concentration of 15% of fungicide gave higher contamination and the concentration of 25% of fungicide had negative effect on the response of explants (Table 3.2). It was found that the uses of the ethanol for longer time more than 30 seconds reduce the rate of responsive explants. Absence of using 70% of ethanol during sterilization increase the rate of contamination. Using higher percentage of HgCl2 damaged the explant also. The duration of the running tap water also effected the contamination rate. The tap washed for 30 minutes gave low number of contamination but the rate of responsive explants also reduces. 15 minutes washed of running tap water with 5 to 6 times washed with autoclaved distilled water in the shaking condition gave a good rate of responsive explants with low number of contamination. (Table 3.1). Washed with tap water for 15 mins. With 0.2% HgCl2 and 70% ethanol for 20 sec give best sterilization and the contamination rate was 10% with 40% of responsiveness. On the wet season best result was getting by washed the explants in tap water for 30 mins. along with 0.1% HgCl2 and 70% ethanol for 30 sec. with 20% fungicide wash gave 7% of contamination with 40% responsiveness.

Table 3.1 Optimization of sterilization process with the relation of the rate of responsive explants

Combination of treatment	No. of explants	No. of contaminated explants	Contamination rate (%)	No. of responsive explants	Responsive explants (%)
(A)	20	7	35%	9	45%
(B)	20	6	30%	11	55%
(C)	20	4	20%	7	35%
(D)	20	5	25%	7	35%
(E)	20	3	15%	7	35%
(F)	20	2	10%	8	40%
(G)	20	0	0%	6	30%
(H)	20	0	0%	6	30%

Note:

(A) = Tap water for 30 mins. + 0.1% $HgCl_2$ + 70% ethanol for 20 sec.

(B) =Tap water for 15 mins. + 0.1% $HgCl_2$ + 70% ethanol for 20 sec.

(C) =Tap water for 30 mins. + 0.1% $HgCl_2$ + 70% ethanol for 30 sec.

(D) =Tap water for 15 mins. + 0.1% $HgCl_2$ + 70% ethanol for 30 sec.

(E) =Tap water for 30 mins. + 0.2% $HgCl_2$ + 70% ethanol for 20 sec.

(F) =Tap water for 15 mins. + 0.2% $HgCl_2$ + 70% ethanol for 20 sec.

(G) =Tap water for 30 mins. + 0.2% $HgCl_2$ + 70% ethanol for 30 sec.

(H) =Tap water for 15 mins. + 0.2% $HgCl_2$ + 70% ethanol for 30 sec.

Graph 3.1 Optimization of sterilization process with the relation of the rate of responsive explants

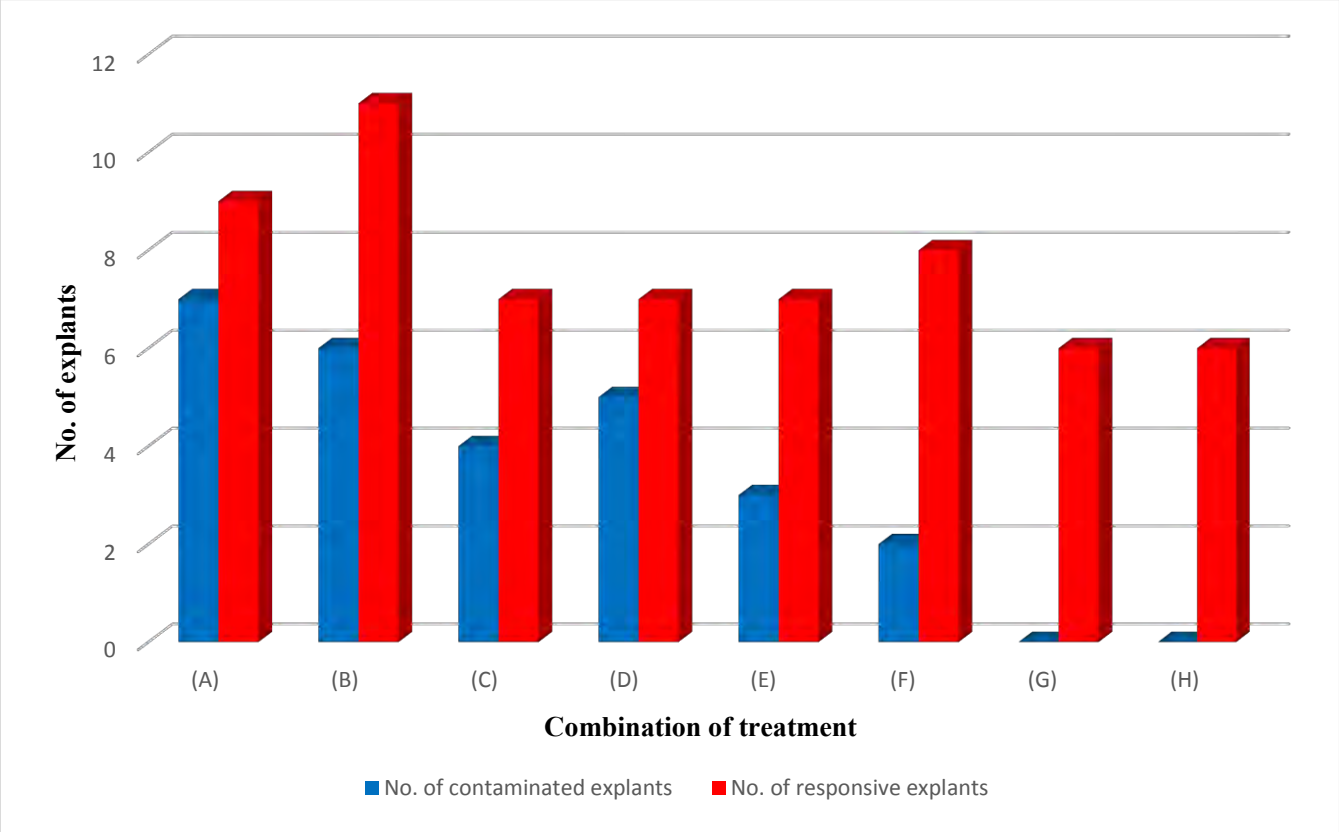
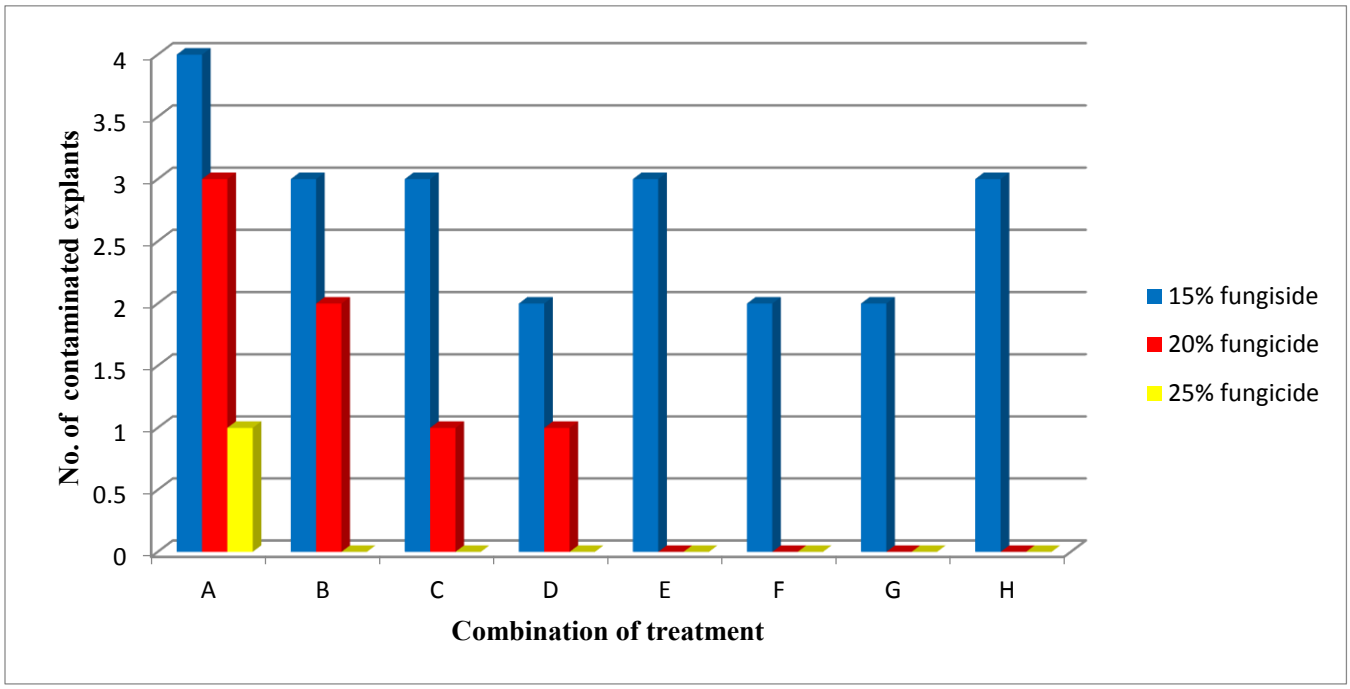


