International Journal of Horticultural Science and Technology
Vol. 3, No. 1; June 2016, pp 51-58

A Simple and Efficient Direct Shoot Organogenesis Method Using Leafy Petiole Explants in *Gerbera jamesonii* 'Royal Soft Pink'

Farzad Nazari[1*], Morteza Khosh-Khui[2] and Pejman Azadi[3,4]

1. Department of Horticultural Science, College of Agriculture, University of Kurdistan, Sanandaj, Iran

2. Department of Horticultural Science, College of Agriculture, Shiraz University, Shiraz, Iran

3. Agriculture Biotechnology Research Institute of Iran, Karaj, Iran

4. Ornamental Plants Research Institute of Iran, Mahallat, Iran

(Received: 10 September 2015, Accepted: 1 May 2016)

Abstract

The gerbera market would benefit from an efficient and simple protocol for high rate regeneration for propagation and genetic engineering. With these objectives, this investigation was done on shoot regeneration via direct organogenesis from leafy petiole explants of *Gerbera jamesonii* 'Royal Soft Pink'. Murashige and Skoog (1962) (MS) medium was supplemented with 0.1 mg L^{-1} indole-3-acetic acid (IAA) and additions of various concentrations and combinations of thidiazuron (TDZ: 0, 0.5 and 1 mg L^{-1}) and N_6-benzyladenine (BA: 0, 2, 4 and 6 mg L^{-1}). Higher values were recorded for a number of shoots on leafy petiole on the MS medium containing BA in combination with TDZ than on media containing BA or TDZ solely. The highest evaluations for percentage of shoot regeneration (85.43 %) and number of shoots per explant (12.88) was recorded in the medium supplemented with 0.1 mg L^{-1} IAA and 1.0 mg L^{-1} TDZ plus 4.0 mg L^{-1} BA. For rooting of the shoots, MS medium supplemented with three concentrations of α-naphthaleneacetic acid (NAA: 0.5 and 1 mg L^{-1}) together with control (MS only) were tried. The optimal results for rooting of shoots were obtained on MS medium containing 1 mg L^{-1} NAA. The *in vitro* raised plantlets were acclimatized and transferred to greenhouse successfully.

Keywords: direct regeneration, gerbera, organogenesis, petiole, TDZ.

Abbreviations: BA, N^6-benzyladenine; **GA_3**, Gibberellic acid; **IAA**, Indole-3-acetic acid; **IBA**, Indole-3-butyric acid; **Kin**, Kinetin; **MS**, Murashige and Skoog medium (1962); **NAA**, α-naphthaleneacetic acid; **PGR**, Plant growth regulator (s); **TDZ**, Thidiazuron.

Introduction

Gerbera (*Gerbera jamesonii* Bolus ex. Hooker. F.) belongs to the Asteraceae family and is one of most prominent cut and pot flowers in the floriculture industry (Dole and Wilkins, 2005). In terms of economic value, gerbera ranks fourth in the global cut flower market, after rose, chrysanthemum, and tulip (Teeri *et al*., 2006). Several methods of *in vitro* multiplication and regeneration of gerbera have recently been developed, such as shoot proliferation from shoot tips (Gantait *et al*., 2010; Cardoso and Teixeira da Silva, 2012; Cardoso and Teixeira da Silva, 2013), direct shoot organogenesis from flower buds (capitulum), explants (Akter *et al*., 2012), or callus culture from different kinds of tissue and cell suspension culture and somatic embryogenesis

