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In vitro multiple shoot proliferation and plant regeneration in rose (*Rosa hybrida* L.)

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ABSTRACT This study was conducted to investigate an optimal condition for shoot proliferation and regenerate shoots from in vitro leaflet and embryogenic calli from in vitro roots in rose. The effect of BAP on shoot proliferation was somewhat different depending upon genotypes or gelling agents. Leaflets with petiole cut from donor shoots which had been cultured in MS medium supplemented with 0.1 $mg \cdot L^{-1}$ NAA for six weeks was effective for regeneration of adventitious buds (ABs) as well as shoot elongation of *Rosa hybrida* cv. Sweet Pink. Culturing seven leaflet explants per petri plate (100 mm × 15 mm) was effective for regeneration of ABs. Embryogenesis was shown in the calli induced from roots of *Rosa hybrida* cv. Sweet Pink cultured in the SH medium supplemented with 11 $mg \cdot L^{-1}$ 2, 4-D for four weeks. Color of calli induced from roots was yellow although their color was a little different as type of basal medium.

Introduction

Recently, genetic transformation or modification takes a position as a breeding technique in crops such as rice, tomato, carnation, and so on. In late 2006, a company of Japan, Suntory Co., announced that new rose cultivar, which was developed by genetic transformation technique, will come into the market. To develop new plant through genetic transformation, first of all, it is prerequisite to set up a regeneration system of that crop. There have been many reports on regeneration through organogenesis (Dubois and de Vries 1995; Ibrahim and Debergh 2001; Pati et al. 2004) or somatic embryogenesis (Hsia and Korban 1996; Marchant et al. 1996; Dohm et al. 2001; Kim et al. 2003a) from various organs in rose. Considering patent, if possible, it is desirable to use Korean cultivars as material to develop transgenic rose plants. So, this study describes shoot regeneration in both leaflet and root explant culture of domestic (Korean) rose cultivars. Also, uniform and consistent supply of material may result in attaining an object in experiment. It was known that shoot proliferation depends on genotype, growth regulators, and so on (Kim et al. 2003b; Pati et al. 2006). So, before executing the above alluded regeneration experiment, at first, we investigate an optimal conditions for shoot proliferation of several cultivars to use as materials.

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Materials and Methods

Plant materials

Domestic (Korean) cultivars (*R. hybrida* L. cvs. Pink Pearl, Red Star, Sharny, Sweet Pink, and White Day) which were bred at National Horticultural Research Institute (NHRI), Rural Development Administration (RDA) and foreign cultivars (*R. hybrida* L. cvs. Landora and Sonia) were used as materials in this study. Lateral shoots with five or seven leaves from donor plants grown in rock wool in a greenhouse were harvested and sterilized. Nodes were placed horizontally in 175 mL glass culture vessels containing 30 mL of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1

$mg \cdot L^{-1}$ BAP. After shoot elongation, they were subcultured at four week interval. Cultures were maintained at 25±2℃ in a 16h photoperiod.

Shoot proliferation

Ten shoots of three cultivars (*R. hybrida* cvs. Sweet Pink, Sharny, and Landora) were placed in five cultural glass vessels of 175 mL containing 30 mL of MS medium supplemented with 0 to 3 $mg \cdot L^{-1}$ BAP and 30 $g \cdot L^{-1}$ sucrose. The pH of the medium was adjusted to 5.8 and the media were solidified with either 0.7% (w/v) agar or 0.24% (w/v) phytagel, respectively. Cultures were maintained at 25±2℃ in a 16h photoperiod.

Regeneration from *in vitro* leaflets

To investigate both an appropriate number of explants incubated in one plate and an appropriate age of donor shoots for shoot regeneration from leaflet, *R. hybrida* cv. Sweet Pink, which showed the highest shoot regeneration in our previousstudy (Lee et al., 2003), were used as materials. Five, seven, ten and fourteen leaflets (with petiole), respectively, which were cut from donor shoots cultured on MS medium supplemented with 0.1 $mg \cdot L^{-1}$ NAA for 5, 6, and 7 weeks, were incubated on petri plates (100 mm × 15 mm) containing 50 mL of a regeneration medium without light for three days and then in light for fifteen days. And then they were transferred to shoot elongation medium. Composition of the regeneration medium as well as shoot elongation medium was the same as that of Dubois and de Vries (1995) except the media were solidified by plant agar (Duchefa P1001). Each treatment was replicated three times.

Regeneration from *in vitro* roots

To obtain embryogenic calli induced from in vitro root, six cultivars (*R. hybrida* cvs. Pink Pearl, Red Star, Sharny, Sweet Pink, Sonia, and White Day) were used as materials. Explants were divided into two parts, one was a part of root including root tip (PRIRT), and the other a part of root without root tip (PRWRT) (1 cm in length). Ten explants were cultured on petri plates (100 mm × 15 mm) containing 50 mL of MS or Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) supplemented with 11 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) for four weeks, and then they were transferred to MS or SH medium without plant growth regulator for four weeks. Then, induced calli were transferred to SH medium supplemented with 3 $mg \cdot L^{-1}$ 2,4-D and 300 $mg \cdot L^{-1}$ L-proline and maintained for five months. Each treatment was replicated three times.

Each medium was supplemented with 30 $g \cdot L^{-1}$ sucrose and was adjusted to pH 5.7. All explants were maintained at 25± 2℃ in a 16h photoperiod.

