Floriculture and Ornamental Biotechnology ©2009 Global Science Books

Agrobacterium-mediated Genetic Transformation of Carnation for Insect Resistance

Jitender Kumar Kanwar • Surinder Kumar*

Department of Biotechnology, University of Horticulture and Forestry, Solan-173 230 (H.P.), India

Corresponding author: * **kanwar_btc@rediffmail.com**

ABSTRACT

Plant regeneration and genetic transformation techniques have been developed in leaf tissue of carnation (*Dianthus caryophyllus* L. cv. 'Indios'). Callus was induced on Murashige and Skoog (MS) medium supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Highest shoot regeneration from callus was obtained with 2 mg/l thidiazuron (TDZ) and 1 mg/l indole-3-acetic acid (IAA). Carnation plantlets were able to regenerate within two months of culture. *Agrobacterium*-mediated genetic transformation was achieved using leaf explants and a synthetic gene encoding the *cry1Ab* δ–endotoxin of *Bacillus thuringiensis*. Highest callus induction was achieved on selective medium containing 100 mg/l kanamycin (Kan) and 500 mg/l cefotaxime (Cef) after 96-hr pre-conditioning following 72-hr co-cultivation. Highest number of shoots per callus was observed when MS medium was supplemented with 2 mg/l TDZ, 1 mg/l IAA, 100 mg/l Kan and 500 mg/l Cef. Shoots were elongated and multiplied on MS medium containing 1 mg/l 6-benzyladenine and solidified with 1% agar. Rooting was accomplished on half-strength MS medium supplemented with 2 mg/l indole-3-butyric acid and 50 mg/l Kan. The putative shoots were hardened with 71% survival in a glasshouse. The transformed shoots were analyzed for the presence of the *cry1Ab* gene by PCR. The expression of the *cry1Ab* gene in the leaves of transgenic shoots was detected by an insect bioassay performed with the larvae of carnation bud borer (*Helicoverpa armigera*).

Keywords: *cry1Ab* gene, *Dianthus caryophyllus*, growth regulators, *Helicoverpa armigera*, leaf
Abbreviations: BA, 6-benzyladenine; ***Bt***, *Bacillus thuringiensis*; **CIM**, callus induction medium; **Cef**, cefotaxime; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kan**, kanamycin; **Kn**, kinetin; **NAA**, α-naphthalene acetic acid; ***npt* II**, neomycin phosphotransferase; **SRM**, shoot regeneration medium; **TDZ**, thidiauron (*N*-phenyl-*N*'-1,2,3-thiadiazol-5-ylurea)

INTRODUCTION

Carnation is one of the world's most important cut flowers. Due to its excellent keeping quality, wide range of forms, ability to withstand long-distance transportation and remarkable ability to rehydrate after continuous shipping, it is preferred by growers to rose and chrysanthemum in several flower exporting countries (Bhatt *et al*. 1989). It ranks next to rose in popularity in the United States (Xia *et al*. 2006). Being an important commercial crop, application of plant tissue culture and plant genetic engineering in carnation cultivars is of special value to obtain improved or desired traits like disease and insect resistance. Carnation is severely affected by diseases and pests. *Helicoverpa armigera* causes considerable economic loss by infesting 40 to 90% of flower buds in carnation (Sood and Kakar 1990). It is also an important pest in cotton (Katageri *et al*. 2007; Tohidfar *et al*. 2008) and chickpea (Gujar *et al*. 2007; Ahid *et al*. 2008)).

Modern agriculture uses a wide variety of insecticides to control insect damage. Most of them are chemically synthesized, notable exceptions are the insect toxins produced by *Bacillus thuringiensis* (*Bt*), which is an entomocidal bacterium that forms insecticidal protenaceous crystals during sporulation. The crystal protein is toxic to Lepidopteran, Dipteran or Coleopteran insects (Aronson *et al*. 1986). Microbial formulations of *Bt* have been applied to agricultural crops as a bio insecticide for over two decades, but poor persistence and high costs incurred by multiple applications have limited product acceptance. Major insect pests are developing resistance to most classes of chemical insecticides and environmental pollution caused by insecticidal chemicals is a serious problem. In view of the environmental hazards, modern biotechnological tools could be of much significance to alleviate the negative effect of such chemicals. Therefore, the use of transgenic crops, expressing foreign genes could be an important aspect of integrated pest management (Boulter 1993). *Agrobacterium*-mediated genetic transformation of elite Indian genotypes of cotton was achieved using shoot apical meristem isolated from seedling as explants and a synthetic gene encoding *cry1Ac* δ-endotoxin of *Bt* (Katageri *et al*. 2007).