*Corresponding author Email: f.nazari433@gmail.com

(Hasbullah *et al*., 2011) from various explants. True-to-type clonal fidelity is one of the most important pre-requisites in the micropropagation of crop species (Bhatia *et al*., 2009). Adventitious shoot regeneration is one of the best methods in economical horticulture for the vegetative propagation and in mutation breeding for the production of solid mutants of gerbera (Jerzy and Lubomski 1991). Shoot regeneration from callus is a valuable technique for gerbera breeding (Elomaa *et al*., 1993), but the method is problematic because of somaclonal variation among produced plantlets (Bhatia *et al*., 2009). Genetic fidelity of *in vitro* raised 45 plants of gerbera derived from three different explants, *viz*., capitulum, leaf, and shoot tips, have been done by 32 ISSR markers, to determine levels of somaclonal variation. The results of this research showed that clones derived from capitulum and shoot tip explants did not have any genetic variation, whereas one of the leaf-derived clones exhibited some degree of variation (Bhatia *et al*., 2009). Overcoming this problem requires the application of an efficient and reliable technique for direct shoot regeneration. There are few reports on direct shoot regeneration from petiole in gerbera. Hedtrich (1979) was the first to observe the regeneration of adventitious shoots from leaf blades of *G. jamesonii* 'Vulkan' on modified MS medium supplemented with 1 mg L^{-1} BA and 0.1 mg L^{-1} gibberellic acid (GA_3). Following this, Jerzy and Lubomski (1991) investigated the different concentrations of BA and kinetin (Kin) on direct adventitious shoot regeneration from leaf petiole of gerbera. They reported that the number of adventitious shoots developed from one petiole explant was mainly dependent on BA concentration and the addition of BA to the MS medium was more effective than the Kin. Also, the highest number of shoots from 8 to 11 was obtained on medium with 10 mg L^{-1} BA, but the shoots were frail, concise, and showed vitrification symptoms. The optimum BA concentrations were 3 and 5 mg L^{-1} and caused formation of four to six shoots with insignificant vitrification symptoms. Moreover, Orlikowska *et al*. (1999) cultured gerbera petioles on induction medium (MS medium containing 2.3 μM TDZ + 0.5 μM NAA) for 3 to 6 days. Then, by transferring explants to three regeneration media (A: 0.2 μM TDZ +0.3 μM IAA, B: 2.2 μM BA + 0.3 μM IAA and C: 4.4 μM BA +4.6 μM Zeatin+ 0.6 μM IAA), they obtained direct shoot regeneration during the first 4 weeks, and after these shoots were discarded, a semi-compact organogenic callus was produced. Thus, in the present study, we would like to introduce an efficient regeneration procedure for *Gerbera jamesonii* 'Royal Soft Pink' through high frequency adventitious shoot proliferation from leafy petioles with the use of various concentrations and combinations of TDZ and BA in MS medium containing 0.1 mg L^{-1} IAA.

Materials and Methods

Plant material and explant preparation

Gerbera jamesonii 'Royal Soft Pink' *in vitro* plants used in the present study were produced from shoot tip culture (Nazari *et al*., 2014). Plants were grown *in vitro* on MS medium supplemented with 3% sucrose, 2.0 mg L^{-1} glycine, 1.0 mg L^{-1} thiamine, 0.5 mg L^{-1} pyridoxine, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} BA, 0.1 mg L^{-1} NAA, pH 5.8 and solidified with 0.8% (w/v) agar. The subculture of these plants was prepared regularly at 4-week intervals in the medium as mentioned above. Small leafy petioles (approximately 1.5 to 2.5 cm in length) detached from *in vitro* stock clusters of 2-month-old plants were used as explant.

Adventitious shoot induction and multiplication

The leafy petiole explants were positioned horizontally with the abaxial side inside glass jars containing 40 ml of MS medium supplemented with 0.1 mg L^{-1} IAA and different concentrations and combinations

of BA and TDZ (Table 1). Media pH was adjusted to 5.8 prior to autoclaving at 121°C, for 20 min. IAA and TDZ were filter-sterilized (0.2-µm pore) and added after autoclaving. For shoot primordial production, jars were incubated in the dark at 23±1°C. After two weeks in the dark, explants were transferred to light for ten days with a 16/8h photoperiod (60 $\mu molm^{-2}s^{-1}$, cool-white fluorescent lamp) at 23±1°C. Shoot clusters derived from petioles were cultured on growth regulator-free MS medium for five days to allow synchronization of clumps. After that period, clumps were cultured for 1 month on jars containing 40 ml of MS medium supplemented with 0.5 mg L^{-1} BA and 0.1 mg L^{-1} NAA for shoot multiplication and growth. Evaluations were made for averages of number of shoots per explant and mean shoot length.

