Note

Plant Biotechnology, **19** (4), 271-275 (2002)

Plant Regeneration from Embryogenic Calli Derived from Immature Seeds in Miniature Rose Cultivar. 'Shortcake'; Somaclonal Variation, Cytological Study and RAPD Analysis

Gyoichi ASANO and Shizufumi TANIMOTO*

Genetic Engineering Laboratory, Faculty of Agriculture, Saga University, Saga 840-8502, Japan
**Corresponding author* E-mail address: tanimoto@cc.saga-u.ac.jp

Received 31 January 2002; accepted 1 July 2002

Abstract

The immature seeds were harvested from self-pollinated miniature rose cv.'Shortcake'. When the immature seeds were cultured on the Murashige and Skoog's (MS) medium without phytohormone for 3 months, the pale yellow and friable embryogenic calli were induced. The calli were subcultured on the MS medium with 1 mg l^{-1} 6-benzyladenine (BA) every 2 to 3 weeks for 3 months, many adventitious shoots were differentiated and average number of shoots per callus was 7.7. These shoots were easily separated and rooted on the half strength MS medium with 0.25 mg l^{-1} IBA.

It was clarified that both original and regenerated plants were tetraploid by the chromosome observation and ploidy analysis. All regenerated plants showed genetic variations from the original plants, such as flower size and color, petal number, simple leaf shape and prickle number. Polymorphic DNA differences were also observed between the original and regenerated plants by the RAPD analysis.

Key words: adventitious shoot, embryogenic callus, Miniature rose cv.'Shortcake', ploidy analysis, RAPD analysis, somaclonal variation.

The direct and/or indirect plant regenerations from various organs of genus Rosa have been studied, such as leaf or petioles (Lloyd *et al.*, 1988; de Wit *et al.*, 1990; Rout *et al.*, 1991; Toyoda *et al.*, 1993; Fukui and Imaida, 1996; Kintzios *et al.*,1999), filament (Noriega and Sondahl, 1991), stem internode or pith tissues (Jacobs *et al.*, 1968; Ishioka and Tanimoto, 1990; Rout *et al.*, 1991; van der Salm *et al.*, 1996), meristems (Fukui and Imaida, 1996), root tips (Matthew *et al.*, 1991; Mottley *et al.*, 1996; van der Salm *et al.*, 1996; Yokoya *et al.*, 1996) and immature seeds (Burger *et al.*, 1990; Kunitake *et al.*, 1993; Visessuwan *et al.*, 1997). Main plant materials of those studies were wild rose and *R. hybrida*. In the present paper, miniature rose cv. 'Shortcake' was chosen for the plant material, because it might be expected *in vitro* that the growth rate and plant regeneration potential of the miniature rose is higher than that of wild rose and *R. hybrida*, due to the small size of the plant.

The organogenesis (Burger *et al.*, 1990) and the embryogenesis (Kunitake *et al.*, 1993) of the immature embryos of rose plants have already been reported. Present paper describes the plant regeneration from the embryogenic calli derived from immature seeds of the miniature rose 'Shortcake', somaclonal variation, cytological study and RAPD analysis of the original and regenerated plant have been carried out.

Plants of miniature rose cv. 'Shortcake' [*Rosa* cv. Polyantha (*R. multiflora* × *R. chinensis*) × *R. chinensis* var. *minima*] were obtained from Keisei Rose Nurseries (Chiba, Japan). These rose plants have been cultivated in a green house for 3 years.

After 3-4 weeks of the self-pollination, developing hips (~10x15 mm) were harvested. Intact immature seeds (3-5 mm) excited from hips were sterilized with 15% (v/v) NaOCl for 5 min, rinsed twice with sterilized water, then longitudinally cut by a scalpel into two pieces. Half-divided embryos of intact immature seeds were placed onto the medium consisted of Murashige and Skoog's mineral salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.25% Gelrite (Merck) (hereafter referred to as MS medium) with no phytohormone, and cultured at 25 ℃ under 16 h light/8 h dark condition for 3 months without any subculture. The induced embryogenic calli were transplanted onto the callus proliferation medium consisted of the MS medium with 0.25 mg l^{-1} 6-benzyladenine

(BA) and 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA), subcultured every 2-3 weeks at 25 ℃ under dark condition.