Regeneration in carnation from callus explants has been reported to be difficult and depends upon the genotype, explant and plant growth regulators (PGRs) (Kallak *et al*. 1997). Efforts devoted to genetic transformation via callus phase in carnation are limited to the work of a few investigators (Shiba and Mii 2005). Several procedures for transferring *Agrobacterium*-mediated marker and desirable genes have been described (Kiss *et al*. 2000; Zuker *et al*. 2001; Bae and Yu 2002; Nontaswastri and Fukai 2005; Kinouchi *et al*. 2006). Before using this technique, it was important to know whether callus mass in *Dianthus caryophyllus* cv. 'Indios' was able to regenerate whole plantlets and conditions required for such plant regeneration and to standardise the technique of *Agrobacterium*-mediated gene transfer in carnation cells with insect resistance (*cry1Ab*) gene. The work described here was initiated to establish a protocol for plant regeneration from callus in cv. 'Indios' using *in vitro* leaf explants and genetic transformation and expression of the *cry1Ab* gene.

Received: 1 June, 2009. Accepted: 6 July, 2009.

Original Research Paper

Table 1 Shoot regeneration frequency of carnation from leaf explants

Treatments (mg/l)						Shoot regeneration (%)	Average number of shoots per callus
BA	Kn	TDZ	Zeatin	NAA	IAA		
2	0	0	0	1	0	24.53 (29.54)	5.5 (2.54)
2	0	0	0	0	1	38.67 (38.44)	4.2 (2.27)
0	2	0	0	1	0	23.13 (28.73)	5.8 (2.61)
0	2	0	0	0	1	42.00 (40.39)	6.8 (2.79)
0	0	2	0	1	0	46.00 (42.70)	12.2 (3.62)
0	0	2	0	0	1	49.33 (44.62)	12.0 (3.58)
0	0	0	2	1	0	32.17 (34.55)	4.2 (2.26)
0	0	0	2	0	1	45.17 (42.23)	4.2 (2.26)
$CD_{0.05}$						(0.31)	(0.04)

Figures in parentheses are arc sine and square root transformed values

MATERIALS AND METHODS

Explant source

Leaves (0.5 cm^2) from aseptic cultures (four-weeks old) of *Dianthus caryophyllus* L. cv. 'Indios' maintained on MS (Murashige and Skoog 1962) medium supplemented with 1 mg/l BA in the Department of Biotechnology, University of Horticulture and Forestry, Solan, India were used as explants.

Culture medium and culture conditions

To achieve callus induction, leaf explants were cultured in 100 ml flasks (Borosil, Bombay, India) with the abaxial surface down on MS medium supplemented with vitamins, 100 mg/l *myo*-inositol, 30 g/l (w/v) sucrose, 8 g/l (w/v) Difco bacto agar (LobaChemie, Bombay, India) and 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D)(callus induction medium, CIM). The concentration of 2,4-D was selected on the basis of preliminary work carried out in the department. The pH of the medium was adjusted with 1 N HCl and/or 1 N NaOH to 5.8. Medium was autoclaved at 1.1 kg/cm^2 for 15 min at 121°C. All the cultures were maintained at 24 ± 2°C with a 16-hr photoperiod (50-60 µmol/m^2/s) provided by white, cool fluorescent tubes (40 W each, Philips). After four weeks of culture, the calli were transferred to shoot regeneration medium (SRM) consisting of MS medium supplemented with various concentrations of PGRs alone or in combination such as 6-benzyladenine (BA), kinetin (Kn), thidiazuron (TDZ), zeatin, indole-3-acetic acid (IAA) and α-naphthalene acetic acid (NAA). In all, a total of 27 concentrations and combinations of PGRs were used, although only the treatments that produced shoots are presented in **Table 1**. The shoots elongated and were multiplied on MS medium supplemented with 1 mg/l BA and solidified with 1% agar. The regenerated shoots were separated and individual shoots were transferred to MS medium (½ strength) containing 2 mg/l indole-3-butyric acid (IBA) and 0.2% activated charcoal (E. Merck (India) Ltd., Bombay) for root induction and elongation to get complete plantlets. The regenerated plantlets were acclimatized by the method described by Kanwar and Kumar (2009).