Root induction from shoots and acclimatization of plantlets

Individual shoots derived from multiple shoots were transferred to MS medium supplemented with two concentrations of NAA (0.5 and 1 mg L^{-1}) along with control (MS only) for root induction. Data that included the root number, root fresh, and dry weight per plantlet, were recorded after 3 weeks of culture. Plantlets with well-developed roots were removed from the culture medium. Once removed, roots were gently washed in sterilized water to remove any trace of medium then plantlets were transferred to plastic boxes containing a mixture of sterilized cocopeat and perlite (1:1 v/v). The potted plants were then transferred to a greenhouse and covered with polyethylene to maintain a condition of high humidity (85% RH), where they were kept for one week. After one week, covers were removed and plants were maintained in the greenhouse for further adaptation and development.

Statistical analysis

This experiment for shoot regeneration was conducted as a completely randomized design (CRD)-based factorial design with two factors and 6 replications. Each replication consisted of a glass jar containing 8 explants. The experiment of rooting of shoots was conducted as a complete randomized design with four replicates, and each replicate (a glass jar) contained 4 shoots. Means were compared using Duncan's New Multiple Range Test (DNMRT) at 5% level of probability using MSTATC program.

Results

The effect of various combinations and concentrations of PGRs on direct shoot organogenesis from leafy petiole explants

In the present study, the organogenic potential of gerbera was restricted to proximal edges of the plant at sites of vascular wounding of the petiole explant (Fig. 1A). Elimination of the proximal region from the petiole of explants caused failure of the plant's ability for shoot regeneration (data not shown). Two months after treatments, no growth was recorded on any of the explants cultured on the plant growth regulator (PGR), free control medium, or MS supplemented with 2 mg L^{-1} BA with 0.1 IAA mg L^{-1} (Table 1). Use of combinations of TDZ and BA showed significantly enhanced shoot regeneration in comparison with using TDZ or BA alone. The highest record for shoot formation was obtained in the medium supplemented with 0.1 mg L^{-1} IAA and 1 mg L^{-1} TDZ plus 4 mg L^{-1} BA (85.43 %), or plus 2 mg L^{-1} BA (84.65 %) (Table 1 and Fig. 1A and B). No shoot production was recorded in the medium containing 2 mg L^{-1} BA alone. With an increase in concentration of TDZ together with BA, there was enhancement in the percentages of explants that produced more shoots per explant. Shoot induction occurred in 4 and 6 mg L^{-1} BA without TDZ. Addition of TDZ showed a significant increase in induction of direct shoot organogenesis. Evaluations for frequency of shoot

organogenesis reduced at a high concentration of BA (6 mg L^{-1}). The highest number of shoots per explant (12.88) was observed in the medium containing 4 mg L^{-1} BA, 1 mg L^{-1} TDZ, and 0.1 IAA mg L^{-1}. Lower average shoot length was observed under increased concentration of BA from 2 to 6 mg L^{-1}. The highest average shoot length (3.87 cm) was recorded in the medium containing 0.5 mg L^{-1} TDZ +2 mg L^{-1} BA+ 0.1 mg L^{-1} IAA. A length of shoots on the media containing 4 mg L^{-1} BA or 0.5 mg L^{-1} TDZ made no significant difference. The lowest average shoot length (1.95 cm) was obtained on the medium containing 1 mg L^{-1} TDZ + 6 mg L^{-1} BA, although no significant difference occurred in the presence of 0.5 mg L^{-1} TDZ and 6 mg L^{-1} BA. In MS medium containing high concentration of BA (4 and 6 mg L^{-1}), reduced values for mean number of shoots per responding explant were observed. In media containing 4 and 6 mg L^{-1} BA alone or in combination with TDZ, hyperhydration was observed (data not shown).