In order to study the effects of cytokinin on the plant regeneration, the embryogenic calli (about 3 g per piece, fresh weight) were transplanted onto the plant regeneration medium consisted of the MS medium with 0, 0.5, 1, or 2 mg l^{-1} BA, and subcultured every 2-3 weeks for 3 months at 25 ℃ under 16 h light/8 h dark condition. Many shoots formed on calli were transplanted onto the rooting medium (Hasegawa, 1980), consisted of the half strength MS medium with 0.25 mg l^{-1} 3-indolbutyric acid (IBA), cultured at 25 ℃ under 16 h light/8 h dark condition for one month. After the acclimation treatment, the plantlets were transplanted on the soil in a green house.

Unopened young leaves (~3 mm) were incubated in 2 mM hydroxyquinolin for 4 h at 20 ℃ under dark condition, then fixed and decolorized in the fixing solution (acetic acid : ethanol = 1:3) for 24 h at room temperature.

The explant was rinsed twice with distilled water, cut with a razor blade into fine pieces and then macerated in a 1.5 ml micro tube carring 100 μl of the enzyme mixed solution consisted of 0.3% Cellulase RS (YAKULT HONSHA Co., LTD.), 0.2% Pectolyase Y-23 (Kikkoman Corp.), 75 mM KCl and 7.5 mM EDTA for 3 h at 37 ℃ . The pellets were rinsed with the buffer consisted of 75 mM KCl and 7.5 mM EDTA then spun down by a centrifuge under 700*g* for 5 min at 4 ℃ . The pellet was rinsed twice with the fixing solution, finally suspended in 100 μl of the fixing solution again, mixed well with a vortex.

In order to spread the pellet uniformly on the clean surface of a slide glass, 10 μl of the pellet solution was dropped down from about 15 cm height onto a slide glass, and then the slide glass was dried in a desiccator overnight.

The slide glass was incubated in 2% (v/v) Giemsa's Solution (MERCK) (Darling and Cour 1960, Ma *et al.*, 1996) and 1/30 mM Na-phosphate buffer for 1 h then incubated in distilled water for 10 min, finally dried in the desiccator again. Chromosome samples were soaked with a drop of xylol, covered with a cover glass and ready for the observation with an optical microscope (magnification = 1000).

A young simple leaf (~5x5 mm) of the plant was placed on a plastic plate, soaked with several drops of nuclei extraction solution (Partec, high resolution DNA kit type-P), chopped with a razor blade into pieces and incubated at room temperature for 3 min. The collected explant was filtered (30 μm), incubated in 4'-6-diamino-2-phenylindole dye solution (Partec, high resolution DNA kit type-P) for 3 min at room temperature.

The peak position of fluorescence light intensity emitted from nuclei in the explant solution was measured with a ploidy analyzer (Partec type-PA).

Young leaves (0.3 g fresh weight) of the original and regenerated plants were pulverized in the liquid nitrogen with a mortar and pestle. The genomic DNA was extracted from pulverized samples and purified with a kit (NIPPON GENE, ISOPLANT Ⅱ). The 20 μl mixed solution consisted of 100 ng μl^{-1} DNA (from original or regenerated plant) 0.5 μl, 10x buffer (250 mM KCl, 100 mM Tris-HCl, 15 mM MgCl, 1% Triton X-100) 3.2 μl, 1.2 mM dNTPs 1.6 μl, 10 pM μl^{-1} each random primer (OPERON TECHNOLOGY, OPA-01~20 and OPD-01~20, in total 40 primers) 1 μl, 5,000 u ml^{-1} r*Taq* DNA polymerase (TOYOBO) 0.1 μl and deionized water 13.6 μl, covered with a drop of mineral oil (SIGMA) were mixed in 200 μl micro tubes for PCR. The DNA samples were amplified with a PCR amplifier (TECHNE, Techgene) under the heat cycle condition consisted of 94 ℃x0.5 min, [94 ℃x0.5 min, 40 ℃x2 min, 72 ℃x3 min] x40 cycles and 72 ℃x8 min (Torres, 1993). All amplified PCR products, 80 products in total, were run in electrophoresis at 100 voltage, 1 h 20 min on the 0.8% agarose gel in TBA buffer (Tris 40 mM, Acetic acid 40 m M, EDTA 1 mM) with 0.8 mg l^{-1} ethidium bromide. The polymorphic DNA on the agarose gel was observed under the UV light and recorded with a CCD-camera (FAS-Ⅲ, TOYOBO).

