Plant Cell Reports (1996) 15:642–646

Plant Cell Reports

Shoot regeneration and *Agrobacterium*-mediated transformation of *Fragaria vesca* L.

Iman El Mansouri [1], José A. Mercado [2], Victoriano Valpuesta [3], José M. López-Aranda [2], Fernando Pliego-Alfaro [1], and Miguel A. Quesada [1]

[1] Departamento de Biología Vegetal, Universidad de Málaga, 29071 Málaga, Spain
[2] Centro de Investigación y Desarrollo Agrario, Finca Cortijo de la Cruz, 29140 Churriana, Málaga, Spain
[3] Departamento de Bioquímica y Biología Molecular, Universidad de Málaga, 29071 Málaga, Spain

Received 7 August 1995/Revised version received 16 October 1995 – Communicated by H. Lörz

Summary. An efficient and reliable method for shoot regeneration from leaf disks of *Fragaria vesca* L. has been developed. This protocol has been successfully employed to obtain transformed plants using *Agrobacterium tumefaciens* as gene vector. Murashige and Skoog basal medium supplemented with benzyladenine (4 mg/l) and indole-3-butyric acid (0.25 mg/l) induced the maximum percentage of shoot regeneration (98%) and the highest number of shoot colonies per explant (4.6) after 8 weeks of culture. Isolated shoots would elongate and proliferate when the benzyladenine concentration was lowered to 0.5 mg/l. The established protocol for shoot regeneration was employed to transform leaf disks using *Agrobacterium tumefaciens* carrying the plasmid pBI121. A 7.7% of the inoculated explants showed kanamycin resistance after 10 weeks of selection in a medium containing 25 mg/l of this antibiotic. The transgenic shoots obtained were rooted in the presence of 25 mg/l kanamycin and successfully acclimatized. The final percentage of transformation obtained based on beta-glucuronidase expression was 6.9%.

Abbreviations: BA:benzyladenine; IBA: indole-3-butyric acid; MS: Murashige and Skoog basal medium; LSD: least significant difference; NOS: nopaline synthase promoter; NPTII: neomycin phosphotransferase (EC 2.7.1.95); CaMV35S: cauliflower mosaic virus promoter; GUS: beta-glucuronidase (EC 3.2.1.31); LB: Luria Broth base; CTAB: hexadecil trimethyl ammonium bromide; PCR: polymerase chain reaction; X-gluc: 5-bromo-4-chloro-3-indolyl-glucuronide

Introduction

In vitro studies in strawberry have followed two main trends. A great amount of effort has been focused towards the development of meristem culture techniques for micropropagation of pathogen free plants (Boxus et al. 1977, Swartz and Lindstrom 1986, Boxus 1992, López-Aranda et al. 1994). There have also been studies dealing with direct and indirect organogenesis from other explant types such as leaves, petioles and runners (Jones et al. 1988, Liu and Sanford 1988, Nehra et al. 1989, Nehra et al. 1990a). Some of the regeneration media developed have been employed to optimize transformation protocols mediated by *Agrobacterium tumefaciens* (James et al. 1990, Nehra et al. 1990bc, James et al. 1993, Mathews et al. 1995) that will facilitate gene manipulation on this species. However, the fact that *Fragaria x ananassa* contains an octoploid genome makes it difficult to use this species as a model for molecular studies and the interpretation of the transformation events. Apparently, only one case of segregation in the R1 transformed progeny has been reported (James et al. 1990). The wild strawberry (*Fragaria vesca*), that contains a diploid genome (2n=2x=14), represents an ideal model to study foreign gene expression in *Fragaria*. Moreover, transformed plants could be used to transfer introduced genes to the octoploid cultivated strawberry by conventional breeding techniques (Bringhurst and Voth 1984). In spite of these advantages, not much effort has been dedicated to set up an efficient system for regeneration and transformation of *Fragaria vesca*. A previous study reports direct shoot formation from leaf explants at low frequency (15%) as well as indirect organogenesis from petiole and leaf derived callus (Greene and Davis 1991). The possible role of cell wall precursors in organogenesis and differentiation processes in callus cultures has also been studied (Bois 1992), but additional research is still needed to clarify the role of these compounds. In this research we report the development of a rapid and reliable method for high-frequency direct shoot regeneration and its application to *Agrobacterium tumefaciens* mediated transformation of the wild strawberry.