The effect of two concentrations of NAA on rooting of shoots

All of the obtained shoots were rooted in MS only and MS supplemented with both concentrations of NAA (Fig. 1C), but the quality of root in shoots treated with NAA was better than control shoots. The highest root number (4.75) per shoot was obtained in MS medium containing 1 mg L^{-1} NAA. In the case of the length of the highest root, root fresh, and dry weight, no significant difference was observed among the two concentrations of NAA (Fig. 2). Plantlets with fully expanded leaves and well-developed roots (Fig. 1D) were successfully acclimatized in one month and over 90% of plantlets survived (data not shown).

Table 1. Effect of different combinations of BA and TDZ on direct adventitious shoot induction from cultured leafy petiole explants of *G. jamesonii* 'Royal Soft Pink'. All media were supplemented with 0.1 mg L^{-1} IAA except control.

PGRs (mg L^{-1})		Measured parameters		
TDZ	BA	Explants with shoots (%) [a]	Mean shoots per responding explant [b]	Average shoot length (cm) [b]
0	0	0.00g*	0.00g	0.00e
0	2	0.00g	0.00g	0.00e
0	4	38.de	6.50d-f	3.40ab
0	6	24.75f	5.00f	2.85c
0.5	0	32.30ef	5.75ef	3.42ab
0.5	2	67.28b	7.00c-e	3.87a
0.5	4	68.21b	9.00b	3.21bc
0.5	6	47.00cd	7.50cd	2.12d
1	0	37.70de	5.95ef	3.12bc
1	2	84.65a	12.23a	3.20bc
1	4	85.43a	12.88a	2.94bc
1	6	54.00c	8.25bc	1.95d

*In each column, means with the same letter (s) are not significantly different at 5% level of significance using DNMRT.

a: The data were recorded after 24 days (14 days at dark+10 days at light)

b: The data were recorded after 35 days of transferring to light condition.

