J Plant Biotechnol (2016) 43:255-260
DOI:http://dx.doi.org/10.5010/JPB.2016.43.2.255

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

In vitro shoot regeneration and genetic transformation of the gerbera (*Gerbera hybrida* Hort.) cultivar 'Gold Eye'

Mi-Young Chung · Min Bae Kim · Yong Mo Chung · Ill-Sup Nou · Chang Kil Kim

Received: 31 May 2016 / Revised: 3 June 2016 / Revised: 8 June 2016 / Accepted: 13 June 2016

Abstract This research was conducted to improve the cold tolerance of the gerbera cv. Gold Eye by introduction of the *Arabidopsis* Ca^{2+}/H^{+} antiporter gene (*CAX1*) via *Agrobacterium*-mediated transformation. Prior to genetic transformation, we optimized a combination of plant growth regulators; 1.0 mgl^{-1} 6-Benzyladenine (BA) and 0.1 mgl^{-1} 3-indole-acetic acid (IAA) were found to lead to proper *in vitro* shoot regeneration from petiole explants. In addition, 50 mgl^{-1} kanamycin was determined to be the minimal concentration useful for selection of putative transgenic plants. In this study, transgenic gerbera expressing the *Arabidopsis* Ca^{2+}/H^{+} antiporter gene (*CAX1*) were obtained using the optimized concentrations. We expect that introduction of the gene to the cultivar will improve cold tolerance, which will be important in the winter months.

Keywords Plant growth regulator, Selective agent, Cold tolerance, Transgenic plants

Introduction

Gerbera is considered as one of the leading ornament plants grown worldwide due to its inclusion in the lists of high demand cut flower for global floral industry. In addition, as it has a long vise life and resistance to transportation damage, no riskiness is necessary to obtain a good market price. Until recently, a large number of new cultivars "*Gerbera hybrida*" have been developed using conventional breeding and introduced to global flower market. Although the conventional breeding has produced numerous elite cultivars with desirable traits such as colour, shape, vase life and resistance against pests and diseases, there are still constraints to this technique due to limited genepool of the genus. Recently, improvement of quality attributes by *Agrobacterium*-mediated transformation has been increasingly used in ornamental plants. In addition, this technique had also been successfully employed in gerbera for many purposes (Elooma et al. 1993; Nowak et al. 1997; Nagaraju et al. 1998)

The gerbera cv. Gold Eye has desirable horticultural traits such as harmonious floret color, long vase life (10.2 days), and it produces high yield of flowers per plant (48.8) in a year (Chung et al 2007). Due to its desirable traits, it has been highly interested by growers and consumers in Korea, however, production of gerbera in winter is expensive and limited as well due to cold stress, thus it is of essence to reduce cold stress suffered by this cultivar using cold stress tolerant gene via *Agrobacterium*-mediated genetic transformation.

Expression of *Arabidopsis* Ca^{2+}/H^{+} antiporter gene, *CAX1*, in *Arabidopsis* enhanced expression of cold resistant genes that improve cold tolerance (Catala et al. 2003). However, its cold tolerant effects had not been reported in any other plant species, thus also, the mechanism by which this gene enhances freezing tolerance is not still clear. Due to the facts, we are interested to generate the gerbera cv. Gold Eye expressing *CAX1* for cold stress tolerance in winter.

For successful genetic transformation, efficient in vitro shoot regeneration is perquisite. Since past a few decades, in vitro regeneration of gerbera using different explants and plant growth regulators (Reynoird et al. 1993; Orlikowska et al., 1999; Aswath and Choudhary, 2002; Tyagi and Kothari, 2004; Chakrabarty and Datta, 2008). However, there have

M.-Y. Chung · M. B. Kim
Department of Agricultural Education , Sunchon National University, Suncheon 57922, Korea

Y. M. Chung
Flower Research Institute, Gyeongsangnam-do Agricultural Research & Extension Services, Changwon 52733, Korea

I.-S. Nou
Department of Horticulture, Sunchon National University, Sunchon 57922, Korea

C. K. Kim (✉)
Department of Horticultural Science, Kyungpook National University, Daegu 41566, Korea
e-mail: ckkim@knu.ac.kr

been no studies reporting in vitro shoot regeneration of this cultivar 'Gold Eye', in addition, a protocol which is suitable for a cultivar is not easily adapted to anther cultivars. Thus, it is necessary to develop efficient in vitro shoot regeneration protocol for genetic transformation of this cultivar.

