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In vitro regeneration studies in gerbera (*Gerbera jamesonii* Bolus)

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Abstract
An efficient protocol was developed on *in vitro* regeneration and acclimatization of gerbera (*Gerbera jamesonii* Bolus). Seeds were used as the primary explants for *in vitro* establishment. Early shoot regeneration from *in vitro* shoots was noticed in MS medium supplemented with 4 mg l^{-1} BAP + 0.3 mg l^{-1} IAA within 12.13 days with maximum number of shoots per clump (4.80), leaves per shoot (10.27) and shoot length (3.85 cm) at six weeks of inoculation. Root initiation was early (10.87 days) when shoots cultured on MS medium containing IBA at 2 mg l^{-1} that also produced highest number of roots per shoot (4.73) and maximum root length (3.47 cm). In acclimatization process highest survival rate was achieved with coco peat alone compared to other media composition. Further tissue cultured plants were subjected to ELISA to confirm that the plants were free from cucumber mosaic virus (CMV).

Keywords: *Gerbera jamesonii* Bolus, 6 benzyl amino purine (BAP), Indole-3- acetic acid (IAA), α-Naphthalene acetic acid (NAA), Indole-3-butyaric acid (IBA), acclimatization

1. Introduction
Gerbera (*Gerbera jamesonii* Bolus) commonly known as Transvaal daisy, Barberton daisy, African daisy, is an herbaceous, perennial plant of the family Asteraceae. It is native to South Africa which is used as cut flower in many countries; also it has gained popularity as an ornamental flower and is in great demand in the floral industry both in the domestic and the international markets. It ranks fifth among the cut flowers after roses, chrysanthemums, tulips and lilium, according Dutch flowers auctions (Raj, 2013) [18] with a value of 131 million Euros. Gerbera contributes with a production of 17,840 MT and stands fourth important cut flower in India. The total area of gerbera in India is 820 ha with a cut flower production of 17, 840 MT and loose flower of 3,960 MT. However, maximum production of gerbera comes from Uttarakhand (7, 200 MT) while, maximum area coverage is from Assam (600 ha). The share of gerbera cut flower production in Karnataka is 200 MT and loose flower production is 580 MT (Anon., 2015) [1].

Planting material of ornamental plants is in great demand for commercial production as well as for domestic gardens and landscaping. Their great ornamental value is due to the typical capitulum inflorescence that displays a great variety of colors, and to the floral stem, which highly valued by consumers as individual vase decorations and bouquet compositions (Mata *et al*., 2009) [12]. The better quality planting material is a basic need of growers for boosting productivity. Gerbera is conventionally propagated by both sexual and asexual methods. Vegetative propagation is commonly practiced through division of clumps, but multiplication rate is very slow (Bose and Yadav 1993) [6]. Plant tissue culture is being widely accepted for its potential in mass multiplication and preservation of elite plants. The most important aspect of plant tissue culture technique is the ability of cultured cells and tissues to regenerate in to complete plants which are being used commercially on a large scale in the ornamental flower industry. Chebet *et al*. (2003) [7] reported the use of biotechnological approaches to improve horticultural crop production. Micropropagation has been recognized as the most reliable, cost and labour effective method for large scale clonal propagation of elite cultivars, leading to systematic development of the floriculture industry. Cut flower trade is increasing exponentially across all the continents and the availability of micropropagated, clonal planting material in sufficient numbers has helped commercial growers to cultivate many commercial varieties for the production of cut flowers. Many new varieties are being introduced every year. To popularise these varieties and also to meet the demand for quality planting material of

elite varieties, there is a need to standardise the technology for rapid multiplication. This could be accomplished through plant tissue culture or *in vitro* techniques. *In vitro* technique is a world-wide applied tool for propagation in the horticultural industry and can be helpful to solve such problems. Standardisation of protocol for *in vitro* propagation depends on several factors and it changes from lab to lab. Therefore, there is an urgent need to standardize the protocol for large scale multiplication of elite planting material. Hence, the present study was conducted to standardize and develop an efficient *in vitro* regeneration protocol for gerbera.

2. Material and methods

The present investigation was carried out at the Plant Tissue Culture laboratory in the Department of Biotechnology, College of Agriculture, Vijayapur, University of Agricultural Sciences, Dharwad during the year 2017-2018. Seeds of *Gerbera jamesonii* Bolus used as a source of explants.