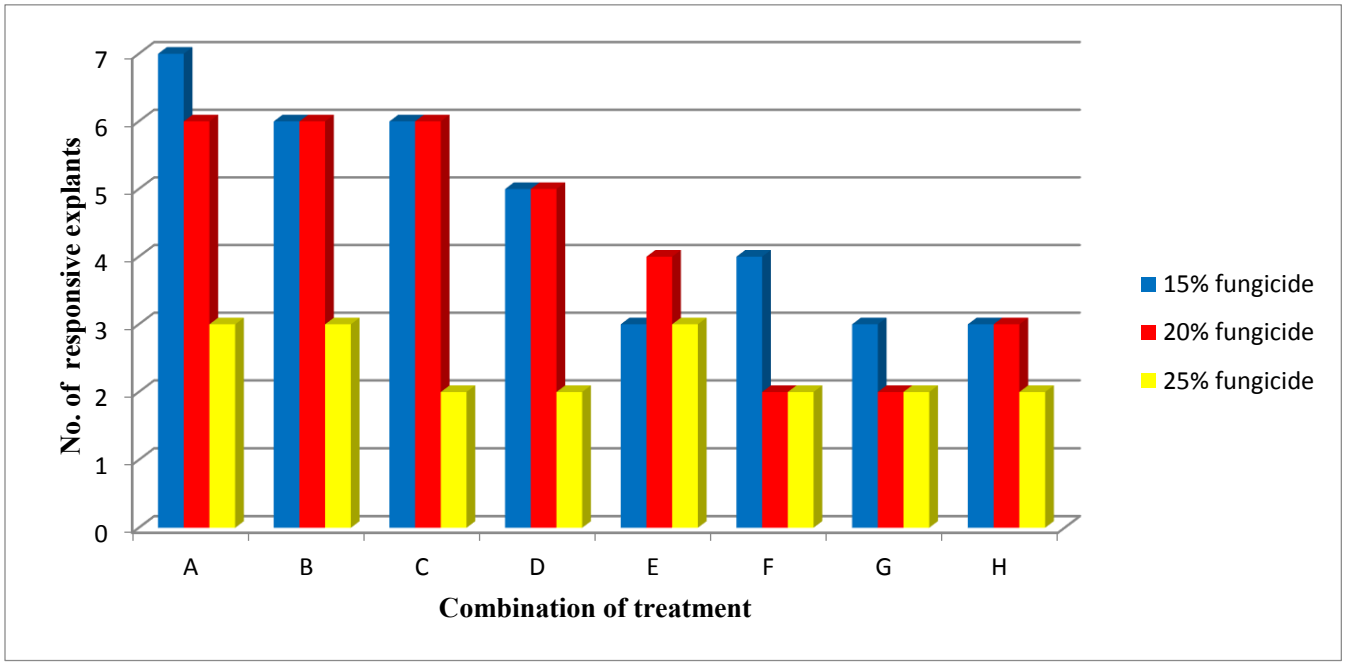
Table 3.2 Optimization of sterilization process (with fungicide) with the relation of the rate of responsive explants

Combination of treatment	Conc. of fungicide	No. of explants	No. of contaminated explants	Contaminated explants (%)	No. of responsive explants	Responsive explants (%)
(A)	15%	15	4	27%	7	47%
	20%	15	3	20%	6	40%
	25%	15	1	7%	3	20%
(B)	15%	15	3	20%	6	40%
	20%	15	2	13%	6	40%
	25%	15	0	0%	3	20%
(C)	15%	15	3	20%	6	40%
	20%	15	1	7%	6	40%
	25%	15	0	0%	2	13%
(D)	15%	15	2	13%	5	33%
	20%	15	1	7%	5	33%
	25%	15	0	0%	2	13%
(E)	15%	15	3	20%	3	20%
	20%	15	0	0%	4	27%
	25%	15	0	0%	3	20%
(F)	15%	15	2	13%	4	27%
	20%	15	0	0%	2	13%
	25%	15	0	0%	2	13%
(G)	15%	15	2	13%	3	20%
	20%	15	0	0%	2	13%
	25%	15	0	0%	2	13%
(H)	15%	15	3	20%	3	20%
	20%	15	0	0%	3	20%
	25%	15	0	0%	2	13%

Graph 3.2 Optimization of sterilization process (with fungicide) with the relation of the rate of contaminated explants



Graph 3.3 Optimization of sterilization process (with fungicide) with the relation of the rate of responsive explants



3.2 Determination of the position of leaf explants and ages of different explants for the suitable regeneration

In the study direct regeneration of shoot and regeneration of shoot from the callus was observed. Different types of explant with different ages were used in the experiment. It was found that 3rd number leaf of the plant (position from the top) was most responsive (Table 3.3) and regarding age of the flower bud and flower stalks 6-7 days were most responsible for the callus induction (Table 3.4), (Table 3.5). All the explants were placed on the MS medium with different hormonal supplements in different concentrations to find the regeneration percentage.

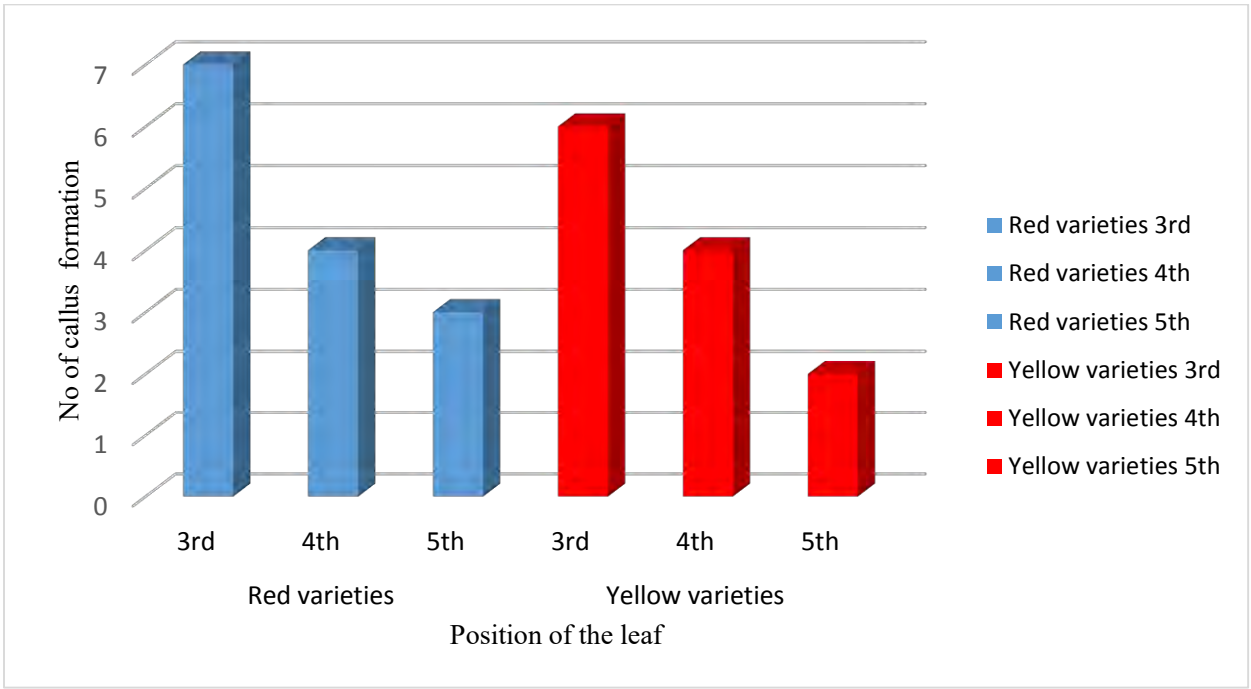
3.2.1 Determination of the position of leaf explants

The age of the leaf was determined by the position of the leaf from the top. During the study 3rd, 4th and 5th no of the leaf from the both varieties were chosen. The 3rd no. of leaf gave the best response (Figure 1). For the red variety and yellow verity of Gerbera, the response rate was 70% and 60 % in respectively observed (Table 3.3).