Agrobacterium strain and plasmid

Disarmed *A. tumefaciens* strain EHA 105 harbouring binary vector pBin Bt1 containing the *cry1Ab* (National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi) and *npt*II (neomycin phosphotransferase) genes driven by CaMV and NOS promoters, respectively (**Fig. 1**).

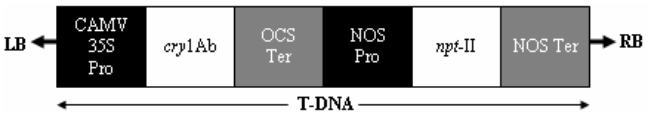

Fig. 1 Structure of expression vector: T-DNA region of pBin Bt1, containing (i) transcriptional fusion of NOS promoter with coding region of *npt-II* and NOS terminator and (ii) transcriptional fusion of CaMV 35S promoter with the coding region of *cry1Ab* and NOS terminator. LB, left border of T-DNA; RB, right border of T-DNA; 35S, CaMV 35S promoter; NOS-ter, nopaline synthase terminator; NOS-pro, nopaline synthase promoter; *npt-II*, neomycin phosphotransferase-II; *cry1Ab*, insect resistance gene; OCS-ter, octopine synthase terminator.

Genetic transformation

The leaf explants were cut into small pieces (0.5 cm^2 in size) and inoculated on CIM and pre-conditioned for 96 hrs. Fresh cultures of *Agrobacterium* were centrifuged for 10 min at 5000 rpm and supernatant was discarded to get a pellet. This pellet was resuspended in MS liquid medium to get a concentration of 10^8 cells/ml. The bacterial suspension and pre-cultured leaf explants were used for co-cultivation experiments. Leaf explants were immersed into bacterial suspension for 5 min and then were blotted dry with a pre-sterilized filter paper and inoculated on CIM for co-cultivation in the dark. After co-cultivation for 72 hrs, explants were transferred to fresh selective callus induction medium containing 100 mg/l Kan (HiMedia, Bombay, India) and 500 mg/l Cefotaxime (Cef; Ranbaxy, India) for the selection of transformed cells and to inhibit further bacterial growth. Leaf explants were subsequently subcultured to fresh selective CIM in order to check excessive bacterial growth. After four weeks, callus pieces (0.8-1 cm^2) were subcultured on selective SRM containing MS salts supplemented with 2 mg/l TDZ, 1 mg/l IAA, 100 mg/l Kan and 500 mg/l Cef. Escapes were removed by subculturing green shoots on selective SRM at an interval of four weeks (two cycles) and regenerated shoots were rooted and acclimatized as explained under 'Culture medium and culture conditions'.

Statistical analysis

Each treatment consisted of at least 15 explants, and each experiment was repeated thrice. Data recorded for different parameters were subjected to completely randomized design (Gomez and Gomez 1984). The statistical analysis based on mean values per treatment was made using ANOVA.

Polymerase chain reaction analysis

Genomic DNAs were isolated from the leaves of Kan resistant shoots following the procedure of Offringa and Lee (1995). PCR analysis was carried out to detect the presence of the *cry1Ab* gene using forward primer 5′-CTCCTCTCCGTCTACGTCC-3′ and reverse primer 5′-GGGCCCCTTCACCGATGTTCC-3′. The primers were designed by Bangalore Genei, Bangalore, India on the basis of DNA sequences of Fujimoto *et al.* (1993). Plasmid DNA used in transformation served as a positive control and DNA isolated from non-transformed (control) plants was used as a negative control.