In addition, efficient selection of putative transgenic plants using optimal concentration of selective agent such as kanamycin or phosphinothricin (PPT), which kills or inhibits growth of surrounding non-transgenic cells, also plays a important role in genetic transformation (Naing et al. 2016).

Therefore, we tested shoot regeneration efficiency using different plant regulators followed by optimal concentrations of selective agent. The optimal concentrations of plant growth regulators and selective agent were then used in genetic transformation of gerbera cv. Gold Eye.

Materials and Methods

Effects of different plant growth regulators on in vitro shoot regeneration

To verify an optimal combination of plant growth regulators for shoot regeneration, petioles from *in vitro* 5-week-old donor plants were segmented into 0.5 ~ 1.0 cm in length and cultured on the Murashige-Skoog (MS) medium containing combinations of various concentrations of 6-Benzyladenine (BA) and 3-indole-acetic acid (IAA) and/or Zeatin (Zn), as presented in Tables 1, along with 3 gl^{-1} of phytagel. Each treatment consisted of 10 explants with three replicates. The explants were cultured at an incubation room setting up with 16 h photoperiod (37 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 5 weeks of culture, a combination of plant growth regulators providing optimal number of shoots per explant was evaluated.

Evaluation of sensitivity of selective agent (kanamycin) to shoot regeneration

Petiole explants segmented as above were cultured on regeneration media containing the combination of 1.0 mgl^{-1} BA and 0.1 mgl^{-1} IAA and various concentrations of kanamycin (Duchefa, The Netherlands) to evaluate the minimal concentration of the selective agent. Each treatment contained 10 explants with three replications. The explants were cultured at the same incubation room described above. After 5 weeks of culture, minimal concentrations of the selective agents inhibiting growth of non-transgenic cells were evaluated by counting the number of shoots per explant.

Plasmid construction

Agrobacterium tumefaciens strains LBA4404 harboring a binary vector pBICaMV was used in this work. The T-DNA region of pBICaMV is constructed with Ca^{2+}/H^{+} antiporter gene, *CAX1* (650 bp), isolated from *Arabidopsis* by placing under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter. The *nptt2* gene conferring kanamycin resistance was used as selectable marker (Wu et al. 2011)

Genetic transformation

Genetic transformation of petiole explants was performed using the protocol described by Naing et al (2016). Briefly, the petiole explants (about 500 explants) were initially pre-cultured on MS medium containing 1.0 mgl^{-1} BA and 0.1 mgl^{-1} IAA for 2 days. The pre-cultured explants were then co-cultivated with *Agrobacterium* suspension (OD_{600} = 0.7) for 30 min. After which, they were blot-dried on a sterile filter paper followed by culturing on MS medium containing 100 μM acetosyringone (pH 5.4) for 2 days under darkness. The explants were then transferred to the regeneration medium containing 250 mg l^{-1} Clavamox and 50 mg l^{-1} kanamycin. After 5 weeks of culture, shoots that showed resistance to kanamycin were transferred to hormone-free MS medium containing the same concentration of kanamycin for rooting.

The rooted plants were transferred to plastic pots filled with the peat based soil (peat moss:perlite 4:1), and then, they were put into a growth chamber for 7 days and moved to a greenhouse.

DNA isolation and polymerase chain reaction (PCR) analysis

Isolation of total genomic DNA from the leaves of kanamycin-resistant and non-transgenic plants (NP) was performed using the HiYield™ Genomic DNA Mini Kit (plant), according to the manufacturer's instructions (Real Biotech Corporation, Taipei, Taiwan). The shoots regenerated from non-transformed explants were used as the control. PCR was performed using the CAX1-specific primers CAX1F 5-ATG TCT TCT TCT TCT TTG AG-3 and CAX1R 5-CAA TGT AGC TGA TCA ACA TAA C-3 in order to amplify a 650-bp fragment. The amplified products were analyzed using electrophoresis in 1% (w/v) agarose gels.