Table 3.3 Selection of the explants (Leaf segments with mid rib)

Name of the varieties	Position of the leaf explant	No. of the explants	No. of the callus formation	Responsive explants (%)
Red varieties	3rd	10	7	70%
	4th	10	4	40%
	5th	10	3	30%
Yellow varieties	3rd	10	6	60%
	4th	10	4	40%
	5th	10	2	20%

Graph 3.4 Selection of the explants (Leaf segments with mid rib)



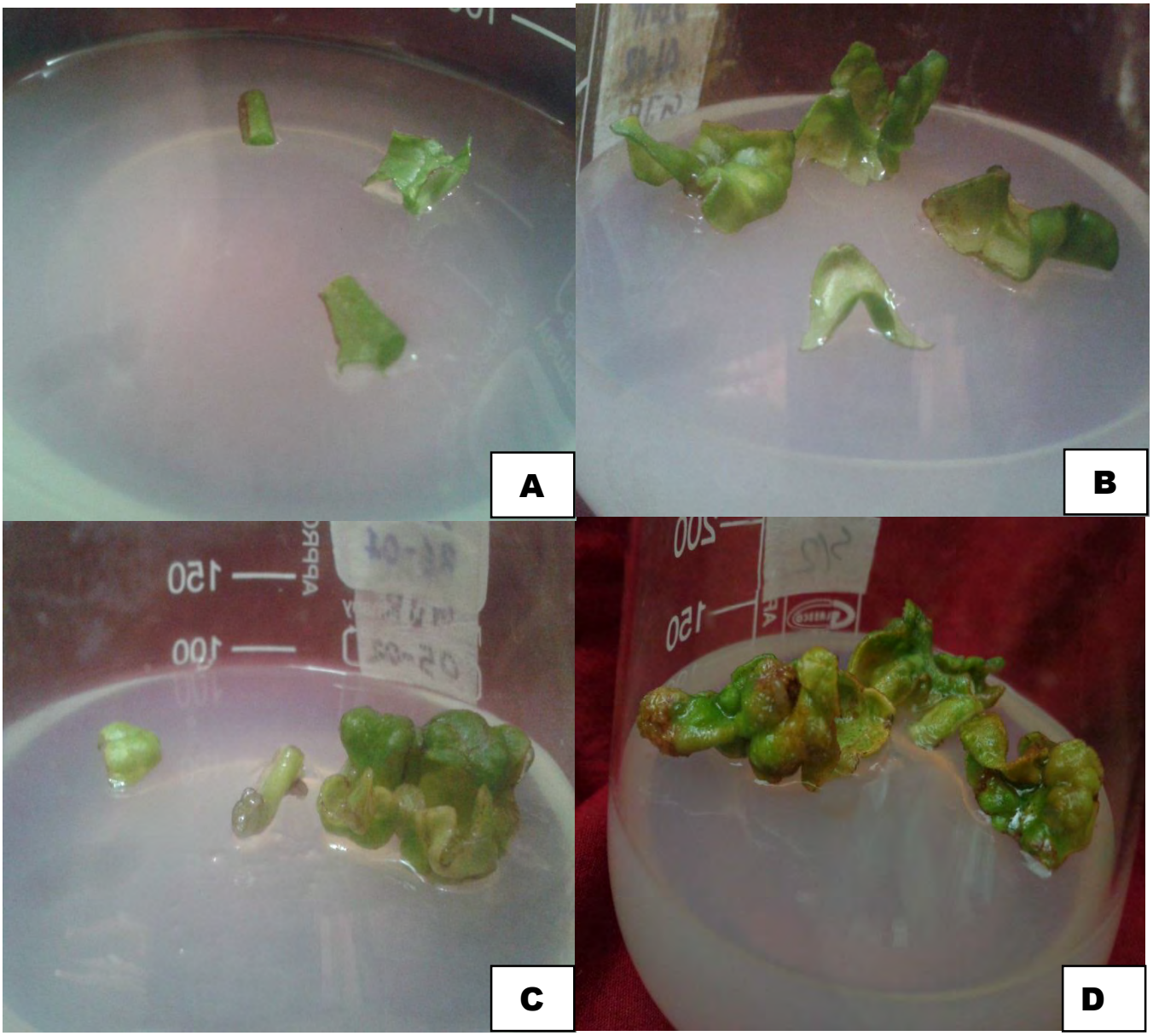


Figure 01 Response of 3rd and 4th position of leaf with mid rib explants to the RM3 medium

A: Response of 4th position of leaf explant of yellow variety after 7 days.

B: Response of 3rd position of leaf explant of yellow variety after 7 days.

C: Response of 4th position of leaf explant of red variety after 7 days.

D: Response of 3rd position of leaf explant of red variety after 7 days.

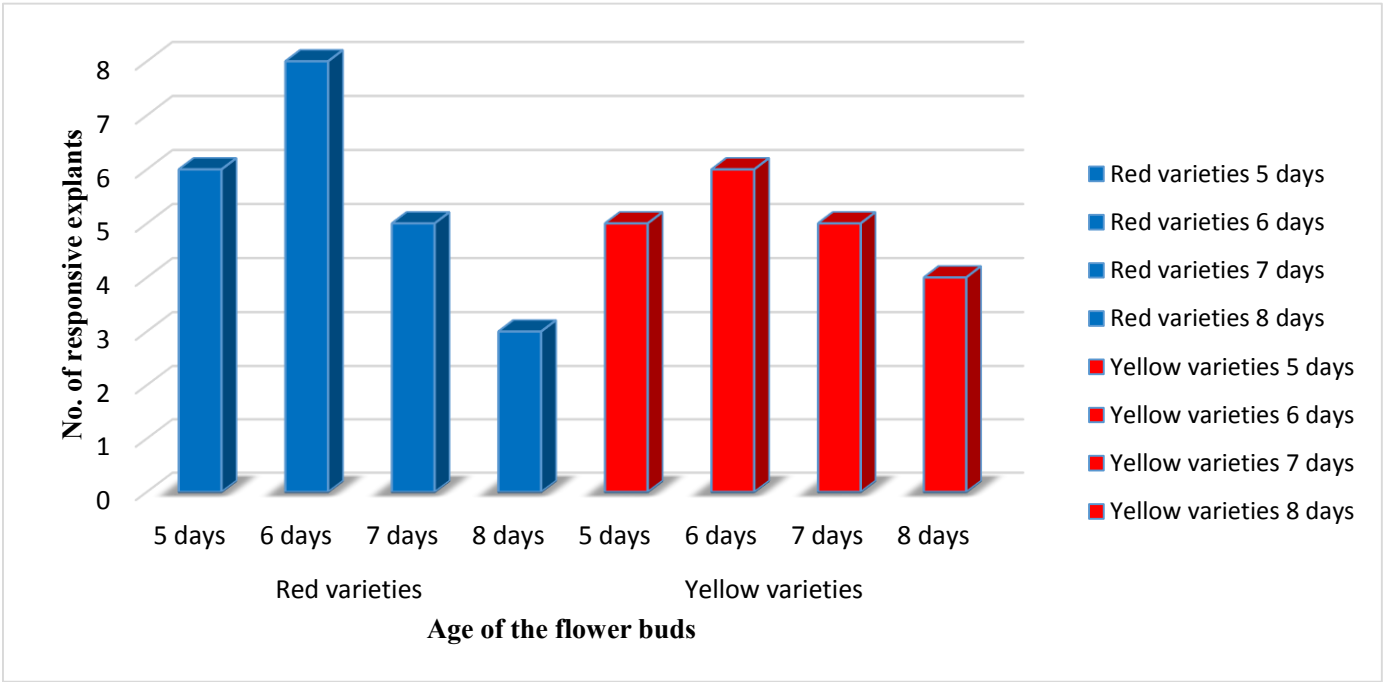
3.2.2 Determination of the age of flower bud explants

In this part of experiment, different ages of the flower bud were taken. It was observed that total size of the flower bud varies from 6 inches to 14 inches with the age of the flower bud (Figure 2). In this study, 4-11 days of flower buds were taken and among them 6-7 days old flower bud gave the best response for the callus regeneration for both varieties (Table 3.4).