2.1 Glassware

All glassware used during the course of experiment like culture bottles, culture tubes, beakers, pipettes and funnels were procured from M/s. Borosil India Limited, Mumbai.

2.2 Tools and equipments

The chemicals used to prepare MS medium was weighed individually using a digital electronic balance (Shimadzu). Elico magnetic stirrer with hot plate was used to adjust the pH of medium and to melt the agar during preparation of medium. Throughout the investigation double distilled water was used and it was collected from quartz double distillation unit. Media bottles and tools like forceps, scalpels *etc.*, were sterilised by using table-top vertical autoclave. All the aseptic manipulations for micropropagation studies were carried out in double chambered laminar air flow.

2.3 Plant material, seed germination and initiation of culture

Seeds of gerbera which are flat and pointed were taken in a beaker containing water and only those seeds sunk in water were used for regeneration. Seeds were washed thoroughly under running tap water for five minutes and then washed in sterile water in laminar-air-flowhood for five minutes and treated with tween 20 for 10 minutes. Then the seeds were washed thoroughly in double distilled water and treated with bavistin (0.1 %) for fifteen minutes and washed again with double distilled water to remove traces of bavistin. Seeds were treated with 2 % sodium hypochlorite for 20 minutes and rinsed twice with double distilled water then treated with 70 % alcohol for 2 minutes followed by 0.1 % mercuric chloride for 2 minutes. The seeds were dried with sterile filter paper and inoculated to the fresh media. The whole process of surface sterilization was performed in a laminar air flow chamber. In the present study, Murashige and Skoog (MS) (1962) [13] basal medium (consisting of salts, vitamins and 3% sucrose) was used after solidifying with 0.8% (w/v) agar. Different plant growth regulators (PGRs) like α- naphthalene acetic acid (NAA), 6 benzyl amino purine (BAP), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA) were added at various concentrations to MS medium and the pH of the medium was adjusted to 5.6- 5.8. Bottles containing medium were autoclaved at 121°C for 15 min with 1.5 $kgcm^{-2}$ pressure. Culture bottles were maintained under artificial conditions at 25 ± 2°C with 60% RH and 16 hours photoperiod (using white fluorescent tubes).

2.4 Shoot initiation.

For shoot initiation, MS medium with 21 different combinations of BAP and IAA were used, along with control (MS medium without adding growth hormone) (Table 1). The trimmed shoot tips were inoculated into the media, dipping approximately up to 0.5 cm for good contact and anchorage. The best combination of growth hormones were identified in terms of its performance over number of days taken for shoot initiation and number of shoots per clump, and number of leaves per shoot and shoot length observed. Data was recorded six weeks after inoculation.

2.5 *In vitro* rooting of shoots

The *in vitro* grown shoots were further transferred to rooting medium for root induction. MS basal medium was supplemented with eight different concentrations of NAA and IBA in which MS medium without any growth hormone was considered as a control (Table 2). The *in vitro* shoots were separated and cultured on fresh rooting medium. The observations were made on number of days taken for root induction, number of roots per shoot and length of roots.

2.6 Hardening off *in vitro* plantlets

Acclimatization procedure was established to maximize the survival rate in an efficient way. The well-rooted healthy plants were taken out of the medium, separated individually and washed carefully under running tap water to remove all traces of medium from the roots and the plantlets were treated with 0.1 percent bavistin for two minutes. In the first phase of acclimatization, the plants were transferred into polypropylene cups containing coco-peat (Figure 4). Polypropylene containers were placed in the growth chamber under 16/8 hours of light/dark. The light intensity was maintained at 3500 lux. For polypropylene cups small holes were made in to the bottom of the upper inverted glass after 12 days of transfer. The holes were made larger after signs of establishment of the plant. Later on, the upper inverted cup was permanently removed when plantlets became acclimatised, a stage indicated by absence of signs of wilting. After 20 days, acclimatised plants were transferred to smaller plastic pots filled with different hardening media and covered with polythene sheet (Figure 5). Four different potting media were studied for hardening off micropropagated plantlets of gerbera. Soil + sand + FYM (2:1:1 v/v), soil + sand + vermicompost (2:1:1 v/v), soil + sand + coco peat (2:1:1 v/v) and coco peat alone were used. Plants were observed for one month and survival rate in each potting medium was recorded.