Correspondence to: M. A. Quesada

Materials and methods

Plant material and culture conditions. Seeds of *Fragaria vesca* were obtained from a pool of wild populations collected in Spain. Seeds were treated with concentrated sulphuric acid as reported by Jelenkovic et al. (1991) and germinated in glass jars containing the MS inorganic formulation at full strength (Murashige and Skoog 1962) supplemented with 0.8% agar (Sigma A-1296). The obtained seedlings were used as the source of explants. In order to develop a shoot regeneration media, young leaves were excised from the seedlings and following removal of the proximal and distal parts of the blade, they were placed abaxial side up in 250 ml glass jars containing 50 ml of MS basal medium (Murashige and Skoog 1962) supplemented with BA (1, 2, 3 and 4 mg/l) and IBA (0.25, 0.5 and 1 mg/l) and solidified with 0.8% agar. Explant regeneration percentage and number of shoot colonies per explant were scored after 4, 6 and 8 weeks of culture. Shoots regenerated from leaf explants were transferred to MS basal medium supplemented with IBA (0.25 mg/l) and different concentrations of BA (0.5, 1, 2, 3 and 4 mg/l) in order to induce shoot proliferation. Afterwards, the effect on rooting of 1 mg/l IBA added to basal medium was studied. In all cases pH was adjusted to 5.74 and media were sterilized for 20 min at 121°C. Cultures were incubated at 25°C under a 16 h photoperiod of 12.5 and 25 $\mu mol.m^2.s^{-1}$ for shoot induction and proliferation respectively. Regeneration experiments were carried out in triplicate with two jars per treatment and 8 explants per jar. Arcsine transformation (Sokal and Rohlf 1981) was applied to all percentages obtained. Mean separation of the transformed percentages was performed by LSD.

Histological procedures. Leaf explants were sampled during the first 4 weeks of culture in the shoot regeneration medium and fixed in Bouin solution (Gabe 1968). The fixed pieces were dehydrated in an ethanol-butanol series and embedded in paraffin. Transverse sections (10 µm) were hydrated and stained with toluidine blue (Gabe 1968).

Effect of kanamycin on shoot regeneration. The kanamycin sensitivity of the explants was tested by culturing leaf explants on shoot regeneration medium containing filter sterilized kanamycin at 0, 10, 25 and 50 mg/l. Callus formation and shoot regeneration were scored after 4, 6 and 10 weeks of culture.

Agrobacterium strain and plasmid. The *Agrobacterium tumefaciens* strain LBA4404 containing the non-oncogenic plasmid pAL4404 and the binary vector pBI121 was employed in the transformation experiments. The pBI121 plasmid contains the NOS/NPTII gene for kanamycin resistance, and the CaMV35S/GUS chimaeric gene as reporter (Jefferson et al. 1987). This binary vector was mobilized into *Agrobacterium* by the freeze-thaw method (Holsters et al. 1978). Bacteria were grown at 28°C in LB medium containing 50 µg/ml of kanamycin.

Transformation procedure and characterization of transformants. Routinely, transformation experiments were initiated with 150-200 explants. Leaves were excised and cultivated 2 days on Petri dishes containing 25 ml of the shoot regeneration medium. After this induction period, explants were placed in 50 ml tubes, inoculated with an overnight grown *Agrobacterium* culture diluted 1/10 in MS basal medium and gently shaken for 20 min. The infected explants were blotted dry on sterile filter paper and cocultivated on the shoot regeneration medium for 3 days. Explants were then transferred to a selection medium (the medium found to be optimum for shoot regeneration) supplemented with 25 mg/l kanamycin and 500 mg/l carbenicillin. After 4 weeks on the selection medium, the carbenicillin concentration was lowered to 250 mg/l. Shoots regenerated after 10 weeks of culture were elongated in the shoot proliferation medium and rooted in MS basal medium, both media containing 25 mg/l kanamycin. GUS enzyme activity in the transgenic shoots was determined by histochemical and fluorogenic assays as reported by Jefferson (1987). *In vitro* leaves (50-100 mg) were used to extract genomic DNA by the CTAB method as modified by Lassner et al. (1989) and used to amplify the *nptII* gene by PCR according to Lipp Joao and Brown (1993).

