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Plant Regeneration from Cell Suspension Culture Derived from Immature Embryo of Rose

Rongrong VISESSUWAN, Takashi KAWAI and Masahiro MII

Laboratory of Plant Cell Technology, Faculty of Horticulture, Chiba University, Matsudo, Chiba 271, Japan

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Abstract

Fine cell suspension cultures with plant regeneration ability were established from leaf-like structures which were produced on friable green calli originated from an immature embryo of rose (*R. hybrida* cv. Gelb Dagmer Hastrap × *R. chinensis* var. *mutabilis*). The leaf-like structures were initially cultured in liquid MS medium containing 2 mg/*l* 2, 4-D for inducing calli, which were then transferred to the medium containing a reduced concentration of 2, 4-D (0.01 mg/*l*) for establishing rapidly proliferating fine cells. The cells thus obtained showed the ability to regenerate embryo-like structures on gellan gum-solidified MS medium containing 1 mg/*l* BA. The embryo-like structures developed into normal plantlets through adventitious shoot formation on MS medium containing 0.1 mg/*l* TDZ. The plantlets were successfully established on the soil.

1. Introduction

For the successful application of plant biotechnologies such as somatic hybridization and genetic transformation for plant breeding , it is essential to establish plant regeneration systems from tissue, cell and protoplast cultures. In rose, however, it is still far from applying these technologies to the breeding of roses and only a few studies have been reported on somatic hybridization [10] and genetic transformation [3, 15]. One of the main reasons for the limitation is the difficulty in regenerating plants from the calli with embryogenic potential which have commomly been induced from different tissue sources of roses, because the embryogenic calli are easily lose the ability to regenerate normal somatic embryos during the subculture and, instead, embryo- or leaf-like structures with no apical meristems are produced predominantly. Therefore, it is essential to establish an efficient method of plant regeneration from these abnormal structures.

In protoplast culture of rose, cell suspension cultures with embryogenic potential have been used as a source of protoplasts [6, 9, 10, 14] due to the difficulty in culturing protoplasts from other sources. However, regeneration of plants from protoplasts through somatic embryogenesis has also been difficult due to the loss of normal embryogenic potential during culture and only Matthews *et al.* [10] have succeeded in regenerating whole plants from protoplasts of *R. persica* × *xanthina*. Establishment of fine cell suspension culture with high plant regeneration ability is also a prerequisite for the efficient production of transgenic plants by *Agrobacterium*-mediated or direct gene transfer method.

In this paper, we report the successful establishment of homogeneous cell suspension cultures from immature embryo-derived leaf-like structures (LLS) and the regeneration of plantlets in rose.

2. Materials and Methods

2. 1 Callus material

The friable calli with the potential to produce embryo-like structures (ELS) were used in this study. The calli were initially induced from an immature embryo obtained from cross pollination between *Rosa hybrida* L. cv. Gelb Dagmer Hastrap and *R. chinensis* Jacq. var. *mutabilis* (RHC) on 2 g/*l* gellan gum (Gelrite; Kelco, Division of Merck and Co.Inc., San Diego, CA)-solidified MS medium [11] containing 68 g/*l* sucrose in a growth chamber at 25°C under continuous illumination of cool-white fluorescent light at 38 μmol m^{-2} s^{-1}. The calli were then routinely subcultured on the same medium but containing 30 g/*l* sucrose at 1 month intervals for 2 years.

2. 2 Shoot regeneration from embryogenic callus

The calli were cultured on MS media containing 30 g/*l* sucrose, 0-1 mg/*l* *N*-1, 2, 3-thiadiazol-5-yl-*N'*-phenylurea (thidiazuron, TDZ; Wako Pure Chemical, Japan) and 0-0.5 mg/*l* α-naphthaleneacetic acid (NAA) for testing the shoot regeneration ability in a

growth chamber under the same environmental conditions as used for routine subculture.

2. 3 Establishment of cell suspension cultures

In our preliminary experiments, the friable calli did not proliferate in liquid medium with the same composition as used for the callus subculture. Therefore, we tried to induce embryogenic calli with suitable properties to proliferate in a liquid medium from leaf-like structures (LLS) which had been produced on the friable calli.