2.7 Virus indexing

The tissue cultured plants were tested at Plant Virology Laboratory, Department of Plant Pathology, College of Agriculture, GKVK, University of Agricultural Sciences, Bengaluru. The samples of hardened gerbera plantlets were subjected to Enzyme Linked Immuno-Sorbent Assay (ELISA) to ascertain the plantlets free from virus, particularly cucumber mosaic virus (CMV). ELISA procedure followed in the present investigation is given below:

1. Crude extract was prepared in coating buffer-using dilution of 1:10 (1 gm of leaf sample/9 ml of buffer). Extract was dispensed into each well of microlitre plate at the rate of 200 µl per well using a micro pipette and the plates were incubated at 37°C for 2-2.5 hr.
2. The contents of the plates were poured off and rinsed in PBS-Tween. This was followed by washing the plates in

three changes of PBS-Tween buffer allowing 3 min. for each wash.
3. Addition of antibody diluted in conjugate buffer to a concentration of 1:1000 dilution at the rate of 200 µl per well. Then the plates were incubated at 37°C for 2 to 2.5 hr and were washed in PBS – Tween as in step -2.
4. Alkaline phosphatase labelled antirabbit IgG diluted in antibody buffer to a concentration of 1:8000 dilutions. The plates were incubated at 37^0C for 2hrs and were washed in PBS-Tween as in step-2.
5. Substrate buffer containing 0.5-1mg/ml of PNPP was then dispensed to each well @200 µl/well and incubated at room temperature for 10-30 minutes. Light orange to yellow color, development indicated a weak to strong positive reaction.

2.6 Experimental design and statistical analysis
After inoculation, data on shoot proliferation was recorded six weeks after inoculation. In case of root induction data was recorded after four weeks of inoculation. The survival percentage was calculated from the number of plants survived out of total number of plants potted. Observations on cultures were carried out every day. The experiment was based on completely randomized design with three replications. Each treatment was consisting of 20 explants. The data collected from the investigation was subjected to statistical analysis by adopting the completely randomised design as described by Panse and Sukhatme (1986) [15].

3. Results
The findings of the present study have been presented in the following sub heads:

3.1 Influence of hormones (BAP and IAA) on shoot regeneration at 6 weeks of inoculation
The effect of different concentration of BAP and IAA were tested on initiation of *in vitro* culture. Of the various hormonal combinations tried for culture establishment, it was observed that the optimum concentration of BAP and IAA for culture initiation of *in vitro* shoots was MS + BAP 4.0 mg l^{-1}+ IAA 0.3 mg l^{-1}, which gave early shoot initiation (12.13 days). The number of shoots regenerated varied from 1.87 to 4.80. MS medium supplemented with BAP 4.0 mg l^{-1}+ IAA 0.3 mg l^{-1} proved to be the best combination for shooting as it produced maximum number of shoots per clump (4.80), maximum number of leaves per shoot (10.27) (Figure 1) and larger shoot length of 3.85 cm(Figure 2) at six weeks of inoculation (Table 1).

3.2 Influence of hormones (NAA and IBA) on root regeneration
After four weeks of inoculation on to the MS medium supplemented with NAA or IBA, the observations on number of days taken for root initiation, number of roots per shoots and root length were recorded. Although, root formation was obtained in all concentrations of NAA or IBA, addition of IBA (2 mg l^{-1}) significantly increased the root numbers. The data given in the table 2 revealed that MS medium containing 2 mg per liter IBA rooted early (10.87), produced maximum number of roots per shoot (4.73) and maximum root length (3.47 cm) (Figure 3) while, shortest root length was observed with the control (0.95 cm).

3.3 Standardization of media for hardening off
Acclimatization is very necessary before planting them in to field condition because it may face extreme change in their environmental condition as they were grown in laboratory condition. Therefore, *in vitro* regenerated plantlets were tested for suitable carrier substrates viz., sand + soil + FYM (2:1:1), sand + soil + vermicompost (2:1:1), sand + soil + coco peat (2:1:1) and coco peat (100 %) alone. The data presented in Table 4 clearly reveled that, cent percent survival rate was noticed with the treatment containing 100 percent coco peat whereas, in potting media comprising soil + sand + FYM and soil + sand + vermicompost (2:1:1 v/v) only 40 percent survival was recorded, after four weeks of transfer to hardening medium (Table 3).