The initiation frequency of embryogenic callus was 3.3% as shown in **Table 1**. This value was almost similar to previous report (Kunitake *et al.*, 1993). The feature of embryogenic callus was friable, globular and/or leafy structure, so it is easy to separate them from non-embryogenic callus as shown in **Fig. 1**. The growth rate of the embryogenic callus was about two times greater than that of non-embryogenic callus.

Table 1 Frequency of embryogenic callus induction from immature seeds on MS medium without phytohormone for 3 months.

Experiment	No. of seeds	No. of embryogenic callus	No. of non-embryogenic callus
1 st.	20	1 (5 %)	0
2 nd.	20	1 (5 %)	1 (5 %)
3 rd	20	0	1 (5 %)
Total	60	2 (3.3%)	2 (3.3%)

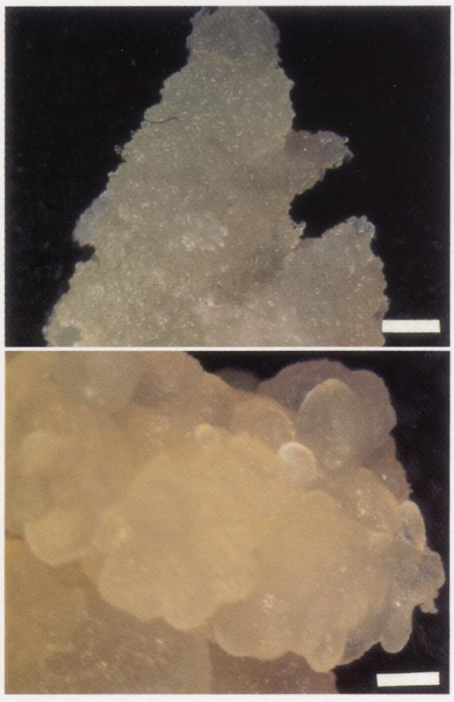

Fig. 1 Morphological features of non-embryogenic callus (top) and embryogenic callus (bottom) of miniature rose cv. 'Shortcake'. (bar = 1 mm)

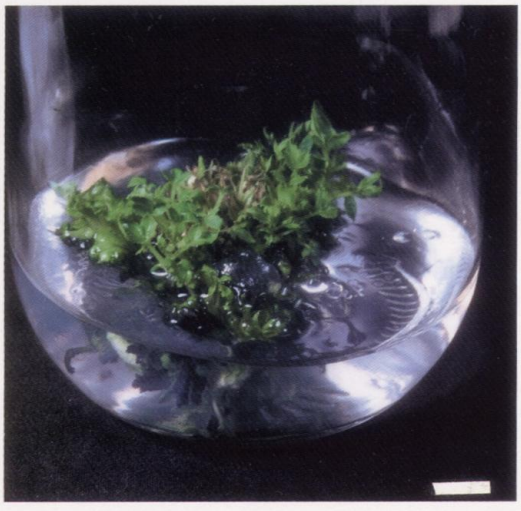

Fig. 2 Shoots regenerated on an embryogenic callus subcultured every 2-3 weeks on MS medium with 1 mg l^{-1} BA for 3 months. (bar = 1 cm)