Each PCR reaction mixture (20 µl) consisted of 10.2 µl Milli Q water, 0.4 µl of 2 U *Taq* polymerase, 2 µl of 10X *Taq* DNA polymerase buffer, 1.8 µl of 25 mM $MgCl_2$, 1.5 µl of 200 mM dNTPs, 1 µl of 5 pM each primer and 2 µl of 50 ng genomic DNA. The conditions for PCR reactions were; one cycle a 94°C for 4 min as pre-heating, 32 cycles at 94°C for 60 sec (denaturation), 55°C for 90 sec (annealing), 72°C for 120 sec (extension) and one cycle at 72°C for 5 min (final extension). The amplified products were separated on 1.4% agarose gel and visualized with ethidium bromide using a UV transilluminator and photographed using gel documentation system (Alpha Imager, USA).

Insect bioassay

Laboratory bioassays were performed using second instar larvae of *Helicoverpa armigera* reared on an artificial diet consisting of chickpea soaked in water for 48 hrs. The tests were conducted using excised young apical leaf tissues from control and transformed plants. Visual observations on the feeding behaviour (leaves consumed by larvae) and mortality rate of larvae were recorded daily up to four days.

RESULTS AND DISCUSSION

After two weeks of inoculation, the size of the leaf segments had increased. Callus was observed on the cut ends of the explants and the entire surface of the explants was covered with callus after four weeks of inoculation. Shoots started originating from calli subcultured on SRM after four weeks. Per cent calli forming shoots and average number of shoots formed per callus were recorded, with the highest response on MS medium supplemented with 2 mg/l TDZ and 1 mg/l IAA. Shoot elongation and multiplication were achieved on MS medium supplemented with 1 mg/l BA. Shoots (2-3 cm length) were excised and cultured on ½-strength MS medium supplemented with 2 mg/l IBA and 0.2% activated charcoal. The rooted plantlets were transferred to pots containing a mixture of sand: soil: FYM (1: 1: 1) and acclimatized.

Among various PGRs tested in MS medium for shoot regeneration, 2 mg/l each of BA, Kn, TDZ and zeatin in combination with NAA or IAA were able to form shoots from callus. 2 mg/l TDZ in combination with 1 mg/l IAA was more effective in inducing shoots than any other combination (**Table 1**). Kumar *et al.* (2006) achieved 27 shoots from callus mass on MS medium supplemented with 0.6 mg/l TDZ and 0.6 mg/l zeatin in carnation cv. 'Candy'. Frey and Janick (1991) achieved maximum proliferation from petal explant-derived calli with 0.05 µM TDZ and 0.5 µM NAA in cvs. 'Scania', 'White Sim' and 'Sandra'. TDZ was more effective than BA in inducing shoot formation and proliferation in heat tolerant carnation 'German Red' (Sankhla *et al*. 1995). However, in our study, continuous culture on TDZ medium resulted in abnormal shoots and higher rate of hyperhydricity. Similar results were reported by Ahmad *et al*. (2006) in carnation 'Finest Mix'. Therefore, shoots were elongated and multiplied on MS medium supplemented with 1 mg/l BA and solidified with 1% agar. In our experiment, 27 growth regulators combinations were used for shoot regeneration (full data set not shown), but only a few treatments (**Table 1**) responded indicating that 'Indios' is recalcitrant. These findings supported the results of other researchers (Nakano and Mii 1992; Shiba and Mii 2005) who reported difficulty in regenerating plants from highly de-differentiated callus cultures.