Table 3.4 Selection of the explants (Flower buds)

Name of varieties	Age of Flower buds	No. of Explants	No. of Responsive Explants	Responsive Explant (%)
Red varieties	4-5	10	6	60%
	6-7	10	8	80%
	8-9	10	5	50%
	10-11	10	3	30%
Yellow varieties	4-5	10	5	50%
	6-7	10	6	60%
	8-9	10	5	50%
	10-11	10	4	40%

Graph 3.5 Selection of the explants (Flower buds)



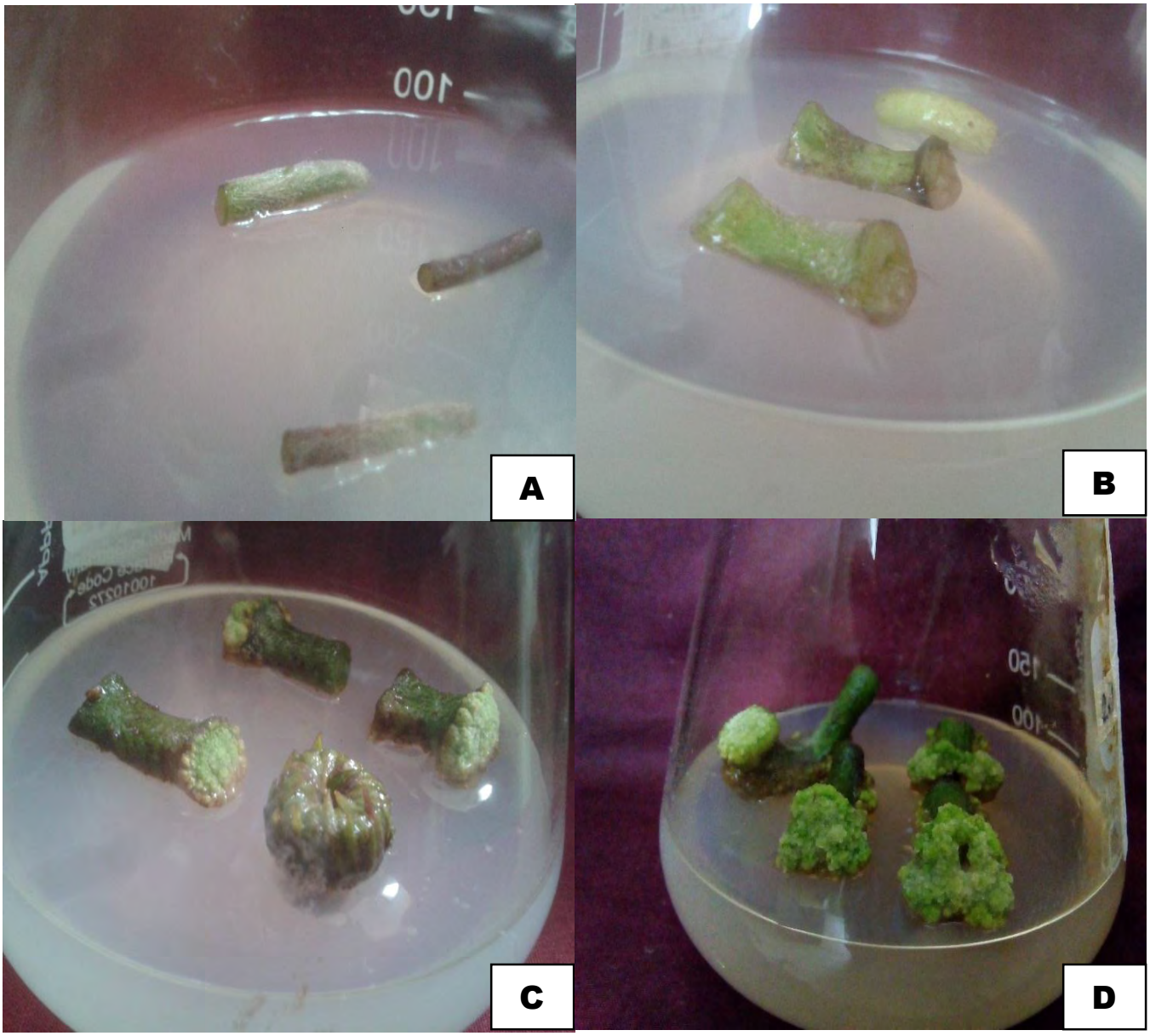


Figure 02 Response of 4-5 days old and 6-7 days old flower bud explants in RM3 medium

A: Response of 4-5 days old flower buds explant of yellow variety after 7 days.

B: Response of 6-7 days old flower buds explant of yellow variety after 7 days.

C: Response of 4-5 days old flower buds explant of red variety after 7 days.

D: Response of 6-7 days old flower buds explant of red variety after 7 days.

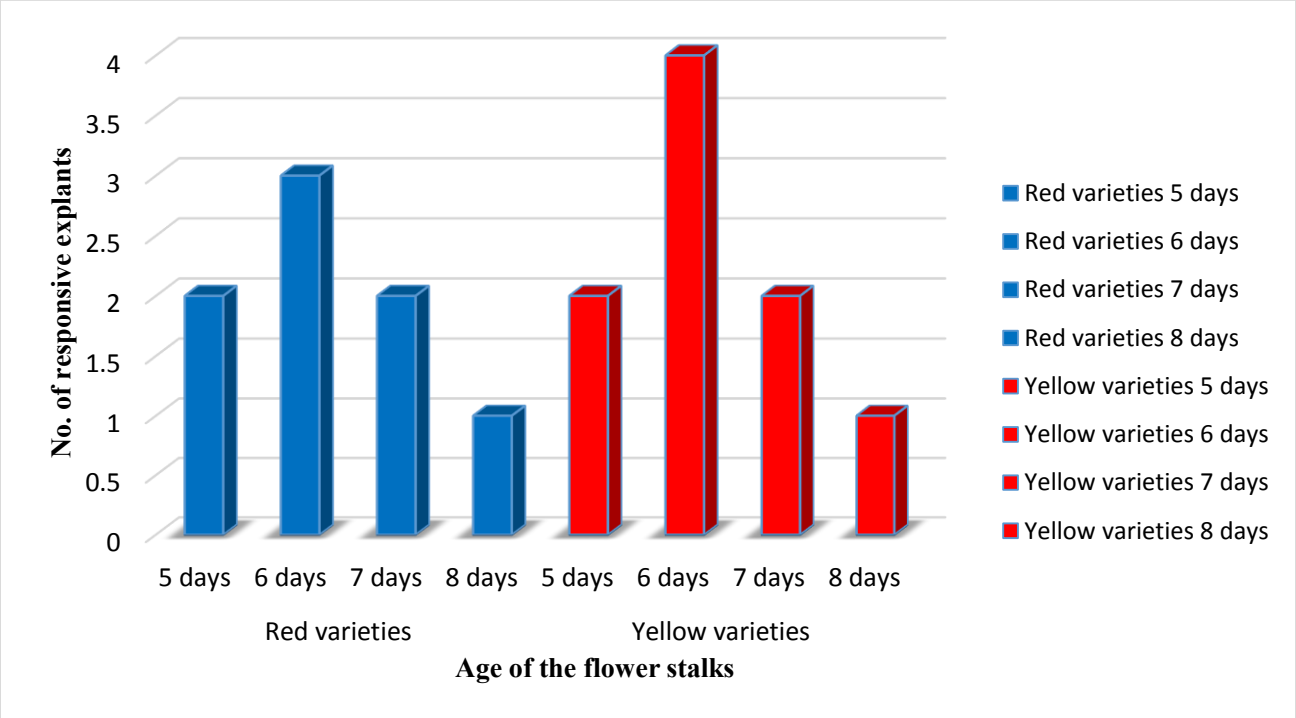
3.2.3 Determination of the age of flower stalks explants

During the study, the capitulam of the flower stalks were taken and the ages of those explants were 4 to 11 days. Among them ages between 6-7 days of flower stalks gave good response for callus regeneration (Figure 3). In red and yellow varieties response rate 38% and 50% was respectively observed (Table 3.5).