3.4 Virus indexing
Gerbera plantlets after hardening off subjected to Enzyme linked immune sorbent assay (ELISA) test, to ascertain the plantlets raised through tissue culture are free from viruses, particularly cucumber mosaic virus (CMV). It is evident from the ELISA, the *in vitro* gerbera plants were free from CMV contamination as there was no colour development with the samples placed in the wells (Figure 6).

4. Discussion
Growth and morphogenesis of *in vitro* plants are regulated by the interaction and a balance between the growth regulators supplied in the medium and the growth substances produced endogenously by the cultured cells (George and Sherrington, 1984) [9].

4.1 Shoot proliferation
In gerbera cytokinins are the most crucial factors affecting the shoot proliferation. Different combinations of cytokinins and auxins have been examined to achieve shoot induction in gerbera. In the present study, among the different concentrations of BAP alone and BAP in combination with IAA, the MS medium supplemented with 4 mg l^{-1} BA + 0.3 mg l^{-1} IAA showed significantly superior with respect to number of shoots per clump, number of leaves and shoot length compared to other combinations. The observations in parallel to this outcome were made by Nazari *et al*. (2016) [14], where highest number of shoots per explants was observed in the MS medium containing 0.1 mg per litre IAA + 4 mg per litre BA + 1 mg per litre TDZ. Similarly, Aswath and Survey (2004) [2] obtained more number of shoots with 3.0 mg per litre BAP and 0.5 mg per litre IAA concentrations in gerbera selection GJ-23. Shailaja *et al*. (2004) [19] as well, stated BAP (3 mg/l) was the best growth hormone for obtaining maximum shoots per explants in gerbera. Various combinations of auxins and cytokinins have also been tried to achieve multiple shoot induction in gerbera (Barbosa *et al*., 1993) [5]. Among the cytokinins, BAP was found to be a better cytokinin for shoot proliferation in gerbera (Hempel *et al*., 1985) [11]. BAP increases the cytokinin biosynthesis by coding those enzymes which are involved in the conversion of dimethylallyl diphosphate (DMAPP) and adenosine-mono-phosphate (AMP) to iso-pentenyle adenosine-5-monophosphate (iPMP) through the action of ipt genes, thus causing a marked increase in shoot length (Haberer and Kieber, 2002) [10]. The presence of IAA slightly reduced the incidence of hyperhydricity maintaining a high number of developing shoots (Aswath and Survey, 2004) [2].
The results obtained in the present investigation are contrary to the results of Pierik *et al*. (1975) [16] 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^{-1}. Based on the results of this study, it

can be concluded that BA stimulates shoot proliferation in gerbera, but at the same time, especially at higher concentrations, it reduces the growth and multiplication of plantlets. Consequently, it may be suggested that for obtaining better growth and development of plantlets lower concentration of BAP (4 mg l^{-1}) should be used (Table 1). Sharma and Mohan (2006) [20] indicated that using of BAP in high concentrations on the MS medium caused the vitrification phenomenon in *Chlorophytum borivilianum.*

4.2 Root regeneration

The rooting of gerbera is easy and possible in MS media without plant growth regulators (PGRs). There are numerous reports about using different kinds of auxins such as IBA, NAA and IAA for obtaining rapid rooting, best rooting percentage, and the highest number of adventitious roots per shoot (Cardoso and Silva, 2013) [7]. In this study all the explants which were inoculated to the fresh rooting medium showed rooting even in MS medium alone (control). The best rooting was observed in MS medium containing 2 mg per litre IBA. These findings were supported by Aswath and Choudhary (2001) [3] with the similar outcome when cultured gerbera on MS medium containing 1.5 mg per litre IBA.

4.3 Hardening off gerbera

Highest survival rate was observed in coco peat alone. Aswath and Choudhary (2002) [4] obtained almost 100 percent survival rate when plantlets after root development were transferred to plastic pots filled with coco peat, red soil and sand in a 3:1:1 ratio.