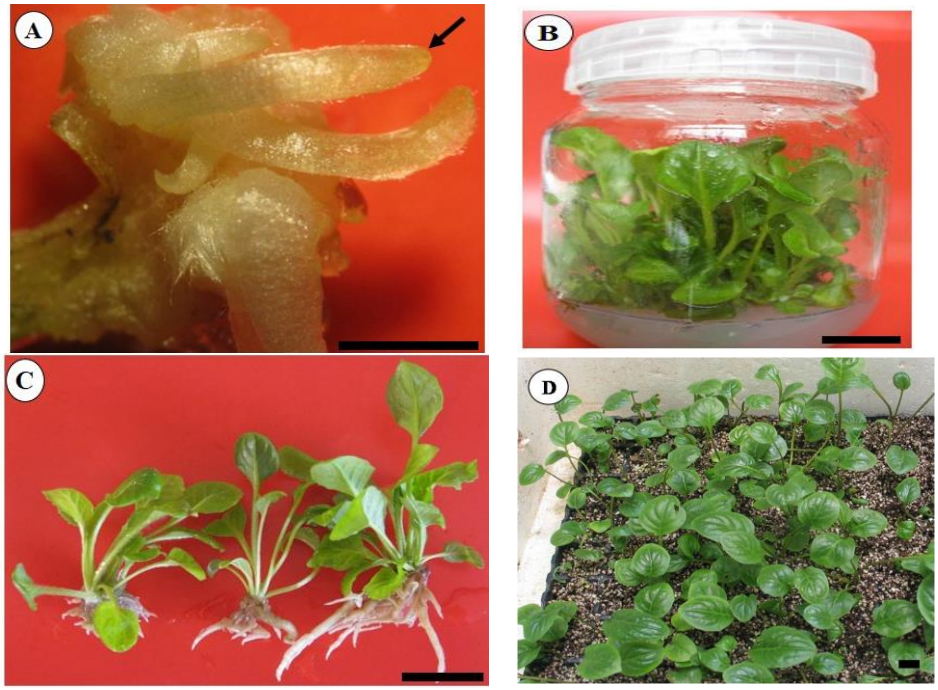

Fig. 1. Multiple shoot bud induction and shoot regeneration from petiole explants of *G. jamesonii* 'Royal Soft Pink'. (A) Multiple shoot bud induction directly formed on the proximal surface of petiole (black arrows) after two weeks cultivation of detached leaf explants on MS medium supplemented with 1.0 mg L^{-1} TDZ + 4.0 mg L^{-1} BA + 0.1 mg L^{-1} IAA in dark condition, (B) multiple shoot proliferation and elongation on MS medium with 0.5 mg L^{-1} BA plus 0.1 mg L^{-1} NAA, (C) plantlets rooted *in vitro* with stout and healthy roots on MS medium containing 1 mg L^{-1} NAA after removal from glass jar (D) two-week-old acclimatized plant growing in greenhouse (Scales bar: 1cm).

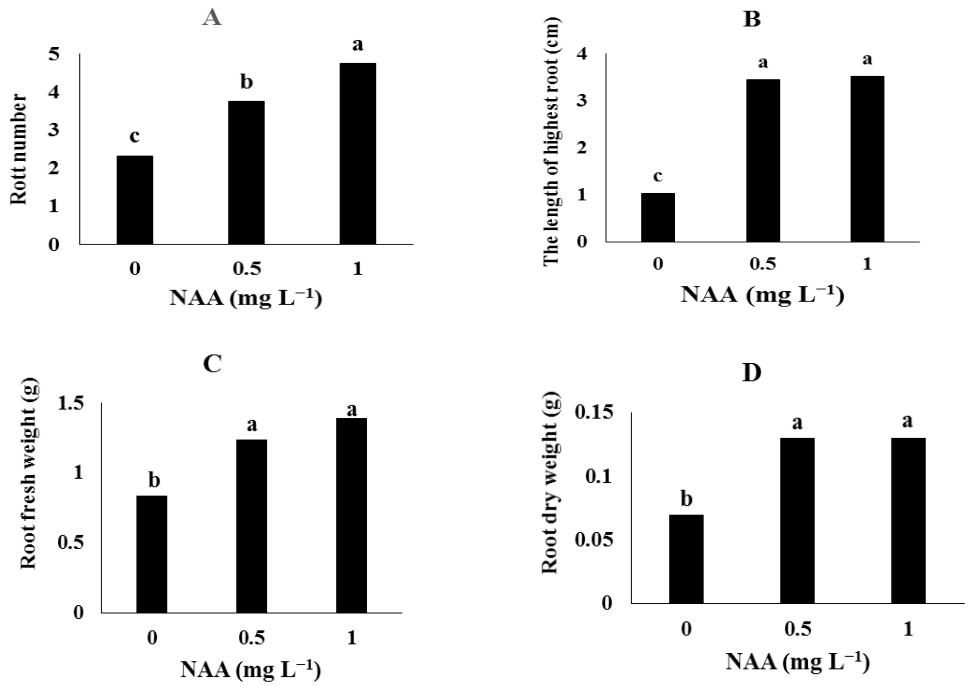

Fig. 2. The effect of two concentrations of NAA (0.5 and 1 mg L^{-1}) along with control (0=MS only) on root number (A), the length of highest root (B), and root fresh (C) and dry weights (D) of individual *in vitro* shoots of *G. jamesonii* 'Royal Soft Pink' (means with the same letter are not significantly different at 5% level of probability using DNMRT).