Table 3.5 Selection of the explants (Flower stalks)

Name of varieties	Age of Flower buds	No. of Explants	No. of Responsive Explants	Responsive Explant (%)
Red varieties	4-5	10	2	25%
	6-7	10	3	38%
	8-9	10	2	25%
	10-11	10	1	13%
Yellow varieties	4-5	10	2	25%
	6-7	10	4	50%
	8-9	10	2	25%
	10-11	10	1	13%

Graph 3.6 Selection of the explants (Flower stalks)



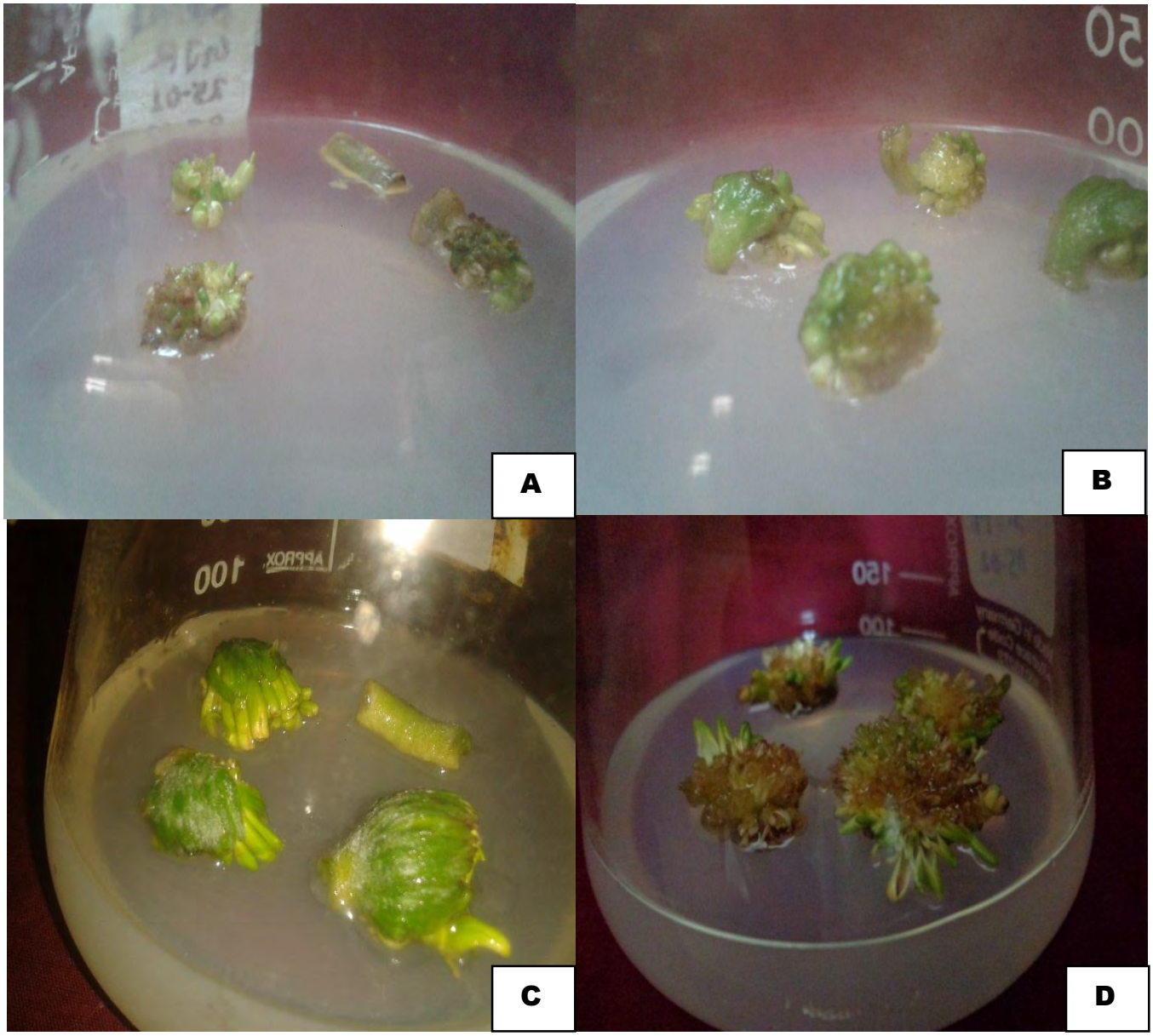


Figure 03 Response of 4-5 days old and 6-7 days old flower stalks explant in RM3 medium

A: Response of 4-5 days old flower stalks explant of yellow variety after 7 days.

B: Response of 6-7 days old flower stalks explant of yellow variety after 7 days.

C: Response of 4-5 days old flower stalks explant of red variety after 7 days.

D: Response of 6-7 days old flower stalks explant of yellow variety after 7 days**.**

3.3 Suitable hormonal medium for explant regeneration

MS medium with the different combination of growth hormones were used to get regeneration response of the explants. The medium contains different concentration of BAP and BAP along with different concentration of Kn and NAA. In this study three different types of explants (leaf segments with mid rib, flower stalks and flower buds) were used. Those explants were culture in MS medium with different hormone composition and concentration to get the best explant for multiple shoot regeneration. In this part of experiment shoot proliferation of different explants of Gerbera was observed.

3.3.1 Effects of different hormonal combination for shoot regeneration from leaf segments with mid rib explants

Leaves are one of the most available explants for the in vitro regeneration throughout the year. For the both varieties (red and yellow) the 3rd number of the leaf gave the best response in MS medium (Table 3.1). The MS contain BAP (2 to 5 mg/l) combined with NAA (0.5 to 1 mg/l) or Kn (0.5 to 1 mg/l) was used to get the regeneration. Those combinations were RM1-2 mg/l BAP + 1 mg/l NAA, RM2- 3 mg/l BAP + 1 mg/l NAA, RM3- 5 mg/l BAP + 1 mg/l NAA RM4- 2 mg/l BAP + 0.5 mg/l NAA RM5- 3 mg/l BAP + 0.5 mg/l NAA, RM6- 5 mg/l BAP + 0.5 mg/l NAA, RM7- 2 mg/l BAP + 0.5 mg/l Kn, RM8- 3 mg/l BAP + 0.5 mg/l Kn, RM9- 3 mg/l BAP + 0.5 mg/l Kn. RM3 gave the callus within 4 weeks and the rate response was 80% on the other side RM9 gave the second best response (75%) within 6 weeks for both (red and yellow) varieties (Table 3.5). It was observed that more than 5 mg/l BAP was the cause for decreasing the response rate. BAP less than 2 mg/l with other Hormonal combination was responsible only for the callus induction no shoot was generated after subculture the callus. All the calluses and explants were subculture in the same hormonal combination for the better growth.

Multiple shoot regeneration was found within 4-5 weeks after callus formation. The highest mean no of shoot per explants were 7.75 which was cultured in the MS medium with RM3. Those explants who were cultured in MS with RM9 gave 5.27 mean number of shoot from per explants (Table 3.6).

Table 3.6 Effects of different hormonal combination for shoot regeneration from leaf segments with mid rib explants

Hormonal combination (mg/l)			No. of explants	Days required for callus induction	No. of responsive explants	Responsive explants (%)	Days to shoot initiation	Mean no. of shoots /Explant
BAP	NAA	Kn						
2	1	-	20	32-37	11	55	30-35	3.19
3	1	-	20	30-35	14	70	27-32	4.5
5	1	-	20	27-32	17	85	22-27	7.75
2	0.5	-	20	-	No Response		-	-
3	0.5	-	20	27-32	6	30	22-27	3
5	0.5	-	20	30-35	11	55	30-35	2.29
2	-	0.5	20	-	No Response	-	-	-
3	-	0.5	20	42-47	12	60	30-35	4.47
5	-	0.5	20	40-45	15	75	27-32	5.27

Name of the hormone composition	**Hormone composition**
RM1	2 mg/l BAP + 1 mg/l NAA
RM2	3 mg/l BAP + 1 mg/l NAA
RM3	5 mg/l BAP + 1 mg/l NAA
RM4	2 mg/l BAP + 0.5 mg/l NAA
RM5	3 mg/l BAP + 0.5 mg/l NAA
RM6	5 mg/l BAP + 0.5 mg/l NAA
RM7	2 mg/l BAP + 0.5 mg/l Kn
RM8	3 mg/l BAP + 0.5 mg/l Kn
RM9	5 mg/l BAP + 0.5 mg/l Kn