LLS (about 0.4 g FW) excised from the friable calli were cultured in MS liquid medium containing 2 mg/*l* 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.4 g/*l* proline, 1 g/*l* glutamine and 30 g/*l* sucrose (modified from Huang *et al.* [4] and Chowdhry *et al.* [2]) and continuously transferred into the same medium every 7 days for at least 3 months until small calli were induced. For the establishment of fine homogeneous cell suspension cultures, the calli were then filtered through a 1 mm stainless steel sieve, and 0.2 g cells in fresh weight were cultured in 30 m*l* liquid MS medium containing 0.01 mg/*l* 2, 4-D, 1 g/*l* glutamine and 30 g/*l* sucrose in 50 m*l* Erlenmeyer flask. The cells were routinely subcultured every 7 days by transferring 1 g of cells in fresh weight into 30 m*l* medium in 50 m*l* flask until becoming fine homogeneous cell suspensions. All of the cultures were kept on a gyratory shaker at 120 rpm at 25°C under continuous illumination with cool-white fluorescent light at 38 μmol m^{-2} s^{-1}.

2. 4 Plant regeneration from cell suspension culture

Cells in suspension culture were collected on a nylon sieve and 10 inoculums of the cells (60 mg each) were placed onto Petri-dishes (90 mm × 15 mm) containing the media for inducing ELS. For the differentiation media, MS medium containing 30 g/*l* sucrose was supplemented with various kinds of plant growth regulators (PGR) such as NAA, benzyl-aminopurine (BA), and thidiazuron (TDZ) singly or in combination at different concentrations. The medium containing 100 m*l*/*l* coconut water (CW) (BIOBRID, Coconut Water Natural 100%, WEST TIGER (S) PTE. LTD) was also used as the comparison. The cell aggregates-derived calli cultured on differentiation media were subcultured at 2 month-intervals by transferring 200 mg of calli to the same media.

For shoot regeneration from ELS, the nodular calli with ELS induced on medium with 1 mg/*l* BA were transferred to MS medium containing no PGR, 100 m*l*/*l* CW, 1 mg/*l* BA or 0.1 mg/*l* TDZ and cultured for 1 month. All of the media contained 30 g/*l* sucrose and were solidified with 2 g/*l* gellan gum. The cultures were incubated in a growth chamber at 25°C under continuous illumination of cool-white fluorescent light at 38 μmol m^{-2} s^{-1}.

The shoots of 2-3 cm long were removed from the calli and transferred to PGR-free MS medium for rooting. *In vitro* rooted plants were gently removed from the culture medium, washed for removing gellan gum and transplanted to vermiculite in pots. They were kept in an incubator at 25°C under continuous illumination (45 μmol m^{-2} s^{-1}) for about 2 weeks and then transferred to a greenhouse for further growth.

3. Results

3. 1 Shoot regeneration from immature embryo-derived calli

The immature embryos produced two types of calli: compact green calli and friable green calli on MS medium containing 68 g/*l* sucrose. Although both of the calli proliferated well on the medium containing 30 g/*l* sucrose without adding PGR, only the friable green calli showed the ability to produce embryo-like structures (ELS) which eventually differentiated into leaf-like structures (LLS) (**Fig. 1A**). However, no normal embryos with germination ability were produced from the calli on any media tested. Instead, shoot formation was observed from the friable green calli with LLS on media containing 0.1 mg/*l* TDZ at high frequencies (90%). The friable calli retained the regeneration ability of ELS and LLS after subculture at 1 month intervals for 2 years. However, shoot regeneration ability on TDZ-containing media was lost after 3-4 subcultures.

3. 2 Establishment of cell suspension cultures

LLS excised from the friable calli gradually expanded and increased the fresh weight 3 times after culture for 1 month in liquid MS medium containing 2 mg/*l* 2, 4-D, 0.4 g/*l* proline and 1 g/*l* glutamine. As the liquid medium turned brown during the culture, LLS were routinely transferred into fresh medium every 7 days. Although LLS turned dark-brown and stopped growth thereafter, yellow compact calli were initiated on the surface of browned LLS after 3 months of culture (**Fig. 1B**) and slowly proliferated as compact cell aggregates with some browning. The cells initiated to proliferate vigorously as small fine cell aggregates (**Fig. 1C**) by transferring to liquid medium containing 0.01 mg/*l* 2, 4-D and 1 g/*l* glutamine after filtering through a 1 mm sieve. In this medium with reduced 2, 4-D concentration, fresh weight of the cells increased approximately 10 times during the 4 weeks of culture and browning of the cells was also reduced. The suspension culture finally obtained consisted of homogeneous compact cell clumps (*ca.* 200 μm in diameter) in which the cells were round, small (20-25