Results and discussion

Effects of different plant growth regulators on *in vitro* shoot regeneration

In this study, types and concentrations of plant growth regulator used significantly affected *in vitro* shoot formation from petiole (Table 1). The explants exhibited initiation of shoot bud formation after 10 days of culture on the medium containing different concentrations of BA and IAA combinations, however, increase of BA concentration higher than 1 mgl^{-1} showed to negatively affect percentage of shoot formation and number of shoots per explant, thus, the maximum percentage of shoot formation (81%) and number of shoots per explant (5.0) were achieved with a combination of 1.0 mgl^{-1} BA and 0.1 mgl^{-1} IAA after 5 weeks of culture. In addition, shoots obtained from this combination also exhibited to be better in plant growth (Fig. 1A) than those obtained from other combinations. It seemed that inclusion of high concentrations of BA not only affected shoot regeneration efficiency but also shoot quality. Many researchers had applied the high concentrations of BA for *in vitro* shoot regeneration of gerbera from different explants; however, they did not report the adverse affect. In earlier report done by Barbosa et al (1994), among different combinations of BA (0 ~ 4 mgl^{-1}) and IAA (0.1 mgl^{-1}) maximum regeneration rate was obtained on 1 mgl^{-1} BA, irrespective of the IAA concentration used. In addition, efficient regeneration for four gerbera genotypes was achieved with 1 mgl^{-1} BA and 0.1 mgl^{-1} NAA (Xi and Shi (2003). Therefore, our result supports the findings of the previous studies.

Addition of Zeatin to the combinations of BA and IAA distinctly suppressed shoot regeneration. Specifically, approximately 50 % of shoot regeneration rate were declined in the media containing 1.0 mgl^{-1} BA and 0.1 mgl^{-1} IAA, and only 3.7 shoots per explant were induced after 5 weeks of culture. On the media containing the combination of 3.0 mgl^{-1} BA and 0.1 mgl^{-1} IAA inclusion of Zeatin inhibited shoot regeneration rate from 38% to 17%, this result being different from previous report on other species of gerbera (Hasbullah et al (2008), in which the combination of 2.0 mgl^{-1} and 0.5 mgl^{-1} IAA gave reasonable shoot regeneration rate (73.2) and number of shoots per explant (5.6) from petiole explants. Possible explanation for this difference is that tt might be due to having no synergic effect of BA and Zeatin on shoot regeneration from petiole, irrespective of IAA. Another reasonable explanation for this is that would be due to different genotypes or different concentrations used in this study. In gerbera, application of Zeatin in shoot regeneration is still rare and its effect on shoot regeneration is also unknown yet. Thus, more researches on in vitro shoot regeneration of gerbera using Zeatin along with

Table 1 Effects of combinations of various concentrations of plant growth regulators on shoot regeneration from petiole explants of gerbera cv. Gold Eye

Plant growth regulators (mgl^{-1})		Rate of regeneration (%)	No. of shoot per explant
Primary culture	Subcultrue		
BA 1.0 + IAA 0.1	BA 0.5 + IAA 0.1	81a	3.0a
	BA 1.0 + IAA 0.1	69b	2.1b
	BA 2.0 + IAA 0.1	67b	1.2d
BA 2.0 + IAA 0.1	BA 1.0 + IAA 0.1	57cd	1.8c
	BA 2.0 + IAA 0.1	50e	1.0d
	BA 3.0 + IAA 0.1	55d	0.7de
BA 3.0 + IAA 0.1	BA 1.0 + IAA 0.1	61c	1.1d
	BA 2.0 + IAA 0.1	53de	0.7de
	BA 3.0 + IAA 0.1	38f	0.5g
BA 1.0 + IAA 0.1+ Zeatin 1.0	BA 0.5 + IAA 0.1+ Zeatin 1.0	46e	0.7de
	BA 1.0 + IAA 0.1+ Zeatin 1.0	39f	0.7de
	BA 2.0 + IAA 0.1+ Zeatin 1.0	40f	0.5g
BA 2.0 + IAA 0.1+ Zeatin 1.0	BA 1.0 + IAA 0.1+ Zeatin 1.0	48e	0.6fg
	BA 2.0 + IAA 0.1+ Zeatin 1.0	27g	0.4cd
	BA 3.0 + IAA 0.1+ Zeatin 1.0	18h	0.4g
BA 3.0 + IAA 0.1+ Zeatin 1.0	BA 1.0 + IAA 0.1+ Zeatin 1.0	36f	0.6fg
	BA 2.0 + IAA 0.1+ Zeatin 1.0	26g	0.4g
	BA 3.0 + IAA 0.1+ Zeatin 1.0	17h	0.3h

Means marked with the same letter in the same column are not significantly different by DMRT at the 5% level.