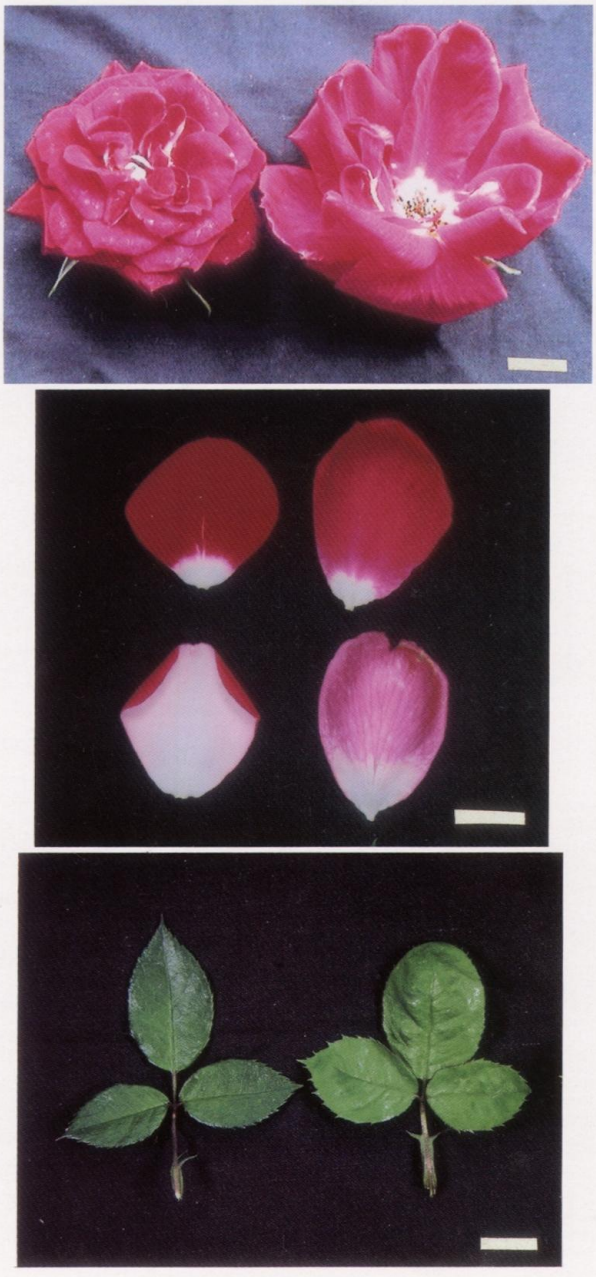

Fig. 3 Flowers (top), adaxial (middle top) and abaxial (middle bottom) surface of petal and trifoliate leaves (bottom) of original plant (left), regenerated plant (right) of miniature rose cv. 'Shortcake'. (bar = 1 cm)

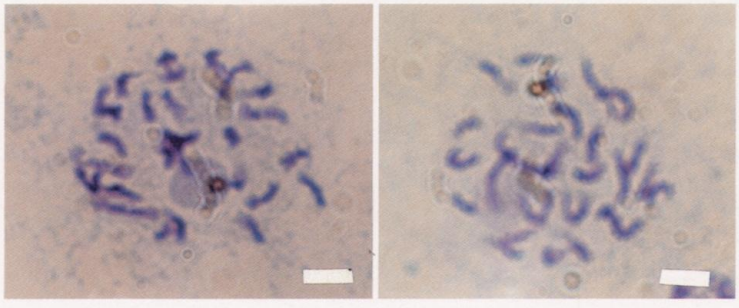

Fig. 4 Somatic chromosome configuration of miniature rose cv. 'Shortcake', original plant (left) and regenerated plant (right). (Both plants are tetraploid., 2n = 4X = 28). (bar = 2 μm)

The effects of cytokinin, BA, on the plant regeneration was shown in **Table 2**. The number of adventitious shoots emerged from embryogenic calli were counted after 3 months from the transplantation onto the regeneration medium with 0, 0.5, 1, or 2 mg l^{-1} BA. The optimal concentration of BA was 1 mg l^{-1}. As shown in **Fig. 2**, many adventitious shoots were come up from an embryogenic callus. After one month later from the transplantation of shoots onto the rooting medium, 1/2 MS medium with 0.25 mg l^{-1} IBA, 80% of shoots developed roots successfully. After the acclimation treatment, plantlets were transplanted onto the soil in a green house and 70% of them grew successfully.