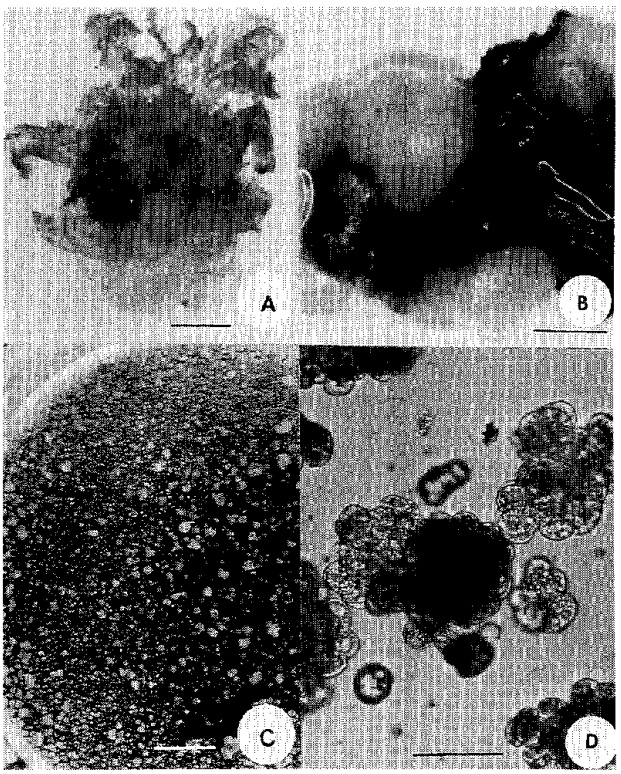

Fig. 1 Establishment of cell suspension culture of rose (*Rosa hybrida* cv. Gelb Dagmer Hastrap × *R. chinensis* var. *mutabilis*).
(A) leaf-like structures (LLS) induced from immature embryo-derived callus on medium containing 68 g/*l* sucrose (bar=2 mm). (B) Compact calli induced on LLS in liquid medium containing 2 mg/*l* 2, 4-D, 0.4 g/*l* proline and 1 g/*l* glutamine after 4 months of culture (bar=1 mm). (C) Initial stage of cell suspension culture containing cell aggregates (1-2 mm) in liquid MS medium containing 0.01 mg/*l* 2, 4-D and 1 g/*l* glutamine (bar=5 mm). (D) Fine cell clumps in suspension culture established in the same medium after 2 months of culture (bar=200 μm).

μm diameter), rich in cytoplasm, contained few vacuoles and were thin-walled (**Fig. 1D**).

3. 3 Regeneration of ELS from suspension culture-derived cells

The cell aggregates inoculated proliferated as friable calli in all the media tested except for the media containing 1 mg/*l* BA alone or 2 mg/*l* NAA and 5 mg/*l* BA on which yellow nodular calli were produced (**Fig. 2A**). Some of the nodular calli developed into ELS after 2 months of culture (**Fig. 2B**). Although the percentage of the cell aggregates with ELS formation was relatively low (7%) on medium containing 1 mg/*l* BA, it remarkably increased with repeating the subculture of the calli onto the same media at 2 month-intervals; 24% and 76% after 4 and 6 months of culture, respectively (**Table 1**). ELS formation was also observed on media containing 1 mg/*l* TDZ, where