Leaf segments were pre-cultured on CIM for 96 hrs, then co-cultivated with *A. tumefaciens* for 72 hrs and then transferred to the fresh selective CIM containing antibiotics. Callus formed after two weeks at the cut edges of the leaf segments and also at the wound site, where the tissue was damaged during inoculation (**Fig. 2A**). The non-transformed (control) tissue did not survive on selective medium containing 100 mg/l Kan and 500 mg/l Cef. The developing transformed calli were transferred to selective SRM. The explants gave rise to green callus from which shoots developed (**Fig. 2B**); these shoots were subjected to two more cycles (four weeks per cycle) on the same medium to remove escapes. Three independent transformed lines (T_1, T_2 and T_3) were selected in the presence of Kan; these were elongated and multiplied. The Kan-resistant shoots were transferred onto rooting medium for rooting (**Fig. 2C**) and acclimatized. The transformed plants were carefully nurtured in the glasshouse (**Fig. 2D**). PCR analysis showed the presence of the *cry1Ab* gene in these shoots (**Fig. 3**). The transformation frequency (percentage of total number of PCR-positive shoots to total number of infected explants) was low (2.5%) (**Table 2**). Probably the recalcitrant nature

Table 2 Transformation efficiency of *in vitro*-derived leaf explants.

Number of explants infected	120
Number of Kan resistant shoots	3
Number of PCR positive shoots	3
Transformation efficiency* (%)	2.5

*Transformation efficiency is percentage of total number of PCR positive shoots to total number of explants infected

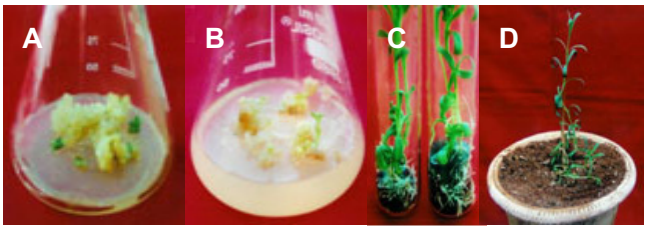

Fig. 2 Plant regeneration and genetic transformation studies in carnation (*Dianthus caryophyllus* L. cv. 'Indios') leaf tissue. (**A**) callus formation on selective callus induction medium in leaf explants; (**B**) shoot regeneration on selective shoot regeneration medium after four weeks; (**C**) rooting of regenerated shoots after three months; (**D**) hardened transformed plant of carnation.

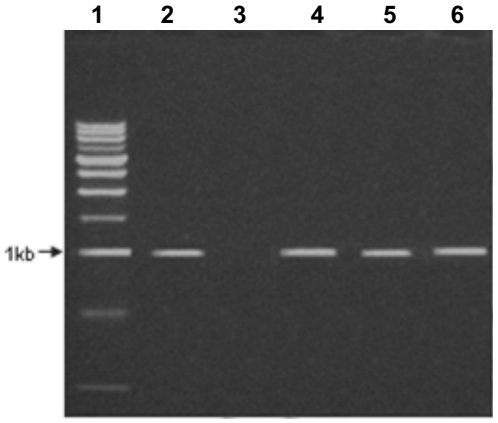

Fig. 3 PCR amplification of *cry1Ab* gene fragment in transformed carnation plants. Lane 1, a marker (500 bp DNA ladder); Lane 2, plasmid DNA (positive control); Lane 3, DNA sample of non-transformed control plants; Lanes 4-6, DNA sample from transformants T_1, T_2, T_3 (1 kb DNA fragment corresponding to *cry1Ab* gene).

of the carnation cells affects transformation. Franklin *et al*. (2008) reported that the recalcitrant nature of cells affected transformation in *Hypericum perforatum*. Shiba and Mii (2005) reported that there have been no reports on the use of callus or cell suspension cultures for transformation because of the difficulty in regenerating plants from highly de-differentiated cultures in carnation. Therefore, a regeneration system applied widely in transformation studies have been restricted to inducing direct adventitious shoot formation from excised plant tissues such as petals, leaf, apical meristem and internode segments (Lu and Chandler 1995). Kinouchi *et al*. (2006) transformed potted carnation cv. 'Lillipot' with cDNAs for ACC oxidase in sense or antisense orientation or mutated carnation ethylene receptor cDNA by *Agrobacterium* mediated gene transfer and reported transformation frequency of 4% in leaf explants. Van Altvorst *et al*. (1996) reported transformation frequency of 2.5% in *Agrobacterium* mediated transformation of petal explants of carnation cv. 'White Sim'. The expression of the gene was further confirmed by an insect bioassay where 100% mortality was observed within four days of initiation of the bioassay (**Table 3; Fig. 4**).