Results

Shoot Regeneration

The results obtained with different combinations of IBA and BA are shown in Fig. 1. Shoot regeneration occurred in all treatments, but the percentages observed were higher in the range 3-4 mg/l BA. The best responses were obtained when 0.25 mg/l IBA was present in the medium, whereas higher auxin levels decreased shoot regeneration. Small amounts of friable and transparent callus appeared at the edge of the explants in all treatments. The highest percentage of explants regenerating shoots (98%) and the highest number of shoot colonies per explant (4.6) were induced by the treatment including BA 4 mg/l and IBA 0.25 mg/l (Fig.1).

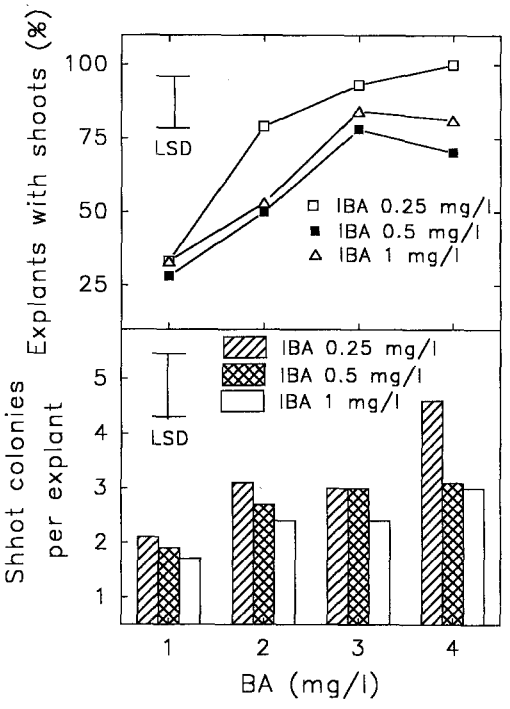

Fig.1. Effect of BA and IBA concentration on shoot regeneration percentage and number of shoot colonies per explant, in leaf disks of *Fragaria vesca* after 8 weeks of culture.

Histological studies revealed active proliferation of subepidermal cells at 2 weeks (Fig. 2A), afterwards meristem-like structures could be observed (Fig 2B). Shoot regeneration along the periphery of the explants was visually detected within 4-6 weeks (Fig. 2C). Most explants showed well developed shoots (5mm) within 8 weeks (Fig. 2D). Elongation of the regenerated shoots was improved by lowering the BA concentration, and the best results were obtained at BA and IBA concentrations of 0.5 and 0.25 mg/l, respectively. Four weeks after transfer to this medium, the final shoot lengths observed ranged