Discussion

Direct organogenesis is one of the most important morphogenetic occurrences in plant tissue culture because of direct shoot development without an intervening callus phase that produces a significant reduction of soma clonal variation (Koné *et al*., 2013). Leafy petiole explant is one of the most suitable plant tissues for direct organogenesis in gerbera. Preliminary results of these tests indicated that two things were important in terms of preparation of leafy petiole explants for shoot regeneration in gerbera. The first showed that the proximal end of explants as a critical region should not be cut from the petiole, and the second determined that the explant should be a petiole with a whole leaf (data not shown). Jerzy and Lubomski (1991) concluded that leaves with petioles shortened to one-half of their length while leaves without petioles failed to form adventitious shoots. Axillary shoot apical meristems usually developed in the axils of leaves; the axil being the junction between leaf and stem (McConnell and Barton, 1998). Tran Thanh Van (1973) reported that cells associated with vascular tissue were most often the origins of meristemoids and produced primordial organs. Moreover, Ananthakrishnan *et al*. (2005) reported that plants were incapable of developing adventitious shoots after removal of the proximal region from cotyledonary explants from cashew plants. Plant growth regulators (PGRs) play a decisive role in process potentials of dedifferentiation and redifferentiation. Shoot induction and regeneration responses of leafy petiole of gerbera were greatly influenced by treatments that combined TDZ and BA. TDZ is a synthetic phenylurea and is considered as one of the most active cytokinins for shoot induction in plant tissue culture (Huetteman and Preece, 1993). Successful shoot regeneration using TDZ has been reported in other research (Thomas and Puthur, 2004; Husain *et al*., 2007; Lata *et al*., 2009). Thiruvengadam *et al*. (2010) reported that use of TDZ in combination with NAA was significantly advantageous to indirect bud formation from leaves of bitter melon. These regenerative processes in cell and tissue cultures may be stimulated by TDZ alone or in combination with other plant growth regulators (Guo *et al*., 2011). Guo *et al*. (2011) showed that TDZ treatment induced the regeneration process by increasing accumulations of mineral ions (such as iron), other metabolites, as well as storage and passage of endogenous plant signals that predisposed the explant to stress. In micropropagation of Philodendron, experiments showed that explants from petiole were more responsive than those from leaf laminas and that direct shoot formation was achieved with application of TDZ (Chen *et al*., 2012). Liu *et al*. (2003) reported that cultures grown in the medium supplemented with TDZ produced the maximum number of shoots per intact seedling in *Artemisia judaica* L. TDZ has been used extensively in tissue culture studies. It exhibits strong cytokinin-like activity, promotes regeneration of axillary shoots, invigorates adventitious organ regeneration, and induces somatic embryogenesis (Huetteman and Preece, 1993). Nielsen *et al*. (1995) reported that the synergistic effect of exogenous cytokinins BA and TDZ heightened shoot regeneration in *Miscanthus×ogiformis*. This could contribute to enhanced axillary shoot formation from application of a combination of BA and TDZ, which is possibly due to active binding to both cytokinin-binding protein (CBP) sites. Nielsen *et al*. (1995) reported that media containing two different cytokinins might alter the number and quality of shoot production compared to media with only one cytokinin. On the medium containing both BA and TDZ, some *Vitis rotundifolia* cultivars formed more axillary shoots than on the medium containing only one type of cytokinin (Sudarsono and Goldy, 1991). High concentration of cytokinins produced significantly lower numbers and smaller

sized shoots. Moreover, some shoots were short, vitrified, and had abnormal leaf morphology (data not shown). Similar results were reported in other plants (Malik and Saxena, 1992; Lu, 1993; Nielsen *et al*., 1995; Polisetty *et al*., 1997; Sreekumar *et al*., 2001). The rooting of gerbera is easy and possible in MS without 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 Teixeira da Silva, 2013). In our experiment, the MS supplemented with 1 mg L^{-1} NAA was better than 0.5 mg L^{-1} NAA because the highest root number was obtained in this medium.

Conclusions

Leafy petiole explant is one of the most suitable plant tissues for direct organogenesis in gerbera and the organogenic potential of gerbera was restricted to proximal edges of the plant at sites of vascular wounding of the petiole explant. Also, shoot induction and regeneration responses of leafy petiole of gerbera were greatly influenced by treatments that combined TDZ and BA, and the highest number of shoots per explant (12.88) was observed in the MS medium containing 0.1 mg L^{-1} IAA+ 4 mg L^{-1} BA + 1 mg L^{-1} TDZ.

Acknowledgements

We would like to thank Mr. Lachmann from Takii Europe BV (Netherlands) for providing the seeds of gerbera, and many special thanks to Mr. F. Nikbakht and my wife (Mrs. T. Jamshidi) for their assistance during this experiment.