Some morphological variations were observed on the regenerated plants. For example, the flower size of regenerated plant was a little bit larger than that of the original plant. However, the petal number of the regenerated plant was less than that of the original one as shown in **Fig. 3** (top). The average number of petals was 17.9 and 25.6 respectively. The color of the adaxial petal surface of the original plant is red but the color of the abaxial one is white. However the white color of the abaxial petals surface of the regenerated plant is almost diminished, subsequently the flower color is rather bright pinkish red. The size of petals of the regenerated plant was a little bit larger than that of original plant but the petal shape was almost same as shown in **Fig. 3** (middle). The simple leaf shape of regenerated plant is rather round, different from that of the original one which is rather elliptic, as shown in **Fig. 3** (bottom). The mean value of the ratio in length/width of a simple leaf is 1.22 and 1.75 and prickle number of regenerated plants is reduced from 1.26 cm^{-1} (shoot length) of original plant to 0.22 cm^{-1} as shown in **Table 3**. The comparison of mean values in **Table 3** was studied by using Student's test (Brandt, 1970).

Moreover the prickle shape of the regenerated plant is thinner than that of the original one. The cytological studies of genus Rosa have been reported, for instance, *R. chinensis* Jacq was diploid(Saitoh, 1969), *R. damascena* was tetraploid (Tabaeezadeh and Khosh-khui, 1981), *Rosa wichuraiana* Creep × *R. roxburghii* Thratt. was diploid, *R. hybrida* 'Sunflare' and 'Angel Face'were tetraploid (Ma, *et al.*, 1996), *R. hybrida* was tetraploid, and R. rugosa was diploid (Souq *et al.*, 1996) . Photos of chromosome of the original and regenerated plant of miniature rose'Shortcake'were shown in **Fig. 4** (left) and **Fig. 4** (right). The chromosome number of both original and regenerated plant was 2n = 28 (at least 30 cells were observed), therefore the original and regenerated plant was obviously tetraploid. It was also studied that the ploidy index comparison of three regenerated, one original plant with a wild rose *R. chinensis* Jacq. (2x = 14), as a reference by using the ploidy analyzer.

The result was shown in **Table 4**. All ploidy index of the three regenerated plants and original plant was almost same but not just 2. The reason of the slight index deviation from 2 is not clear but it might be caused by slight fluctuations of fluorescence intensity emitted from nuclei of each plant or the effect of heteroploid (Saitoh, 1969) however the ploidy analyzer PA has not enough resolution power

Table 2 Effects of cytokinin (BA) on plant regeneration from embryogenic calli derived from immature seeds after 3 months transplantation on MS medium with BA.

BA (mg l^{-1})	No. of embryogenic callus	Total no. of shoots regenerated	No. of shoots per embryogenic callus[1)]
0	21	0	0
0.5	21	23	1.1
1	21	161	7.7
2	21	36	1.7
4	21	5	0.2

[1)] about 3 g fresh weight.

Table 3 Morphological variations (somaclonal variation) in regenerated plants

Plant	Flower diameter (cm)	Petal number	Simple leaf (l/w)[1)]	Prickle no. per cm (shoot length)
Regenerated	5.4 ± 0.69[2)]	17.9 ± 2.7[3)]	1.22 ± 0.09[4)]	0.22 ± 0.09[5)]
Original	4.6 ± 0.57[2)]	25.6 ± 2.6[3)]	1.75 ± 0.2[4)]	1.26 ± 0.53[5)]

[1)] (l/w) = (length/width) of simple leaf

[2)] α (level of significance) = 0.25

[3), 4), 5)] α = 0.01

Table 4 Ploidy analysis of an original plant and plants regenerated from embryogenic calli derived from immature seeds.