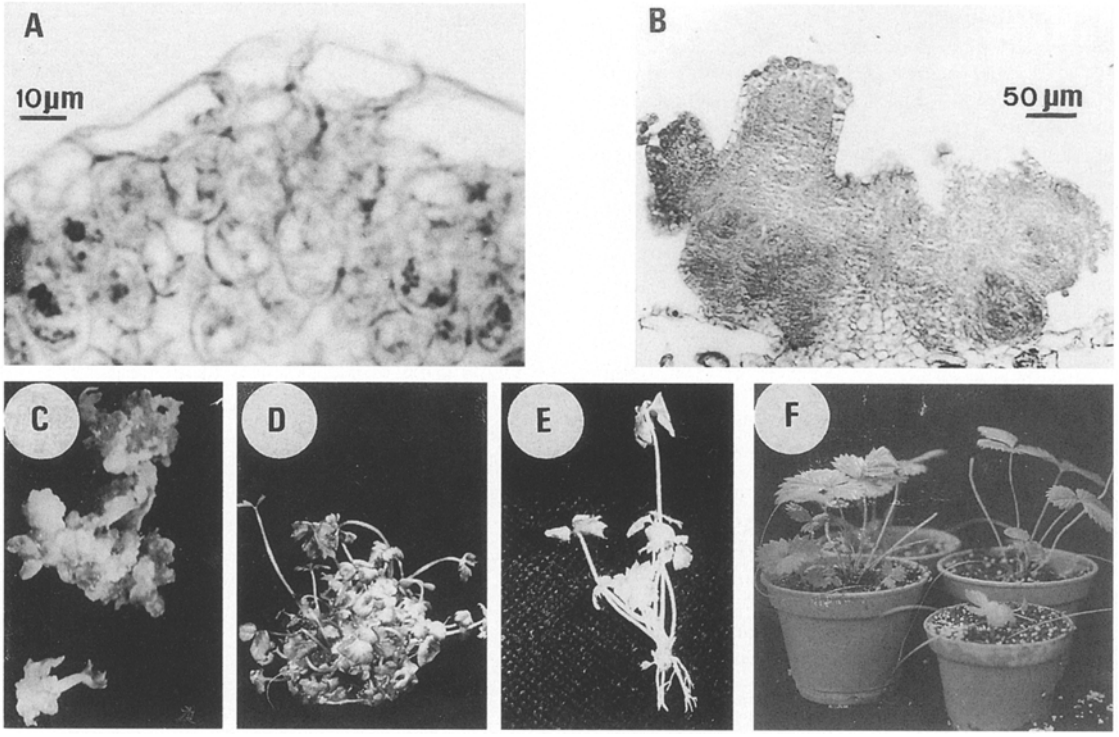

Fig.2. A and B: cross sections of leaf explants after 2 and 4 weeks of culture in the induction medium. C-F: different phases of *Fragaria vesca* regeneration from leaf disks. Shoot induction after 6 weeks of culture (C), elongation after 8-10 weeks (D), shoot rooting (E) and acclimatized plants (F).

from 12 to 20 mm, contrasting with shoot lengths of 4 -6 mm in media with BA in the range 2-4 mg/l. Shoots were isolated and rooted in MS basal medium within 2 weeks. The presence of IBA (1 mg/l) decreased the number of rooted shoots from 87.5 to 37.5%, and affected the morphology of the roots that were shorter, thicker and more abundant. Roots also appeared in shoots elongated in the medium containing 0.5 mg/l BA and 0.25 mg/l IBA (Fig. 2E), so in this case transfer to basal medium was not necessary. Rooted shoots could be successfully acclimatized following the procedure reported by López-Aranda et al. (1994) (Fig. 2F).

Once the best regeneration medium was known, the sensitivity of the explants to kanamycin was determined. Shoot induction and callus proliferation were strongly decreased at all concentrations assayed. Even at 10 mg/l kanamycin, shoots viable at 4 weeks became necrotic at 6 weeks (Table 1). Thus, although a concentration in the range 25-50 mg/l could be used for selection, 25 mg/l of kanamycin was chosen because of the high sensitivity of *Fragaria vesca* leaf disks to this antibiotic.

Agrobacterium-mediated transformation

Table 2 summarizes the results obtained according to the established transformation protocol. Six weeks after inoculation with *Agrobacterium* only 40 explants showed cell proliferation, the remaining disks were bleached and some of them turned brown and necrotic. As expected, all the non-infected disks showed green meristematic regions.

Table 1. Effect of kanamycin concentration on shoot organogenesis in *Fragaria vesca*. Mean separation of the arcsine transformed percentages in columns by LSD. Different letters within a column indicate significant differences at the 5% level.

	Weeks		
	4	6	10
Kanamycin (mg/l)	Explants with shoots (%)		
0	94.7a	98.3a	98.3a
10	7.6b	7.6b (dead)	7.6b (dead)
25	0c	0c	0c
50	0c	0c	0c

The surviving infected explants with meristematic regions were fragmented and subcultured in the same regeneration medium supplemented with 250 mg/l carbenicillin. Only 25 of these explants had a healthy appearance and regenerated shoots after 4 additional weeks of selection. Ninety percent of the kanamycin resistant shoots showed GUS activity as detected by the histochemical and fluorogenic assays (Fig. 3A). The staining with X-gluc was observed throughout the entire rooted plant (results not shown). The presence of the *nptII* gene in the DNA of the transgenic plants was confirmed by PCR (Fig. 3B). The absence of *Agrobacterium* contamination was shown

Table 2: Frequency of transformation in explants of *Fragaria vesca* after continuous selection on 25 mg/l kanamycin, using the disarmed binary vector pBI121. The results correspond to one experiment but are representative of the percentages observed in subsequent experiments.

Initial number of explants	144	100%	
Explants with microcalluses after 6 weeks of selection	40	27.8%[a]	
Transfer to new media and fragmentation of explants			
Number of fragmented explants with microcalluses	90	---	100%
Fragmented explants regenerating shoots after 10 weeks of selection	25	7.7%[a]	27.7%
Final percentage of transformation (GUS positive)	23	6.9%[a]	25.5%

[a]Final percentages are related to the initial number of explants and represent independent transformation events.