References

1. Akter, N., M.I. Hoque, and R.H. Sarker. 2012. *In vitro* Propagation in Three Varieties of Gerbera (*Gerbera jamesonii* Bolus.) from Flower Bud and Flower Stalk Explants. Plant Tissue Cult. Biotech. 22*:*143-152.
2. Ananthakrishnan, G., R. Ravikumar, R. Prem Anand, G. Vengadesan, and A. Ganapathi. 1999. Induction of Somatic Embryogenesis from Nucellus Derived Callus of *Anacardium occidentale* L. Scientia Hort. 79:91-99.
3. Bhatia, R., K.P. Singh, T. Jhang, and T.R. Sharma. 2009. Assessment of Clonal Fidelity of Micropropagated Gerbera Plants by ISSR Markers. Scientia. Hort. 119:208-211.
4. Cardoso, J. C. and J. A. Teixeira da Silva. 2012. Micropropagation of Gerbera Using Chlorine Dioxide (ClO_2) to Sterilize the Culture Medium. In Vitro Cell. Dev. Biol. Plant. 48:362–368.
5. Cardoso, J. C. and J. A. Teixeira da Silva. 2013. Gerbera Micropropagation. Biotechnol. Adv. 31:1344-1357.
6. Chen, F.C., C.Y. Wang, and J.Y. Fang. 2012. Micropropagation of Self-Heading *Philodendron* via Direct Shoot Regeneration. Scientia Hort. 141:23–29.
7. Dole, J.M. and H.F. Wilkins. 2005. Floriculture Principles and Species. Prentice- Hall, Inc. USA, 1023 pp.
8. Elomaa, P., J. Honkanen, R. Puska, P. Seppiinen, Y. Helariutta, M. Mehto, M. Kotilainen, L. Nevalainen, and T.H. Terri. 1993. *Agrobacterium*-Mediated Transfer of Antisense Chalcone Synthase cDNA to *Gerbera hybrida* Inhibits Flower Pigmentation. Biotechcol. 11:508-511.
9. Gantait, S., N. Mandal, S. Bhattacharya, and P.K. Das. 2010. An Elite Protocol for Accelerated Quality-Cloning in *Gerbera jamesonii* Bolus cv. Sciella. *In Vitro* Cell. Dev. Biol. Plant 46:537-548.
10. Goldy, R.G. 1991. Growth Regulator and Axillary Bud Position Effects on *In Vitro* Establishment of *Vitis rotundifolia*. HortScience 26:304-307.
11. Guo, B., B.H. Abbasi, A. Zeb, L.L. Xu, and Y.H. Wei. 2011. Thidiazuron: a Multidimensional Plant Growth Regulator. Afr. J. Biotechnol. 10:8984-9000.
12. Hasbullah, N.A., A. Saled, and R.M. Taha. 2011. Establishment of Somatic Embryogenesis from *Gerbera jamesonii* Bolus ex. Hook F. Through Suspension Culture. Afr. J. Biotechnol. 10:13762–13768.
13. Hedtrich, C.M., 1979. Sprossregeneration Aus Blatern Und Vermehrung Von *Gerbera jamesonii*. Gartenbauwissenschaft 44:1-3.
14. Huetteman, C.A. and J.E. Preece. 1993. Thidiazuron: a Potent Cytokinin for Woody

Plant Tissue Culture. Plant Cell Tissue Organ Cult. 33:105-119.

15. Husain, M.K., M. Anis, and A. Shahzad. 2007. *In Vitro* Propagation of Indian Kino (*Pterocarpus marsupium* Roxb.) Using Thidiazuron. In Vitro Cell. Dev. Biol. Plant 43:59–64.

16. Jerzy, M. and M. Lubomski. 1991. Adventitious Shoot Formation on *Ex Vitro* Derived Leaf Explants of *Gerbera jamesonii*. Scientia Hort. 47:115–124.