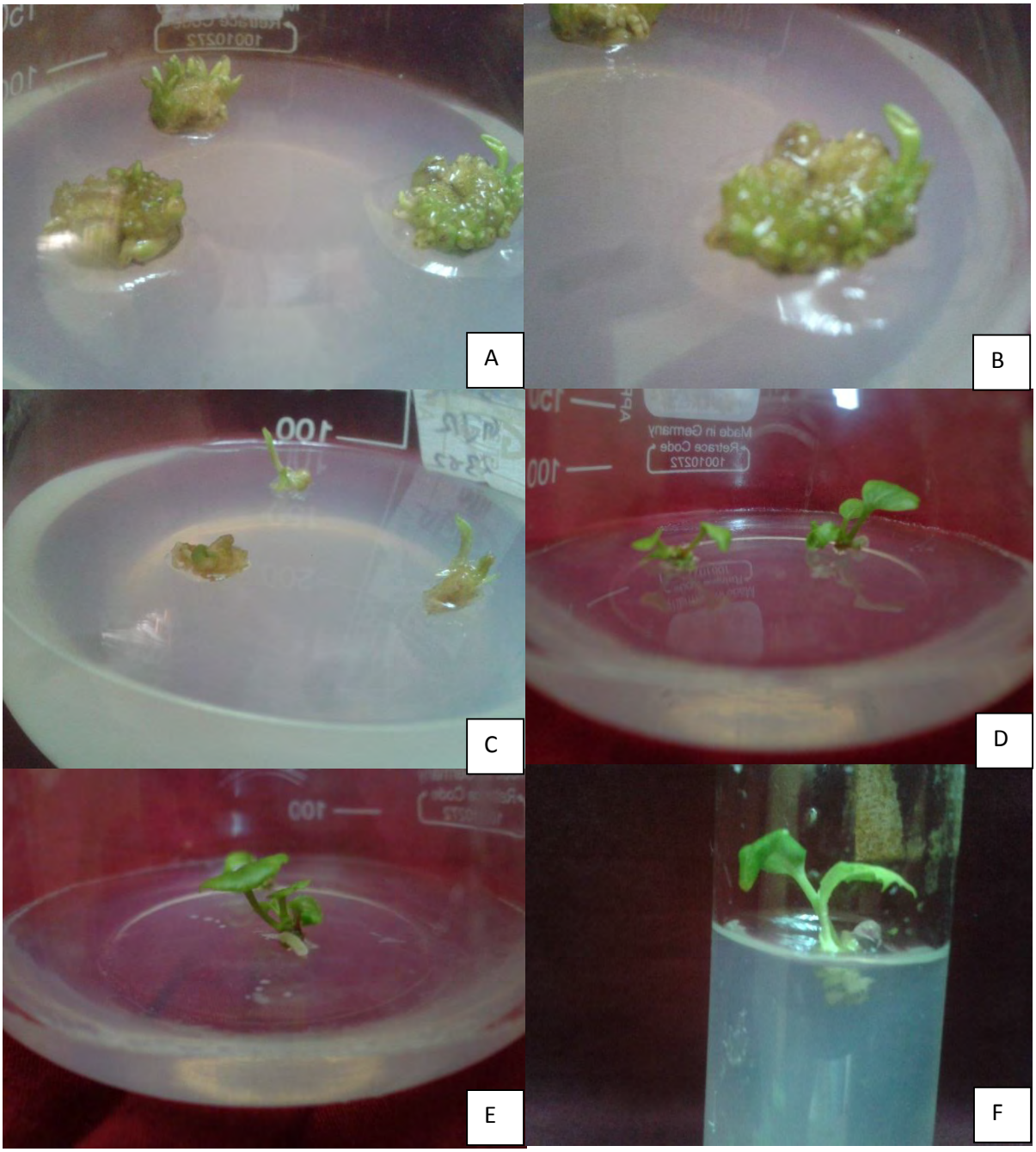


Figure 04 Response of leaf with mid rib in RM3 medium for shoot regeneration

A and B: Shoot initiation from leaf with mid rib after 30-35 days.

C, D and E: Shoot elongation from the leaf with mid rib.

F: Cultured the single shoot to the rooting medium.

3.3.2 Effects of different hormonal combination for shoot regeneration flower buds and flower stalks

Flower buds and flower stalks explants were also responsive towards in vitro regeneration of Gerbera. The rate of responsive explants was lower than the leaf explants in this study. In this experiment it was found that the ages between 6 to 7 days of the flower buds and flower stalks were high responsive for regeneration (table 3.4), (table 3.5). All the explants were inoculated in the MS medium containing BAP (2 to 5 mg/l) along with NAA (0.5 to 1 mg/l) or Kn (0.5 to 1 mg/l) to get the regeneration. The observation showed that the hormonal combination of RM3 gave highest rate of response (60%) for flower bud. The second highest response rate was 30% which was found with MS medium with RM9. The lowest response rate was 25% which was founded for the hormonal combination of RM6. The mean number of the multiple shoot regeneration was 5.13 for the hormonal combination of RM3. The mean number of shoot regenerate from the flower bud in the hormonal combination of RM2 was 4.17which was second best result getting from the flower buds (Table 3.6).

Table 3.6 Effects of different hormonal combination for shoot regeneration flower buds

Hormonal combination (mg/l)			No. of explants	Days required for callus induction	No. of responsive explants	Responsive explants (%)	Days to shoot initiation	Mean no. of shoots /Explant
BAP	NAA	Kn						
2	1	-	20	-	No Response	-	-	-
3	1	-	20	30-35	6	30	28-33	4.17
5	1	-	20	30-35	12	60	25-30	5.13
2	0.5	-	20	-	No Response	-	-	-
3	0.5	-	20	-	No Response	-	-	-
5	0.5	-	20	30-35	5	25	32-37	3.19
2	-	0.5	20	-	No Response	-	-	-
3	-	0.5	20	-	No Response	-	-	-
5	-	0.5	20	-	No Response	-	-	-

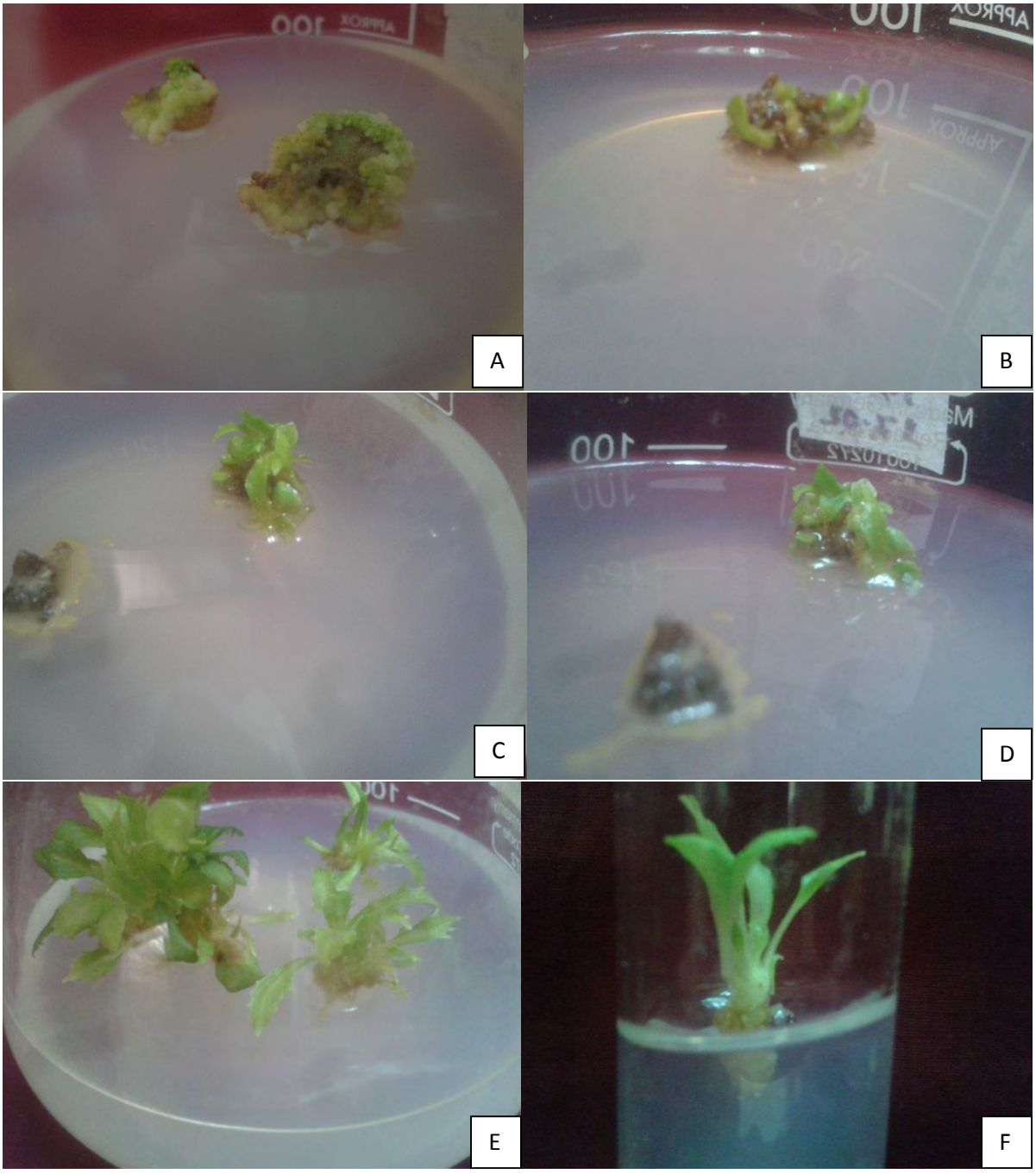


Figure 05 Response of Flower buds in the RM3 medium for shoot regeneration

A: Callus cultured to the RM3 medium after 15-20 days. B, C and D: Shoot initiation from the callus after 30-35 days. E: Multiple shoot regeneration from the callus after 50days. F: Single shoot cultured to the rooting medium**.**

3.3.2 Effects of different hormonal combination for shoot regeneration flower buds and flower stalks

For the flower stalks explants RM3 gave the highest response which was 40%. Other explants in different medium gave only the callus and after sub cultured no shoot regeneration was observed. In this study there was only one successful regeneration from the flower stalks. All the explants were taking under observation for 6 to 7 weeks. Only the explants cultured in the MS hormone combination with RM3 gave shoot regeneration. But the mean no of shoot regenerated from per explant was very low then the flower buds and leaf explants. In average only 3 shoots were observed from per explant in this experiment (Table 3.7).