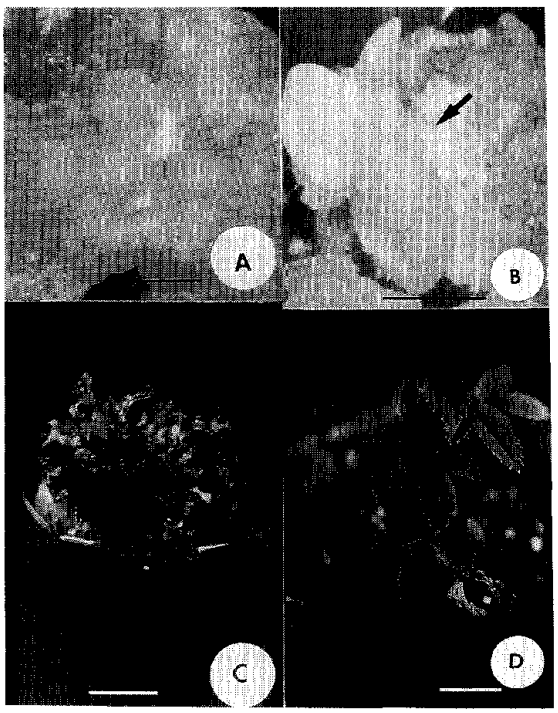

Fig. 2 Plant regeneration from cell suspension-derived nodular calli of rose (*Rosa hybrida* L. cv. Gelb Dagmer Hastrap × *R.chinensis* var. *mutabilis*).
(A) Nodular calli produced from suspension culture-derived cells on medium containing 1 mg/*l* BA (bar=1 mm). (B) Embryo-like structures (ELS) (arrow) produced from nodular calli on medium containing 1 mg/*l* BA (bar=3 mm). (C) Multiple shoots developing from ELS on medium with 0.1 mg/*l* TDZ (bar=50 mm). (D) Plantlet successfully transferred to vermiculite (bar=75 mm).

56% of the cell aggregates produced ELS although no ELS was produced before the subculture (**Table 1**).

3. 4 Shoot and plant regeneration from ELS

By transferring ELS onto PGR-free MS medium, they turned green, expanded and finally developed into leaf-like structures (**Table 2**). However, the leaf-like structures failed to develop into normal shoots on this medium. In contrast, adventitious shoots were induced from ELS on MS medium with 100 m*l*/*l* CW, 0.1 mg/*l* TDZ or 1 mg/*l* BA. Especially, multiple shoot formation (about 5-8 shoots/ELS mass) was observed in medium with 0.1 mg/*l* TDZ.

These regenerated shoots of 2-3 cm long were individually transferred to culture tubes containing PGR-free MS medium. The leaves in all shoots turned yellow and fell down during the initial 2 weeks, but eventually they produced new leaves. Some of the shoots successfully produced roots and were established in pots after acclimatization.

Table 1.
Effect of plant growth regulators on differentiation of embryo-like structures from the cells derived from an immature embryo of rose (*R. hybrida* cv. Gelb Dagmer Hastrap × *R. chinensis* var. *mutabilis*).

PGR (mg/*l*)*1	No. of cell aggregates cultured	No. of cell aggregates with ELS (%)*2 Period of culture (months)*3		
		2	4	6
BA 1.0	42	3 (7.1)	10 (23.8)	32 (76.2)
NAA 1.0, BA 0.5	30	0	—*5	—
NAA 1.0, BA 1.0	32	0	—	—
NAA 1.0, BA 2.0	34	0	—	—
NAA 1.0, BA 5.0	32	0	—	—
NAA 2.0, BA 0.5	32	0	—	—
NAA 2.0, BA 1.0	32	0	—	—
NAA 2.0, BA 2.0	35	0	—	—
NAA 2.0, BA 5.0	35	1 (2.9)	—	—
TDZ 1.0	32	0	6 (18.8)	18 (56.3)
CW*4	30	0	—	—

*1 Basal medium: MS medium containing 3% sucrose and 0.2% gellan gum.
*2 (number of cell aggregates with ELS/total number of cell aggregates cultured) × 100.
*3 The cells were subcultured at 2 month-intervals by transferring 200 mg of calli to the same media.
*4 10% coconut water.
*5 not tested.

Table 2.
Effect of CW, BA and TDZ in MS medium on the initiation of shoot from nodular calli with ELS of rose (*R. hybrida* cv. Gelb Dagmer Hastrap × *R. chinensis* var. *mutabilis*) after 2 months of culture.

Media composition	Growth Response
1. MS	ELS proliferation and green LLS formation
2. MS+10%CW	Shoot formation.
3. MS+1 mg/*l* BA	Shoot formation.
4. MS+0.1 mg/*l* TDZ	Multiple shoot formation.

4. Discussion

In the previous studies, shoot regeneration could be induced from leaves, roots and calli in *R. persica* × *xanthina* [8] and from cotyledon-derived calli in *R. hybrida* [1] on media containing BA and NAA. However, ELS produced in the present study could not develop into normal shoots or plantlets but only turned to leaf-like structures (LLS) in most of the combinations of PGR including NAA and BA probably because they lacked apical meristem [7]. Such abnormal ELS formation has been observed in some rose genotypes [7, 13] and was considered to be a recalcitrant problem for the application of plant biotechnology for rose breeding and micropropagation.