17. Koné, M., T. Koné, H.T. Kouakou, S. Konaté, and J.S. Ochatt. 2013. Plant regeneration *Via* Direct Shoot Organogenesis from Cotyledon Explants of Bambara groundnut (*Vigna subterranea* L.). Biotechnol. Agron. Soc. Environ. 17:584-592.

18. Lata, H., S. Chandra, I. Khan, and M.A. ElSohly. 2009. Thidiazuron-Induced High-Frequency Direct Shoot Organogenesis of *Cannabis sativa* L. In Vitro Cell. Dev. Biol. Plant 45:12-19.

19. Liu, C.Z., S.J. Murch, E.L. Demerdash, and P.K. Saxena. 2003. Regeneration of the Egyptian Medicinal Plant *Artemisia judaica* L. Plant Cell Rep. 21:525-530.

20. Lu, C. 1993. The Use of Thidiazuron in Tissue Culture. In Vitro Cell. Dev. Biol. Plant. 29:92–96.

21. Malik, K.A. and P.K. Saxena. 1992. Thidiazuron Induces High Frequency Shoot Regeneration in Intact Seedling of Pea (*Pisum sativum*) Chickpea (*Cicer arietinum*) and Lentil (*Lens culinaris*). Austral. J. plant physiol. 19:731–740.

22. McConnell, J.R. and M.K. Barton. 1998. Leaf Polarity and Meristem Formation in *Arabidopsis*. Dev. 125:2935-2942.

23. Nazari, F., M. Khosh-Khui, P. Azadi, H. Salehi, and A. Niazi. 2014. Growth regulators Affected *In Vitro* Propagation of Pot Gerbera (*Gerbera jamesonii* cv. Royal Soft Pink). Intl. J. Agr. Biosci. 3:185-189.

24. Nielsen, J. M., J. Hansen, and K. Brand. 1995. Synergism of Thidiazuron and Benzyladenine in Axillary Shoot Formation Depends on Sequence of Application in *Miscanthus* × *ogiformis* cv. Giganteus. Plant Cell Tissue Organ Cult. 41: 165–170.

25. Orlikowska, T., E. Nowak, A. Marasek, and D. Kucharska. 1999. Effects of Growth Regulators and Incubation Period on *In Vitro* Regeneration of Adventitious Shoots from Gerbera Petioles. Plant Cell Tissue Organ Cult. 59:95–102.

26. Polisetty, R., V. Paul, J.J. Deveshwar, S. Khetarpal, K. Suresh, and R. Chandra. 1997. Multiple Shoot Induction by Benzyladenine and Complete Plant Regeneration from Seed Explants of Chickpea (*Cicer arietinum.* L.). Plant Cell Rep. 16:565–571.

27. Sreekumar, S., S. Mukunthakumar, and S. Seeni. 2001. Morphogenetic Responses of Six *Philodendron* Cultivars *In Vitro*. Indian J. Exp. Biol. 39:280–1287.

28. Sudarsono, A., and R.G. Goldy. 1991. Growth Regulator and Axillary Bud Position Effects on *In Vitro* Establishment of *Vitis rotundifolia.* HortScience. 26:304-307.

29. Teeri, T.H., P. Elomaa, M. Kotilainen, and V.A. Albert. 2006. Mining Plant Diversity: Gerbera as a Model System for Plant Developmental and Biosynthetic Research. Bioassays 28:756–767.

30. Thiruvengadam, M., K.T. Rekha, C.H. Yang, N. Jayabalan, and I.M. Chung. 2010. High Frequency Shoot Regeneration From Leaf Explants Through Organogenesis in Bitter Melon (*Momordica charantia* L.). Plant Biotechnol. Rep 4:321–328.

31. Thomas, T.D. and J.T. Puthur. 2004. Thidiazuron Induced High Frequency Shoot Organogenesis in Callus From *Kigelia pinnata* L. Bot. Bul. Acad. Sinica 45:307–313.

32. Tran Thanh Van, M. 1973. *In Vitro* Control of *De Now* Flower, Bud, Root and Callus Differentiation From Excised Epidermal Tissues. Nature 246:44-45.