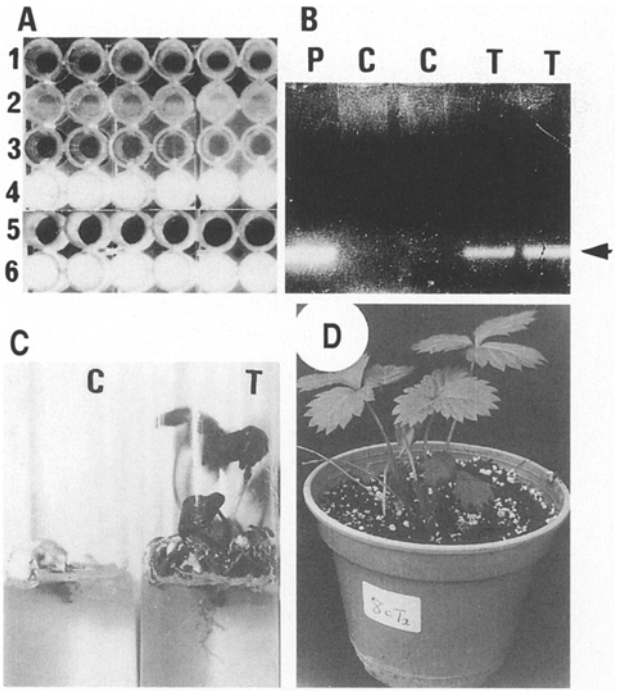

Fig.3. A: Fluorogenic assay of the GUS activity. Row 1: control extracts plus incubation medium without fluorogenic substrate. Row 2: idem including the substrate. Rows 3 and 5: extracts of putative transformed plants plus incubation medium without fluorogenic substrate. Rows 4 and 6: idem including the substrate. Each well represents an independent plant. B: PCR amplification of the nptII gene electrophoresed on 2% agarose gel, arrow indicates the 255 bp fragment amplified. Line P: pBI121 plasmid; lines C: control plants; lines T: transgenic plants. C: Transgenic (right) and control (left) shoots rooted in a medium with 25 mg/l of kanamycin. D: Acclimatized transgenic plant.

by cultivation of leaf fragments on LB medium. These shoots were able to root in the presence of 25 mg/l kanamycin (Fig. 3C) and after rooting were acclimatized and transferred to the greenhouse (Fig. 3D). Transgenic shoots have also been maintained for several subcultures in the proliferating medium including kanamycin but lacking carbenicillin, without any sign of *Agrobacterium* contamination. As shown in Table 2, the final efficiency of the transformation experiment evaluated by kanamycin resistance was 7.7% whereas it was 6.9% when determined by the GUS expression assay.

Discussion

Regeneration of several cultivars of *Fragaria x ananassa* has been found to be enhanced as result of supplementary nitrogen in the medium (Liu and Sanford 1988, Sorvari et al. 1993). In the case of *Fragaria vesca*, excellent regeneration was obtained using Murashige and Skoog's mineral salts (1962) without any additional nitrogen. In accordance with previous results obtained with the cultivated strawberry (Liu and Sanford 1988, Barceló et al. 1993, Sorvari et al. 1993) a high BA/IBA ratio yielded excellent results in *Fragaria vesca* regeneration. The high BA content of the organogenesis medium inhibited shoot elongation so the concentration was lowered to 0.5 mg/l to induce elongation and proliferation of shoots. The histological study indicated that the adventitious shoots mainly developed from subepidermal cells, but a single cell origin could not be established. Callus development was very low and the organogenesis can be defined as *quasi-direct* (Buiatti and Morpurgo 1990) with a low risk of somaclonal variation.

Fragaria vesca was extremely sensitive to kanamycin as reported for others berries (Graham et al. 1990). This sensitivity enabled us to use a low concentration of kanamycin (25 mg/l) for selection with a very low percentage of escapes. Similar levels of kanamycin have been used in transformation of *Fragaria x ananassa* (Nehra et al. 1990bc, Mathews et al. 1995). The regeneration protocol developed was successfully employed to introduce foreign genes in this species via *Agrobacterium tumefaciens*, with a percentage of transformation of 7.7 based on kanamycin resistance and 6.9 based on GUS activity. This percentage was as high as the best percentage reported for *Fragaria x ananassa* by Nehra et al. (1990b) using the same vector pBI121, and higher than those reported by James et al. (1990) using the binary plasmids pBIN6 and pSS1. The persistence of resistance to kanamycin after several subcultures of the transgenic shoots might indicate the integration of the T-DNA in the plant genome. Moreover, GUS activity was observed throughout the whole regenerated plantlets. However, presence of the T-DNA in the plant genome was only detected by PCR analysis in one of the clones

obtained when using newly formed young leaves due to the difficulty to extract DNA suitable for molecular analysis from leaves of elongated shoots of this species. Studies to optimize the DNA extraction method are currently under way.