4.4 Virus indexing

There was no colour development in the wells where hardened gerbera plant samples are used. This might be due to use of seed as explants to generate plantlets that will not carry any virus. Further, *in vitro* shoots were used for the entire investigation and it is well established fact that generally, *in vitro* plantlets will be free from viruses.

Pradhan *et al*. (2016) [17] reported that orchid tissue cultured plants were 100 percent free from cucumber mosaic virus, when subjected to ELISA.

Table 1: Influence of hormones (BAP and IAA) on shoot regeneration at 6 weeks of inoculation

Treatments BAP + IAA (mg l^{-1})	No. of days for shooting	No. of shoots/ clump	No. of leaves / shoot	Shoot length (cm)
Control	21.20	1.87	2.33	1.71
BAP 2	16.40	3.53	6.27	2.71
BAP 4	15.73	4.73	8.33	3.18
BAP 6	16.60	2.87	6.53	2.61
BAP 8	17.40	2.60	4.67	2.50
BAP10	18.20	2.47	4.33	2.29
2 + 0.1	16.67	4.07	6.67	2.68
4 + 0.1	16.27	4.33	8.73	3.25
6 + 0.1	17.13	3.47	6.40	2.57
8 + 0.1	17.47	3.27	5.33	2.24
10 + 0.1	18.13	2.67	4.20	1.83
2 + 0.2	16.53	4.40	8.33	2.69
4 + 0.2	15.87	4.67	9.53	3.43
6 + 0.2	17.20	3.53	6.67	2.59
8 + 0.2	17.60	3.27	5.67	2.19
10 + 0.2	18.33	2.67	5.27	1.76
2 + 0.3	16.40	4.13	8.47	3.11
4 + 0.3	12.13	4.80	10.27	3.85
6 + 0.3	17.33	3.53	5.53	2.38
8 + 0.3	18.00	3.27	5.33	2.07
10 + 0.3	18.60	2.87	4.47	1.64
S. Em. ±	0.112	0.071	0.077	0.046
C.D at 5 %	0.321	0.203	0.220	0.131
C.D at 1 %	0.1429	0.272	0.294	0.175

Table 2: Influence of hormones (NAA and IBA) on root regeneration at four weeks of inoculation

Treatments (mg l^{-1})	No. of days for rooting	No. of roots/ shoot	Root length (cm)
Control	21.47	1.47	0.95
NAA 0.5	17.13	2.33	2.21
NAA 1.0	15.60	3.47	2.38
NAA 1.5	13.60	3.73	2.66
NAA 2.0	12.20	4.33	3.11
IBA 1.0	13.67	3.47	2.89
IBA 2.0	10.87	4.73	3.47
IBA 3.0	13.60	3.87	3.04
S. Em. ±	0.175	0.067	0.046
C.D at 5 %	0.524	0.200	0.137
C.D at 1 %	0.722	0.275	0.189

Table 3: Standardization of media composition for hardening of gerbera

Treatments	Percent survival (%)	Percent mortality (%)
M_1	40	60
M_2	40	60
M_3	80	20
M_4	100	0

M_1 - soil + sand + FYM- 2: 1: 1
M_2 - soil + sand +vermicompost - 2: 1: 1
M_3 - soil + sand + coco peat- 2: 1: 1
M_4 - coco peat

Fig 1: Maximum shoot regeneration with 4 ppm BAP+ 0.3 ppm IAA 6 weeks after inoculation

Fig 2: Maximum shoot length with BAP at 4 ppm+IAA at 0.3 ppm 6 weeks after inoculation

Fig 3: Maximum root length observed with IBA 2ppm

Fig 4: Hardening off gerbera *in vitro* platelets covered with polypropylene

Fig 5: Hardening off Gerbera *in vitro* plantlets covered with polyethene

Fig 6: Magnified view of ELISA showing no colour (yellow) development for CMV

5. Conclusion

An efficient mass propagation protocol which can be easily adopted for large-scale micropropagation to fulfil the demand of planting material for commercial cultivation was developed. Among different treatments, MS medium supplemented with 4 mg per litre BAP + 0.3 mg per litre IAA was found best for shoot regeneration, MS medium supplemented with 2 mg per litre IBA was noticed as best hormonal concentration for root regeneration and coco peat alone was the best hardening media. Plantlets produced were free from cucumber mosaic virus.

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