The genetic transformation of plants offers a new perspective to introduce new insect control genes into crop's gene pool. Insecticidal crystal proteins of *Bt* have long been used to control insect pests (Tabashnik 1997). In recent years, transgenic cultivars expressing the *cry* gene have been shown to defend insect attack in a wide variety of

Table 3 Insect bioassay performed with larvae of control and transgenic (T_1, T_2, T_3) plants (n = 30) of carnation using second instar larvae of *Helicoverpa armigera*.

Genotype	Number of dead larvae died			
	24 hrs	48 hrs	72 hrs	96 hrs
Control	1	0	0	0
T_1	0	2	7	21
T_2	1	3	8	18
T_3	0	2	8	20

* One replication means one larvae feeding on leaf disc of the selected and control plants

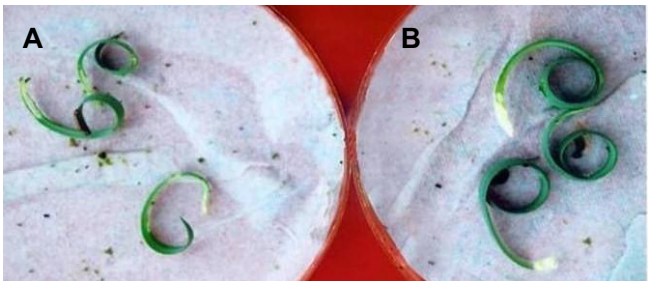

Fig. 4 Insect toxicity bioassay of transformed and non-transformed plants of carnation with second instar larvae of *Helicoverpa armigera*. (**A**) Insect larvae died after feeding the leaves of transformed plants; (**B**) insect larvae survived in non-transformed plants, after four days.

crops (Sharma and Ortiz 2000; Chakrabarty *et al*. 2002; Cao *et al.* 2005; Li *et al*. 2006). However, no work has been done on genetic transformation of carnation using insect resistance gene (*cry1Ab*), except for the present investigation. *Agrobacterium*-mediated genetic transformation of elite Indian genotypes of cotton was achieved using apical meristems/shoot apices for insect resistance (Satyavathi *et al.* 2002; Katageri *et al*. 2007).

The results demonstrated that it is possible to transform carnation by adopting *Agrobacterium*-callus mediated technique whereas direct shoot regeneration system was used earlier in carnation (Van Altvorst *et al*. 1995, 1996; Estopa *et al*. 2001; Zhang *et al.* 2005). The degree of insect protection conferred by the expression of the *cry1Ab* protein in transformed carnation was significant as shown by insect bioassay. The callus-mediated regeneration system seems to be promising and may help in carnation improvement programs.

ACKNOWLEDGEMENTS

Authors are grateful to the Director, National Research Centre on plant Biotechnology, Indian Agricultural Research Institute, New Delhi, India for providing the *Agrobacterium* strain. Jitender K. Kanwar is grateful to the Council of Scientific and Industrial Research, Government of India, New Delhi for financial assistance.

REFERENCES

Ahid MAZ, Siam MMI, Eza MH, Rodhan MHZP, Umana Begum MR (2008) Determination of economic injury levels of *Helicoverpa armigera* (Hubner) in chickpea. *Bangladesh Journal of Agricultural Research* **33**, 555-563

Ahmad N, Srivastva R, Anis M (2006) Improvement in carnation shoot multiplication using thidiazuron *in vitro*. *Propagation of Ornamental Plants* **6**, 109-113

Aronson AI, Beckman W, Dunn P (1986) *Bacillius thuringiensis* and related insect pathogens. *Microbial Review* **50**, 1-24

Boulter D (1993) Insect pest control by copying nature using genetically engineered crops. *Biochemistry* **34**, 1453-1466