The procedure reported here makes it feasible to use *Fragaria vesca* as an appropriate model for molecular studies as well as an additional way to introduce agronomical traits in the genome of *Fragaria* species via gene manipulation.

Acknowledgments. The authors thank Ms Mª. Dolores Mota for her technical assistance. Ms Iman El Mansouri was supported by a doctoral fellowship from the ICMA (Spain). Dr Jose A. Mercado was supported by a fellowship from the DGIEA (Junta de Andalucía). Financial support was obtained from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Grant SC93-072.

References

Barceló M, López-Aranda JM, Pliego-Alfaro F (1993) Actas de Horticultura 10: 988-992.

Bois F (1992) Plant Cell, Tissue and Organ Culture 28:91-96.

Boxus P (1992) In: Kurata K, Kozai T (eds) Transplant Production Systems. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 151-162.

Boxus P, Quoirin M, Laine JM (1977) In: Reinert J, Bajaj YPS (eds) Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture. Springer-Verlag, New York, pp 130-143.

Bringhurst RS, Voth V (1984) Iowa State Journal of Research 33:371-381.

Buiatti M, Morpurgo R (1990) In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry. Somaclonal Variation in Crop Improvement. Vol 11. Springer-Verlag, Berlin, pp 400-415.

Gabe M (1968). Techniques Histologiques. Masson, Paris.

Graham J, McNicol RJ, Kumar A (1990) Plant Cell, Tissue and Organ Culture 20:35-39.

Greene AE, Davis TM (1991) In: Dale A, Luby JJ (eds) The Strawberry into the 21st Century. Proceedings of the Third North American Strawberry Conference, Houston Texas, pp 124-125.

Holsters M, de Waele D, Depicker A, Messens E, Van Montagu M, Schell J (1978) Mol Gen Genet 163:181-187.

James DJ, Passey AJ, Barbara DJ (1990) Plant Science 69:79-94.

James DJ, Passey AJ, Dandekar AM, Uratsu SL, Viss P (1993) Acta Horticulturae 336:179-184.

Jefferson RA (1987) Plant Mol Biol Rep 5:387-405.

Jefferson RA, Kavanagh TA, Bevan MW (1987) EMBO J 6:3901-3907.

Jelenkovic G, Chin C, Billings S, Eberhardt J (1991) In: Dale A, Luby JJ (eds) The Strawberry into the 21st Century. Proceedings of the Third North American Strawberry Conference, Houston Texas, pp. 91-97.

Jones OP, Waller BJ, Beech MG (1988) Plant Cell, Tissue and Organ Culture 12:235-241.

Lassner MW, Peterson P, Yoder JI (1989) Plant Mol Biol Rep 7:116-128.

Lipp Joao KH, Brown TA (1993) Plant Cell Reports 12:422-425.

Liu ZR, Sanford JC (1988) HortScience 23:1057-1059.

López-Aranda JM, Pliego-Alfaro F, López-Navidad I, Barceló-Muñoz M (1994) J Hortic Sci 69:625-637.

Mathews H, Wagoner W, Kellogg J, Bestwick R (1995) In Vitro Cell Dev Biol 31:36-43.

Murashige T, Skoog F (1962) Physiologia Plantarum 15:473-497.

Nehra NS, Stushnoff C, Kartha KK (1989) J Amer Soc Hort Sci 114:1014-1018.

Nehra NS, Stushnoff C, Kartha KK (1990a) Plant Science 66:119-126.

Nehra NS, Chibbar RN, Kartha KK, Datla RSS, Crosby WL, Stushnoff C (1990b) Plant Cell Reports 9:293-298.

Nehra NS, Chibbar RN, Kartha KK, Datla RSS, Crosby WL, Stushnoff C (1990c) Plant Cell Reports 9:10-13.

Sokal RK, Rohlf FJ (1981) Biometry. W.H. Freeman, San Francisco, CA.

Sorvari S, Ulvinen S, Hietaranta T, Hiirsalmi H (1993) HortScience 28:55-57.

Swartz HJ, Lindstrom JT (1986) In: Zimmerman RH, Griesbach RJ, Hammerschlag FA, Lawson RH (eds) Tissue Culture as a plant Production System for Horticultural Crops. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, pp 201-220.