Plant		Index (average ± standard error)
Regenerated[1)]	R1	1.925 ± 0.011
	R2	1.927 ± 0.019
	R3	1.883 ± 0.022
Original		1.898 ± 0.019
R. chinensis Jacq (2x, wild rose)		1.000

[1)] R1+R2+R3 = 10

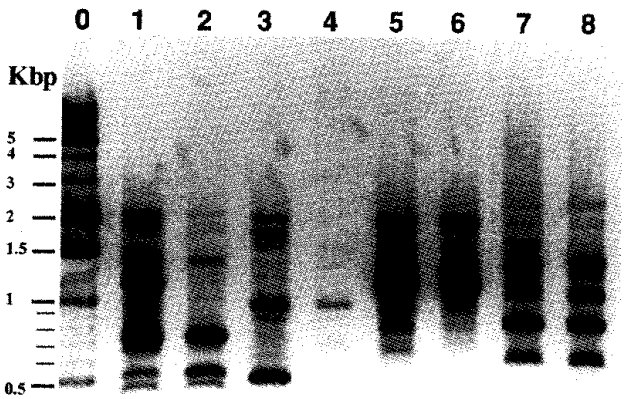

Fig. 5 RAPD of original and regenerated plant of miniature rose cv.'Shortcake'. Lane 1, 3, 5, 7; original plants, lane 2, 4, 6, 8; regenerated plant, lane 0; DNA marker, lane 1, 2; OPA-04 , lane 3, 4; OPA-07, lane 5, 6; OPA-14, lane 7, 8; OPD-08, were used as primers.

to analyze the heteroploid.

It is obvious that the somaclonal variations in the morphology of the regenerated plant have no relation with the ploidy number.

The results of RAPD analysis of the original and regenerated plants were shown in **Fig. 5**. Four random primers (OPA-04; 5'AATCGGGCTG, OPA-07; 5'GAAACGGGTG, OPA-14; 5'TCTGTGCTGG, OPD-08; 5'GTGTGCCCCA) out of 40 (OPA-01~20, OPD-01~20), presented the different DNA patterns between the original and regenerated plants, the others showed the same DNA pattern or no pattern. Some minor variations of the polymorphic DNA were observed in several regenerated plants but the major polymorphic DNA was almost reserved. The results of RAPD analysis suggested that the polymorphic DNA variation of the regenerated plant was caused by the differences of genes.

The regenerated plant from embryogenic calli derived from immature self-pollinated seeds of miniature rose cv.'Shortcake' was a genetic variant which seems to be caused by gene separations. Burger *et al.* (1990) reported on the regenerated genetic variants from the calli derived from cross-pollinated immature seeds of the several *R. hybrida*, which have different petal number from their parent. It requires intensive studies to determine the genes which were related with a certain morphological variation, for instance the round shape of the simple leaf of the regenerated plant from the calli derived from immature seeds of the miniature rose cv.'Shortcake'.

The present system should be available for the *Agrobacterium*-mediated transgenic roses to improve their qualities.

Acknowledgements

We thank Prof. N. Nitoh (Kinki University) and Assoc. Prof. H. Kunitake (Miyazaki University) for great helpful advises and valuable suggestions on this manuscript. We also thank Chief E. Ohyabu (Saga Agriculture Inspection Research Center) for the permission to use the ploidy analyzer and technical advises, Mr. A. Asadi Abkenar (Saga University) for the chromosome observation and Ms. H. Komiya (Saga University) for the measurement of ploidy indexes.

References

Brandt, S., 1970. Statistical and Computational Methods in Data Analysis. North-Holland publishing company-Amsterdam, London

Burger, D. W., Liu, L., Zary, K. W., C. I. Lee, C. I., 1990. Organogenesis and plant regeneration from immature embryos of *Rosa hybrida* L. Plant Cell, Tissue Organ Cult., **21**: 147-152.

Darling, C. D., Cour, L. F., 1960. The Handling of Chromosomes. London Allen and Unwin Ltd.

de Wit, J. C., Esendam, H. F., Honkanen, J. J., Tuominen, U., 1990. Somatic embryogenesis and regeneration of flowering plants in rose. Plant Cell Rep., **9**: 456-458.

Dubois, L. A. M., de Vries, D. P., 1996. The direct regeneration of adventitious buds on leaf explants of greenhouse-grown cut rose cultivars. Acta Hort., **424**: 327-331.