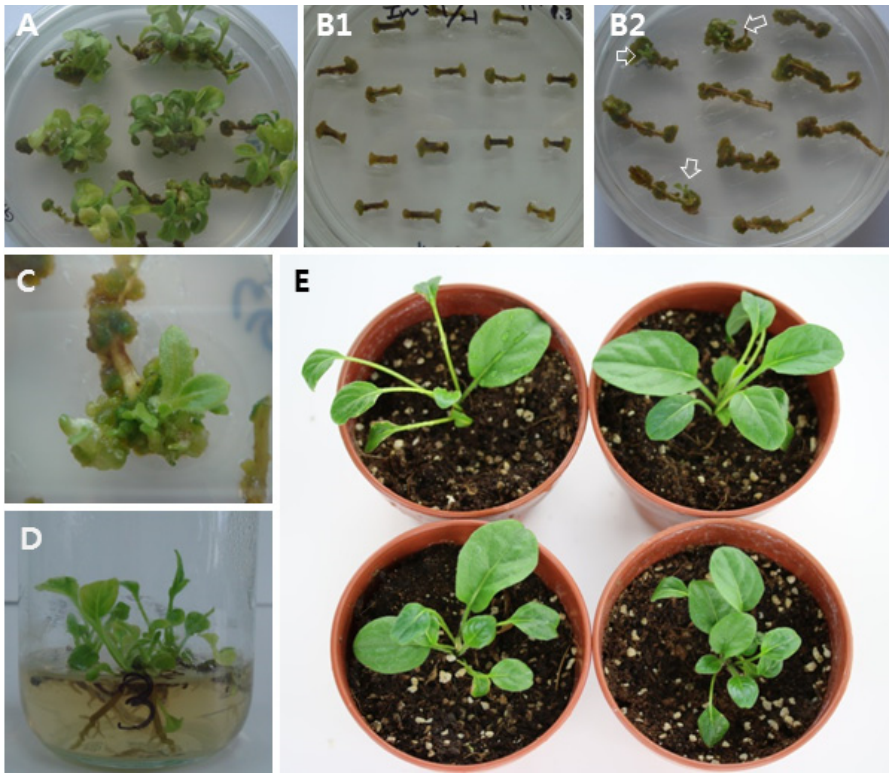

Fig. 1 *In vitro* shoot regeneration and *Agrobacterium*-mediated genetic transformation of gerbera cv. Gold Eye. A) Regenerated shoots derived from medium containing 1.0 mgl^{-1} BA and 0.1 mgl^{-1} IAA; B1) control explants showing no regenerated shoots on medium containing 50 mgl^{-1} kanamycin; B2) co-cultivated explants showing regenerated shoots on the selection medium; C) putative transgenic shoots regenerated from transformed explants cultured on the selection medium; D) transfer of the putative transgenic shoots to PGR-free medium containing kanamycin for rooting; E) transfer of the transgenic plants to pots containing peat-based soil

different plant growth regulators are still necessary.

Sensitivity of selective agent (kanamycin) to shoot regeneration

On the regeneration medium containing 1.0 mgl^{-1} BA and 0.1 mgl^{-1}IAA, explants have been shown to have reasonable shoot regeneration (Table 1), however, addition of various concentrations of kanamycin to the media the regenerability was distinctly inhibited even at the low concentration (25 mgl^{-1}), resulting in no regenerated shoot when concentration was raised to 50 mgl^{-1} (Table 2). More apparently, the explants were seemingly to turn necrotic on the media containing the concentrations of kanamycin higher than 50 mgl^{-1} after 2 weeks of culture (Fig. 1B). Therefore, 50 mgl^{-1} of kanamycin is likely to be the minimal concentration required for the efficient selection of putative transgenic of this cultivar.

Kanamycin as selective agent was often used in genetic transformation of gerbera (Elooma et al 1993; Nowak et al. 1997; Nagaraju et al. 1998), however; their minimal concentrations that kill non-transgenic cells differed from cultivar to cultivar. In addition, minimal concentration suitable for this cultivar has also not been reported yet. Hence, suitable concentration of kanamycin for efficient selection of the transgenic plants of the cultivar could not be predicted. In this study, minimal concentration of kanamycin to be used for gerbera cv. Gold Eye was 50 mgl^{-1}.