Table 3.7 Effects of different hormonal combination for shoot regeneration from flower stalks

Hormonal combination (mg/l)			No. of explants	Days required for callus induction	No. of responsive explants	Responsive explants (%)	Days to shoot initiation	Mean no. of shoots /Explants
BAP	NAA	Kn						
2	1	-	10	-	No Response	-	-	-
3	1	-	10	-	No Response	-	-	-
5	1	-	10	25-30	4	40	28-33	3
2	0.5	-	10	-	No Response	-	-	-
3	0.5	-	10	-	No Response	-	-	-
5	0.5	-	10	-	No Response	-	-	-
2	-	0.5	10	-	No Response	-	-	-
3	-	0.5	10	-	No Response	-	-	-
5	-	0.5	10	-	No Response	-	-	-

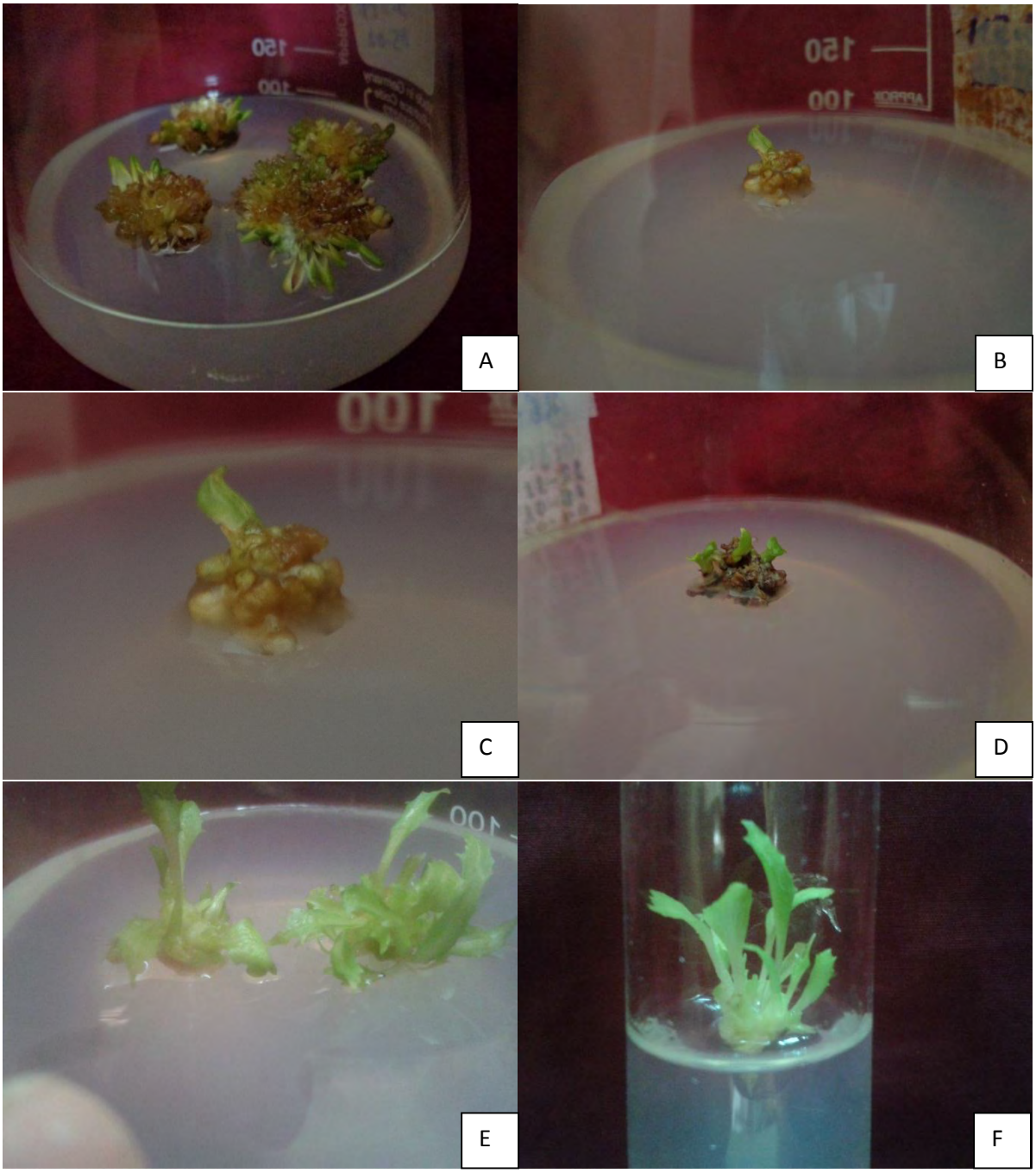


Figure: 06 Response of Flower stalks in the RM3 medium for shoot regeneration

A: Callus cultured to the RM3 medium after 10-15 days. B and C: Shoot initiation from the callus after 30-33 days. D: multiple shoot initiation from the callus after 40 days. E: Shoot elongation to the RM3 medium. F: Shoots cultured to the rooting medium

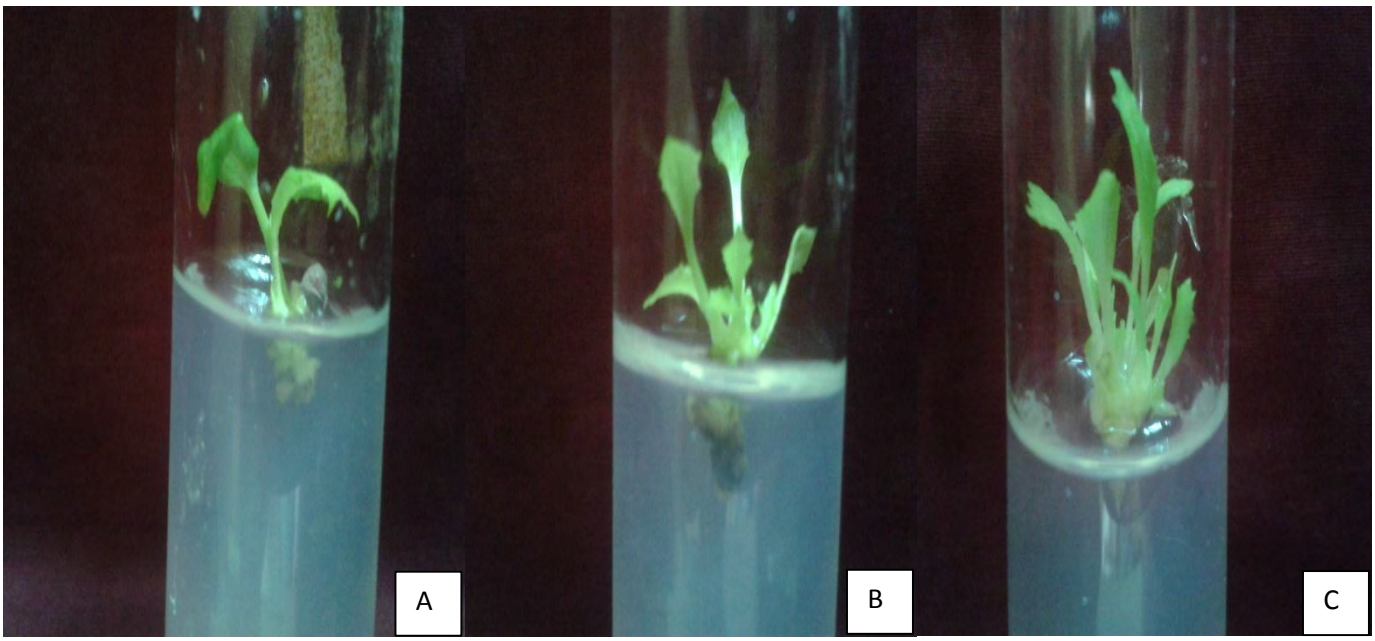


Figure 07 Shoot initiation from different explant of Gerbera

A: Development of shoot from leaf with mid rib explants.