Fukui, H., Imaida, K., 1996. Somatic embryogenesis in *Rosa hybrida* L. cv. Barkarole. Res. Bull. Fac. Agr., Gifu

Univ., **61**: 25-30.

Hasegawa, P. M., 1980. Factors affecting shoot and root initiation from cultured rose shoot tips. J. Am. Soc. Hort. Sci., **105**: 215-220.

Ishioka N., Tanimoto, S., 1990. Plant regeneration from Bulgarian rose callus. Plant Cell, Tissue Organ Cult., **22**:197-199.

Jacobs, G., Bornman, C. H., Allan, P., 1968. Tissue culture studies on rose. Use of pith explants. S. Afr. J. Agr. Sci., **11**: 673-678.

Kintzios, S., Manos, C., Makri, O., 1999. Somatic embryogenesis from mature leaves of (*Rosa* sp.). Plant Cell Rep., **18**: 467-472.

Kunitake, H., Imamizo, H., Mii, M., 1993. Somatic embryogenesis and plant regeneration from immature seed-derived calli of rugosa rose (*Rosa rugosa* Thunb.). Plant Sci., **90**: 187-194.

Lloyd, D., Roberts, A. V., Short, K. C., 1988. The induction *in vitro* of adventitious shoots in *Rosa*. Euphytica, **37**: 31-36.

Ma,Y., Islam-FariDi, M. N., Crane, C. F., Stelly, D. M., Price, H. J., Byrne, D. H.,1996. A new procedure to prepare slides of Metaphase Chromosomes of roses. Hort Sci. **31**: 855-857

Matthew, D., Mottley, J., Horan, I., Roberts, A. V., 1991. A protoplast to plant system in roses. Plant Cell, Tissue Organ Cult., **24**: 173-180.

Mottley, J., Yokoya, K., Matthews, D., Squirrell, J., Wentworth, J. E., 1996. Protoplast fusion and its potential role in the genetic improvement of roses. Acta Hort., **424**: 393-397.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., **15**: 473-497.

Noriega, C., Sondahl, M. R., 1991. Somatic embryogenesis in hybrid tea roses. Bio/Technology, **9**: 991-993.

Rout, G. R., Debata, B. K., Das, P., 1991. Somatic embryogenesis in callus cultures of *Rosa hybrida* L. cv. Landola. Plant Cell, Tissue Organ Cult., **27**: 65-69.

Saitoh, K., 1969. Breeding of flower plants. Seibundoh-shinkoh-sha Tokyo (in Japanese).

Souq, F., Coutos-Thevenot, P., Yean, H., Delbard, G., Maziere, Y., Barbe, J. P., Boulay, M., 1996. Genetic transformation of roses, 2 examples: one on morphogenesis, the other on anthocyanin biosynthetic pathway. Acta Hort., **424:** 381-388.

Tabaeezadeh, Z., Khosh-khui, M., 1981. Anther culture of rose. Scientia Horti., **15**: 63-66.

Torres, A. M., 1993. Identifying rose cultivars using random amplified polymorphic DNA markers. Hort. Sci., **28**: 333-334.

Toyoda, H., Yoshida, K., Ogata, Y., Morikawa, Y., Koreeda, K., Chatani, K., Ouchi, S., 1993. Establishment of a system for efficient callus induction and plant regeneration from leaf explants of auxiliary bud-cultured rose plants. Plant Cell Engineering, **10**: 293-297 (in Japanese).

van der Salm, T. P. M., van der Toorn, C. J. G., Hanisch ten Cate, C. H., Dons, H. J. M., 1996. Somatic embryogenesis and shoot regeneration from excised adventitious roots of the rootstock Rosa hybrida L. 'Moneyway'. Plant Cell Rep., **15**: 522-526.

Visessuwan, R., Kawai, T., Mii, M., 1997. Plant regeneration from cell suspension culture derived from immature embryo of rose. Plant Biotechnol., **14:** 29-33.

Yokoya K., Walker, S., Sarasan, V., 1996. Regeneration of rose plants from cell and tissue cultures. Acta Hort., **424**: 333-337.