Bae KM, Yu SN (2002) Development of viral disease resistance in *Dianthus caryophyllus* by transformation of CaMV CP gene. Development of CaMV CP gene expression system for transformation. *Journal of the Korean Society of Horticultural Science* **43**, 466-470

Bhatt NR, Aswath C, Noorjehan JB, Parthasarathy VA (1989) Carnation. In: Bose TK, Yadav LP (Eds) *Commercial Flowers* (Vol I), Naya Prokash, Calcutta, pp 343-462

Cao J, Shelton AH, Earle FD (2005) Development of transgenic collards (*Brassica oleracea* L. var. Acephala) expressing a *cry1Ac* or *cry1Ac* Bt gene for control of the diamondback moth. *Crop Protection* **24**, 804-813

Chakrabarty R, Viswakarma N, Bhatt SR, Kirti PB, Singh BD, Chopra VL (2002) *Agrobacterium*-mediated transformation of cauliflower. Optimization of protocol and development of *Bt* transgenic cauliflower. *Journal of Bioscience* **27**, 495-502

Estopa M, Marfa V, Mela E, Messeguer J (2001) Study of different antibiotic combinations for use in the elimination of *Agrobacterium* with kanamycin selection in carnation. *Plant Cell, Tissue and Organ Culture* **65**, 211-220

Franklin G, Conceição L, Kombrink E, Dias A (2008) *Hypericum perforatum* plant cells reduce *Agrobacterium* viability during co-cultivation. *Planta* **227**, 1401-1408

Frey L, Janick J (1991) Organogenesis in carnation. *Journal of the American Society of Horticultural Science* **116**, 1108-1112

Fujimoto H, Itoh K, Yamamoto M, Kyozuka J, Shimamoto K (1993) Insect resistant rice generated by introduction of a modified delta endotoxin gene of *Bacillius thuringiensis*. *Biotechnology* **11**, 1151-1155

Gomez KA, Gomez AA (1984) *Statistical Procedures for Agricultural Research*, John Wiley and Sons, New York, pp 328-332

Gujar GT, Khawale RN, Kalia V (2007) Genetic variability of *Helicoverpa armigera* (Hubner) attributable to cadherin gene-specific molecular markers. *Current Science* **92**, 800-808

Kallak H, Reidla N, Hilpus I. Virumae K (1997) Effect of genotype, explants source and growth regulators on organogenesis in carnation callus. *Plant Cell, Tissue and Organ culture* **51**, 127-135

Kanwar JK, Kumar S (2009) Direct adventitious shoot regeneration from leaf and internode explants of *Dianthus caryophyllus. Floriculture and Ornamental Biotechnology* **3**, 31-34

Katageri IS, Vamadevaiah HM, Udikeri SS, Khadi BM, Kumar PA (2007) Genetic transformation of an elite Indian genotype of cotton (*Gossypium hirsutum* L.) for insect resistance. *Current Science* **93**, 1843-1847

Kinouchi T, Endo R, Yamashita A, Satoh S (2006) Transformation of carnation with genes related to ethylene production and perception towards generation of potted carnation with a longer display time. *Plant Cell, Tissue and Organ Culture* **86**, 27-35

Kiss E, Veres A, Galli Z, Nagy N, Toth E, Varga A, Hrazdina G, Heszky L (2000) Production of transgenic carnation with antisence ACS (1-aminocyclopropane-1-carboxylate synthase) gene. *International Journal of Horticultural Science* **6**, 103-107

Kumar SA, Verma A, Singh SK, Raghva SPS, Kumar PA (2006) *In vitro* shoot regeneration from leaf segments of carnation (*Dianthus caryophyllus* L.) via indirect organogenesis. *Plant Cell Biotechnology and Molecular Biology* **7**, 65-68

Li HX, Yin RH, Lu YC, Zhang JH (2006) Inheritance and resistance to insect in *cry1Ac* transgenic cabbage. *Journal of Agricultural Biotechnology* **14**, 546-550

Lu C, Chandler SF (1995) Genetic transformation of *Dianthus caryophyllus* (carnation). In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry,* Springer-Verlag, Berlin-Heidelberg, Germany, pp 156-170