Genetic transformation

When petiole segments co-cultivated with *A. tumefaciens* LBA4404, which harbors the plasmid pBICaMV with *CAX1* gene, were inoculated on the shoot regeneration medium containing 50 mgl^{-1} of kanamycin, untransformed explants (control) turned yellow and gradually died, whereas transformed explants initiated shoot buds from the cut surfaces of the explants after 3 weeks of culture. After 5 weeks of culture, formation of shoots was clearly observed (Fig. 1C), and a total number of shoots (12 shoots) were obtained from about 500 explants co-cultivated with *CAX1*gene and transferred to a PGR-free media with 50 mgl^{-1} of kanamycin (Fig. 1D). Eight out of 12 shoots were successfully rooted in the PGR-free media containing kanamycin after 10 days of culture, and the rooted plants survived well in the greenhouse (Fig. 1E)

Table 2 Effects of different concentrations of kanamycin on shoot regeneration from petiole explants cultured on medium containing 1.0 mgl^{-1} BA and 0.1 mgl^{-1} IAA

kanamycin (mgl^{-1})	Survival rate (%)	No. of shoot per explant
25	45a	2.1a
35	19b	1.3b
50	0c	0c
70	0c	0c
100	0c	0c

Means marked with the same letter in the same column are not significantly different by DMRT at the 5% level.

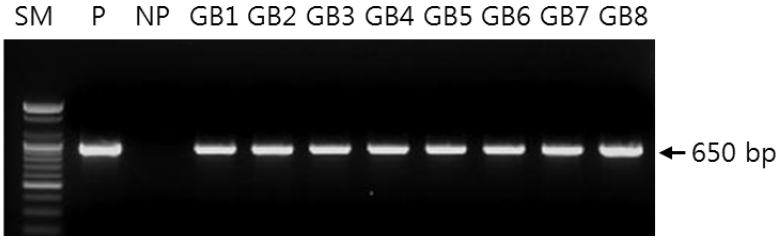

Fig. 2 Detection of the presence of *CAX1* in different transgenic lines (GB1-8) by PCR analysis. SM indicates the size marker, while P and NP stand for plasmid and non-transformed plant (wild type), respectively

PCR analysis was conducted using genomic DNA extracted from leaves of rooted shoots in order to detect the presence of transgenes. In all the transgenic lines, the expected size of *CAX1* (650 bp) were respectively observed, whereas these were not detected in the non-transgenic plant (NP) (Fig. 2).

Clod stress is one of the major environmental factors that adversely affect growth, productivity, physiological, biochemical and molecular changes in plants (Gulzar et al. 2011). Therefore, production of crops genetically improved for cold resistance is necessary. It has been well known that conventional breeding methods have been constraint on successful production of important crops tolerating the cold stress. In addition, improvement of abiotic stress tolerance by induction of in vitro variations did not meet much success. It is important, thus, to find out alternative strategies for production of cold stress tolerant crops. *Agrobacterium*-mediated transformation has been increasingly using as new strategy to produce transgenic plants having improved tolerance to cold stress (Wani et al 2008, 2011; Gosal et al. 2009). Thus far, a number of genes that have been characterized for freezing stress tolerance had been transferred to many crops, suggesting that expressions of those genes are playing important roles for both cold tolerance (Hsieh et al. 2002) and cold acclimation (Knight et al. 1999, Tamminen et al. 2001). Catala et al (2003) claimed that *CAX1* controls induction of *CBF/DREB1* and enhances cold tolerance in *Arabidopsis*. However, its heterologous expression regulating cold tolerance has not been investigated in any important crops. In this study, we could produce the commercially important ornamental plant gerbera cv. Gold Eye expressing the cold tolerant gene *CAX1,* and its functional role as further assessment improving cold tolerance will be investigated in further researches.

Conclusion

In this study, we have optimized a combination of plant growth regulator that induced proper shoot regenerability from petiole explant of the commercially important gerbera cv. Gold Eye. In addition, minimal concentration of kanamycin that is mostly used as selective agent for screening of putative transgenic plants was also optimized. By using these optimized factors, we could successfully produce gerbera transgenic lines expressing *Arabidopsis* Ca^{2+}/H^{+} antiporter gene (*CAX1*) that improves cold tolerance. Despite no further necessary assessments, we expect that expression of the gene will be improving cold tolerance under cold stress condition.