In many woody plants, especially those belonging to Rosaceae such as plum, peach, sour cherry and apple, shoot regeneration from various explants has been induced by TDZ [5]. The result in the present study also suggests the effectiveness of TDZ for shoot regeneration from the friable green calli which were induced from immature embryos. Although reestablished friable calli from LLS through the culture in liquid medium did not respond to TDZ, ELS were induced from the cells after repeating the subculture on medium containing 1 mg/*l* TDZ (**Table 1**). Response of the cells to another cytokinin, BA was also recovered by repeating the subcultures. Although TDZ was less effective than BA for ELS regeneration, it had stronger effect on shoot regeneration from ELS than BA and CW. The difference in the response to TDZ between the primary callus induced from immature embryo and the cells in suspension culture obtained from reestablished callus from LLS may attribute to the fact that the cell suspension culture had been maintained in medium containing 2, 4-D. The accumulation of 2, 4-D in the cells during the subculture might reduce the response to TDZ for shoot induction.

In the present study, we succeeded in establishing finely-dispersed homogeneous cell suspension cultures with plant regeneration ability in RHC. Liquid

medium containing proline and 2, 4-D was effective for embryogenic callus induction in this rose as previously shown in rice [2, 12]. However, reduction of 2, 4-D concentration was required for establishing the cell suspension culture without causing browning of cells after callus induction. The cells of this suspension culture have been maintaining plant regeneration ability for more than 2 years and the protoplasts isolated from the cells have plant regeneration ability (unpublished result). Therefore, the plant regeneration system established in the present study will be utilized for somatic hybridizaton with other genotypes and production of transgenic plants with some important traits such as disease resistance and altered flower color. However it is rather difficult to induce healthy roots from the *in vitro*-produced shoots of RHC. The improvement of *in vitro* as well as *ex vitro* growth conditions for this rose material is now under investigation.

References

[1] Burger, D.W., Liu, L., Zary, K.W., Lee, C.I., 1990. Plant Cell Tissue Organ Cult., **21**: 147-152.

[2] Chowdhry, C.N., Tyagi, A.K., Maheswari, N., Maheshwari, S.C., 1993. Plant Cell Tissue Organ Cult., **32**: 357-361.

[3] Firoozabady, E., Moy, Y., Courtney-Gutterson, N., Robinson, K., 1994. Bio/Technology., **12**: 609-613.

[4] Huang, C., Yan, H., Yan, Q., Zhu, M., Yuan, M., Xu, A., 1993. Plant Cell Tissue Organ Cult., **32**: 19-25.

[5] Huetteman, C.A., Preece, J.E., 1993. Plant Cell Tissue Organ Cult., **33**: 105-119.

[6] Krishnamurthy, K. V., Hendre, R.R., Godbole, D. A., Kulkarni, V.M., Mascarenhas, A.F., Jagannathan, V., 1979. Plant Sci. Lett., **15**: 135-137.

[7] Kunitake,H., Imamizo, H., Mii, M., 1993. Plant Sci., **90**: 187-194.

[8] Lloyd, D., Roberts, A.V., Short, K.C., 1988. Euphytica, **37**: 31-36.

[9] Matthews, D., Mottley, J., Horan, I., Roberts, A. V., 1991. Plant Cell Tissue Organ Cult., **24**: 173-180.

[10] Matthews, D., Mottley, J., Yokoya, K., Roberts, A.V., 1994. In "Biotechnology in Agriculture and Forestry" Vol. 29 Plant Protoplasts and Genetic Engineering (ed. by Bajaj, Y.P.S), p. 146-160, Springer-Verlag, Berlin Heidelberg New York.

[11] Murashige, T., Skoog, F., 1962. Physiol. Plant., **15**: 473-497.

[12] Ozawa, K., Komamine, A., 1989. Theor. Appl. Genet., **77**: 205-211.

[13] Rout, G.R., Debata, B.K., Das, P., 1991. Plant Cell Tissue Organ Cult., **27**: 65-69.

[14] Strauss, A., Potrykus, I., 1980. Physiol. Plant., **48**: 15-20.

[15] van der Mark, F., Pijnacker-Hordijk, J.P., Varga, G.A.I., de Vries, D.P., Dons, J.J.M., 1990. J. Genet. & Breed., **44**: 263-268.