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Nakano M, Mii M (1992) Protoplast culture and plant regeneration of several species in the genus *Dianthus. Plant Cell Reports* **11**, 225-228

Nontaswastri C, Fukai S (2005) *Agrobacterium* mediated genetic transformation of carnation (*Dianthus caryophyllus* L.) using leaf explants derived from *in vitro* plants. *Technical Bulletin of the Faculty of Agriculture, Kagawa University* **57**, 15-19

Nontaswatsri C, Fukai S, Goi H (2004) Revised co-cultivation conditions produce effective *Agrobacterium* modulated genetic transformation of carnation. *Plant Science* **166**, 59-68

Offringa R, Lee F (1995) Isolation and characterization of plant genomic DNA sequence via (inverse) PCR amplification. In: Jones H (Ed) *Methods in Molecular Biology: Plant Gene Transfer and Expression Protocols*, Humana Press Inc., NJ, pp 181-195

Sankhla D, Davis TD, Sankhla N, Upadhyaya A (1995) *In vitro* regeneration of the tolerant 'German Red' carnation through organogenesis and somatic embryogenesis. *Gartenbauwissenschaften* **60**, 228-233

Satyavathi VV, Prasad V, Gita Lakshmi B, Lakshmi Sita G (2002) High efficiency transformation protocol for three Indian cotton varieties via *Agrobacterium tumefaciens*. *Plant Science* **162**, 215-223

Sharma HC, Ortiz R (2000) Transgenics: pest management and the environment. *Current Science* **79**, 421-437

Shiba T, Mii M (1995) *Agrobacterium tumefaciens* mediated transformation of highly regenerable cell suspension culture in *Dianthus acicularis*. *Journal of Horticultural Science and Biotechnology* **80**, 393-398

Sood AK, Kakar KL (1990) Incidence, biology and control of *Helicoverpa armigera* on carnation (*Dianthus caryophyllus*). *Indian Journal of Agricultural Sciences* **60**, 367-368

Tabashnik B (1997) Seeking the root of insect resistance to transgenic plants. *Proceedings of the National Academy of Sciences USA* **94**, 3488-3490

Tohidfar M, Ghareyazie B, Mosavi M, Yazdani S, Golaichain R (2008) *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum*)

using a synthetic *cry1Ab* gene for enhanced resistance against *Heliothis armigera*. *Iranian Journal of Biotechnology* **6**, 164-172

Van Alvorst AC, Koehorst HJJ, De Jong J, Dons JJM (1996) Transgenic carnation plants obtained by *Agrobacterium tumefaciens*-mediated transformation of petal explants. *Plant Cell, Tissue and Organ Culture* **45**, 169-173

Van Alvorst AC, Riksen T, Koehorst HJJ, Dons JJM (1995) Transgenic carnation obtained by *Agrobacterium tumefaciens* mediated transformation of leaf explants. *Transgenic Research* **4**, 105-113

Xia Y, Deng X, Zhou P, Shima K, Teixeira da Silva JAT (2006) The World floriculture industry: dynamics of production and markets. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol IV), Global Science Books, Isleworth, UK, pp 336-347

Xiang Y, Wong WKR, Ma C, Wong RSC (2000) *Agrobacterium* mediated transformation of *Brassica compestris* ssp. *Parachinensis* with synthetic *Bacillus thuringiensis cry1Ab* and *cry1Ac* genes. *Plant Cell Reports* **19**, 251-256

Zhang S, Zhu LH, Li XY, Ahlman A, Welander M (2005) Infection by *Agrobacterium tumefaciens* increased the resistance of leaf explants to selective agents in carnation (*Dianthus caryophyllus* L. and *D. chinensis*) *Plant Science* **168**, 137-144

Zuker A, Shklarman E, Scovel G, Ben MH, Ovadis M, Neta SI, Ben YY, Weiss D, Watad A (2001) Genetic engineering of agronomic and ornamental traits in carnation. *Acta Horticulturae* **560**, 91-94