Acknowledgment

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agri-Bio industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs(MAFRA)(315002-5). This research was supported by Kyungpook National University Bokhyeon Research Fund, 2015

References

Barbosa MHP, Pinto JFBP, Pinto CABP, Innecco R (1994) *In vitro* propagation of *Gerbera jamesonii* Bolus ex Hook cv. Appel Bloesem using young capitulum. Revista Ceres, 41:386-395

Catala R, Santos E, Alonso JM, Ecker JR, Martinez-Zapater JM, Salinas J (2003) Mutations in the Ca2+/H+ transporter CAX1 increase CBF/DREB1 expression and the cold-acclimation response in Arabidopsis. Plant Cell 15:2940-2951

Chakrabarty D, Datta SK (2008) Micropropagation of gerbera: lipid peroxidation and antioxidant enzyme activities during acclimatization process. Acta Physiologiae Plantarum

Chung YM, Cho YC, Kwon OC (2007) A New Yellow Gerbera Cut Flower Cultivar, "Gold Eye" with Strong Peduncle and High Flower Yielding. Korean J. Breed. Sci. 39:86-87

Elooma P, Honkaanen J, Puska R, Seppanen P, Helariutta Y, Mehto M, Nevalainen L, Teeri TH (1993) *Agrobacterium*-mediated transfer of antisense chalcone synthase cDNA to *Gerbera hybrida* inhibits flower pigmentation. Bio/Technology, 11: 505-511

Gosal SS; Wani SH; Kang MS (2009) Biotechnology and drought tolerance. J. Crop Improv., 23, 19-54

Hasbullah NA, Taha RM, Awal A (2007) Growth optimization and

Hsieh TH; Lee JT; Yang PT; Chiu LH; Charng Y; Wang YC; Chan MT (2002) Heterology expression of the Arabidopsis Crepeat/ dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. Plant Physiol., 129:1086-1094

Knight H; Veale EL; Warren GJ; Knight MR (1999) The sfr6 mutation in Arabidopsis suppresses low temperature induction of genes dependent on the CRT/DRE sequence motif. Plant Cell, 11:875-886

Nagaraju V, Srinivas GSL, Lakshmi Sita G (1998) *Agrobacterium*-mediated genetic transformation in *Gerbera hybrida*. Current Science, 71:630-634

Naing AH, Ai TN, Jeon SM, Park KI, Lim SH, Lim KB, Kim CK (2016) Novel antibiotics enhance regeneration and genetic transformation with RsMYB1 gene of recalcitrant chrysanthemum cv. Shinma. Plant Biosystems. doi:10.1080/11263504. 2015. 1103800

Nowak E, Makowska Z, Kucharska D, Orlikowska T (1997) The influence of initial explant on transformation effectiveness of *Gerbera hybrida*. Biotechnologia (Poznan), 4:27-38

Orlikowska T., Nowak E., Marasek A, Kucharska D (1999) Effects of growth regulators and incubation period on *in vitro* regeneration of adventitious shoots from gerbera petioles. Plant Cell Tiss. and Organ Cult, 59:95-102

Aswath C, Choudhary ML (2002) Rapid plant regeneration from *Gerbera jamesonii* Bolus callus cultures. Acta Botanica Croatica, 61:125-134

Reynorid JP, Chriquid D, Noin M, Brown S, Marie D (1993) Plant propagation from *in vitro* leaf culture of several gerbera species. Plant Cell Tiss. and Organ Cult, *33*:203-210

Rganogenesis of Gerbera jamesonii Bolus ex. Hook f. *in vitro.* Pakistan Journal of Biological Science, 11;1449-1454

Tamminen I; Mäkelä P; Heino P; Palva ET (2001) Ectopic expression of ABI3 gene enhances freezing tolerance in response to abscisic acid and low temperature in Arabidopsis thaliana, Plant J., 25, 1-8

Tyagi P, Kothari SL (2004) Rapid *in vitro* regeneration of *Gerbera jamesonii* (H. Bolus ex Hook f.) from different explants. Indian Journal of Biotechnology, 3:584-586

Wani SH; Gosal SS (2011) Introduction of OsglyII gene into Indica rice through particle bombardment for increased salinity tolerance. Biol. Plant. 55:536-540

Wani SH; Sandhu JS; Gosal SS (2008) Genetic engineering of crop plants for abiotic stress tolerance. In: Advanced Topics in Plant Biotechnology and Plant Biology. Malik CP; Kaur B; Wadhwani C; Eds. MD Publications New Delhi, pp. 149-183

Xi M and Shi JS (2003) Tissue culture and rapid propagation of *Gerbera jamesonii*. Journal Wanjing Forestry University, 27: 33-36