B: Development of shoot from flower bud explants.

C: Development of shoot from flower stalks explants.

Discussion

The main purposes of this experiment was to establish a reliable protocol for *in vitro* regeneration of *Gerbera jamesonii.* In this experiment two varieties of gerbera having red and yellow petal had been taken. The whole experiment was divided into three phases, these were establishment of a consistent sterilization protocol for dry season and also for wet season selection of suitable explant and medium for the regeneration, and establishment of an *in vitro* plant regeneration protocol.

In the present study all the explants used for this experiment were collected from BRAC university garden. For those explants, the sterilization process was very important as they were susceptible to many biotic factor. So different chemical like, HgCl2, NaOCl, ethanol etc. were used for the sterilization process.

In the present investigation experiments were conducted to avoid long exposure of the explants with HgCl2, NaOCl and ethanol. For optimizing surface sterilization process different types of pretreating agents which reduced the contamination and also maintained the regeneration ability was tried. For the wet season (July - September) the rate of fungal contamination was high in the air for the moisture. To avoid the contamination rate in that season all the explants were washed through the fungicide named Bavistin with different concentration. Normally the sterilization of explants with 0.2% of HgCl2 and 70% of ethanol gave the least amount of contamination with the high number of responsive rate but if fungicide with 15%, 20% and 25% concentration were used with those sterilization process, the rate of response was fall from 40% to 13%.

During July to September fungicide were effective to reduce the rate of contamination for explant. Used of high concentration of fungicide reduce the response rate up to 13% on the other side less concentration of fungicide increased the contamination rate up to 27%. Evaluated all the result it was found that using 20% of fungicide with 0.1% of HgCl2 and 70% of ethanol gave high responsive rate with low rate of contamination.

Selection of suitable explant for *in vitro* regeneration of *Gerbera jamesonii* was the second phase of this experiment. Different types of explants like leaf, flower buds, flower stalks, flower petals, seeds, stem tips, floral buds, capitulum etc. were used for the *in vitro* regeneration of Gerbera. There was some previous report that using of different explant for *in vitro* regeneration of Gerbera. Researchers used of capitulum (Pierik *et al.,* 1973, 1975), leaves (Hedtrich, 1979) and (Barbosa *et al.,* 1994),

floral buds (Posada *et al.,* 1999), floral bracts (Maia *et al*., 1983), torus (Zhang, 2002) and inflorescence (Schum and Busold, 1985) for the regeneration of Gerbera.

In this experiment, various explants like leaf with mid ribs, flower stalks and flower bud were used for regeneration purposes. Among those explants leaf with mid ribs showed the direct shoot proliferation. On the other side flower buds give tremendous response for indirect regeneration of Gerbera. The rate of shoot proliferation from the flower stalks were not satisfactory on this experiment.

The position of the leaf is an important factor in shoot proliferation. There are different parts of the leaf, like leaf with midrib, leaf blade, petiole, joining point of leaf and petiole etc. in this experiment leaf with mid rib were used for the regeneration. About 1-3 cm long segments of the above explants were cultured to the medium for direct regeneration. For both varieties it was found that the 3rd no. of leaf form the top gave a higher number of response rate for red varieties it was 70% and for yellow varieties it was 60%. The response rate falls from 70% to 40% for red varieties and for yellow varieties it was from 60% to 40% in the 4th no of the leaf. In previous study leaf as an explant, were not give that much satisfactory result.

The age of the flower bud was also an important factor in shoot regeneration. Different ages of explants from 4 days to 11 days were taken in this experiment. Ages between 6 to 7 days of flower bud gave the response rate 80% for red varieties and 60% response rate was found for yellow varieties in that experiment. Older flower bud ages between 6 to 7 days gave lower response for red and yellow varieties. The response rate falls up to 30% when ages were 10 to 11 days. In respect of shoot proliferation, there are some previous reports on using flower bud as an explants for shoot regeneration in gerbera (Pierik *et al.,2005*), (Kumar & Kanwar, 2006,2007) and (Nhut *et al.,* 2007) was found.

In case of flower stalks the result for shoot proliferation was not satisfactory in this experiment. 6 to 11 days old of flower stalks were taken for this experiment. Mainly the capitullam of flower stalks was used for the regeneration but those part were covered with petal so it was difficult to collect the pure explant. The highest rate of response found up to 50% for the yellow varieties when the ages of explants were 6 to 7 days. At the same age the response was 38% for the red varieties.

The development of an efficient and reproductive *in vitro* regeneration protocol depends on several factors including culture condition and regeneration medium with proper hormonal supplements. Previous study showed that different medium with different medium supplements were used to successful *in vitro* regeneration of Gerbera. Researcher used MS medium for shoot regeneration (Pierik *et al.,* 1982; Le *et al*., 1999; Modh *et al.,* 2002; Aswath and Wazneen, 2004; Kumar and Kanwar, 2005, 2006). (Parthasarathy *et al*., 1996; and Verma and Anand, 2006) N6 medium used for culture establishment in Gerbera by (Chu, 1978) and B5 by (Gamborg *et al.,* 1968). (Mandal and Datta, 2002) used LS (Linsmaier and Skoog, 1965) medium for establishing organogenic callus cultures from immature flower buds. (Chen *et al.* 2006) used DKW (Driver and Kuniyuki, 1984) medium for tissue culture studies of Gerbera stem nodes with buds. (Kanwar and Kumar, 2008).

In this experiment all the explant (Leaf with midrib) was cultured in MS medium supplement with (1-6) mg/l of BAP with (0.5-1) mg/l of NAA or (0.5-1) mg/l of Kn. All the callus in MS medium supplement with 5mg/l of BAP and 1mg/l of NAA gave the shoot initiation within 32 to 37 days. The induced callus was sub cultured on the same medium composition for shoot regeneration. The induced callus on sub cultured medium increased their volume. Some report was found about *in vitro* shoot regeneration from different parts of leaf tissue such as, shoot tip and petiole (Hunang and Chu, 1985) and (Orlikowska *et al, 1999*). During this investigation, it was found that callus induction and shoot proliferation responses were significantly influenced by the concentration and combination of cytokinin and auxin. It was observed that when MS medium was supplemented with only BAP then no shoot proliferation was observed. However, proliferation was observed in all varieties of Gerbera when MS medium was supplemented with BAP and NAA or Kn. Combination of auxin and cytokinin induces the formation of adventitious shoots. (Hasbullah *et al*, 2008) reported that addition of auxins together with cytokinins becomes essential for shot induction for Gerbera. When BAP was supplemented with low concentration of NAA or Kn callus was induced but no shoot was proliferated. But when BAP was supplemented with high concentration of auxin like NAA shoot was proliferated. (Pierik *et al,* 1973) also reported that addition of strong auxin NAA with BAP promoted better shoot formation compare to low concentration of NAA.

For callus induction and shoot proliferation, flower bud explant were cultured on MS medium supplement with 5 mg/l BAP and 1mg/l NAA showed best result in respect of higher number of multiple shoots in all varieties of Gerbera. Initiation of callus was found to occur within three to four weeks of inoculation. The percentage of responsive explants in respect of shoot regeneration was around 60% and mean no of shoot was 5.13 in all varieties.

In case of flower stalks explants, very lower number of shoots in all the varieties of Gerbera was observed on MS medium with different supplements. The percentage of responsive explants in respect of shoot proliferation was 40% and it was only found for the MS medium with the supplements with 5 mg//l BAP with 1mg/l NAA. And the mean no of shoot found was 3. For other supplement no result was found.

It was observed that when the regenerated shoots were cultured for longer duration on 5mg/l BAP with 1mg/l NAA supplemented MS medium for shoot multiplication then the shoot were become stunted in growth and vitrified. To overcome these problems shoots were cultured on MS medium containing only BAP (Vardja *et al,* 2001). Similar result was also found by (Chakrabarty & Datta, 2008) for shoot proliferation.

Conclusion:

In the perspective of Bangladesh *in vitro* regeneration of Gerbera is important in recent era because of the demand in the local and international market. The climate and condition is very suitable for Gerbera cultivation in Bangladesh. So in commercially reproducible *in vitro* regeneration protocol is very much essential for large scale plantlet production of Gerbera. This research also opens the windows for further new research like, root induction from *in vitro* regenerated shoot, transformed plantlet production, Gene mutation etc.

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