Results and Discussion

Shoot proliferation

To investigate an appropriate medium for *in vitro* proliferation in rose, shoots were cultured on MS medium with different concentrations of BAP and different types of gelling agents for one month. As Pati et al. (2006) reported that inclusion of BAP (1.0 to 10.0 $mg \cdot L^{-1}$) in the culture medium was essential for bud break and shoot proliferation in rose, in this study also, BAP was essential for shoot proliferation. However, the effect of BAP was somewhat different as genotypes or gelling agents. When agar was used as gelling agent, addition of 2 $mg \cdot L^{-1}$ BAP was effective for shoot proliferation of cv. Sweet Pink (4.7 per explant), whereas treatment of 1 $mg \cdot L^{-1}$ BAP was effective in cv. Sharny (5.8 per explant). On the other hand, when 0.24% phytagel was used as gelling agent, inclusion of 3 $mg \cdot L^{-1}$ BAP was effective in cv. Sweet Pink as well as cv. Landora (6.5 and 7.2 per explant, respectively). Addition of 1 $mg \cdot L^{-1}$ BAP was good for shoot proliferation of cv. Landora regardless of type of gelling agent (Table 1). Kim et al. (2003b) reported that the highest shoot proliferation occurred with 2 $mg \cdot L^{-1}$ BAP, and responsiveness by BAP varied greatly by genotypes. When the same amount of BAP was supplemented, phytagel was more effective for shoot proliferation than agar as gelling agent, Pati et al. (2006) reported that phytagel medium is better for shoot proliferation than agar one because phytagel forms a relatively clear gel and contains no contaminants.

Table 1. Effect of genotype, gelling agent, and BAP on shoot proliferation of *Rosa hybrida* L. after five weeks in culture

Cultivar	Gelling agent	BA ($mg \cdot L^{-1}$)	No. of shoots
Sweet Pink	Agar	0	1.0±0.0[z]
		0.5	3.5±1.4
		1.0	3.6±1.1
		2.0	4.7±0.8
		3.0	4.0±2.1
	Phytagel	0	1.0±0.0
		0.5	3.1±1.1
		1.0	6.1±2.1
		2.0	5.6±1.9
		3.0	6.5±0.7
Sharny	Agar	0	1.0±0.0[z]
		0.5	1.6±0.8
		1.0	5.8±3.6
		2.0	4.8±3.0
		3.0	5.6±1.5
	Phytagel	0	1.0±0.0
		0.5	6.1±2.0
		1.0	7.2±2.4
		2.0	5.2±0.9
		3.0	7.2±3.9
Landora	Agar	0	1.5±1.1[z]
		0.5	5.1±2.9
		1.0	6.8±2.6
		2.0	4.5±2.0
		3.0	5.1±2.6
	Phytagel	0	1.7±1.6
		0.5	6.2±1.1
		1.0	7.0±2.9
		2.0	6.5±2.2
		3.0	5.8±2.4

[z] Data represents mean±standard deviation.

Direct regeneration from *in vitro* leaf

For shoot regeneration, different numbers (5, 7, 10 and 14) of base of leaflets with petiole were cut from different age (5, 6 and 7 weeks) of donor shoot (*R. hybrida* cv. Sweet Pink) and incubated in petri plates. The results were in table 2 and 3 and figure 1. As Pati et al. (2004) as well as Dubois and de Vries (1995) reported that a regeneration response was evinced from petiole in *R. damascene* as well as five cut rose cultivars (*R. hybrida*), in this study, also, adventitious buds (ABs) mainly appeared from petioles. They assumed that regenerative capacity at the petiole could be ascribed to basipetal transport of endogenous auxins and/ or carbohydrates. However, cause of this capacity is not clear till now.

Famiani et al. (1994) reported that treatments applied in the last proliferating subculture such as subculture numbers, hormonal balance in medium, etc., had great influence on organogenesis because of preparing of explants for shoot regeneration. In this study, also, age of donor shoots had influenced on regeneration

Table 2. Effect of ages of donor shoots on regeneration from leaflet explants of *Rosa hybrida* cv. Sweet Pink

Age of donor shoot (weeks)	Explants with adventitious buds (%)
5	34.0±19.5
6	36.0±24.1
7	28.0±19.2

Table 3. Effect of the number of explants incubated per petri plate on regeneration from leaflet explants of *Rosa hybrida* L. cv. Sweet Pink after five weeks in culture

No. of explants incubated per petri plate	Explants with adventitious buds (%)	Adventitious buds elongated to shoots (%)	Mean number of regenerated shoot per explant
5	50.0±28.3	80.2±27.4	1.8
7	59.5±10.8	93.3±10.3	1.7
10	40.0±14.1	61.3±10.3	1.5
14	38.1±4.1	75.6±7.7	1.7

(A) (B) (C)

Figure 1. Shoot regeneration from leaflet explants with petiole of *Rosa hybrida* L. cv. Sweet Pink. A, Base of leaflets with petiole incubated on regeneration medium B, adventitious buds regenerated from explants and C, shoot elongation from adventitious bud.

of adventitious buds (ABs). It was identified that explants from donor shoots of 6 weeks in age was effective for regeneration of ABs, and rate of explants with ABs was 28 to 36% (Table 2). Ibrahim and Debergh (2001) also reported that explants from 4-week-old shoot gave a good regeneration. Furthermore, they reported that green pointed ABs were first observed on regeneration medium after 13 to 15 days in culture. Whereas, in this study, ABs were identified about 1 to 2 weeks after the explants were transferred to shooting elongation medium. That is to say, regeneration was identified at about 4 to 5 weeks in culture. Lloyd et al. (1988) reported that shoots appeared from excised leaves of one hybrid and two species in *Rosa* spp. within six weeks in culture.

In this study, the number of explants placed in one petri plate had an influence on formation of ABs. Ratio of explants with ABs was 38.1 to 59.5%, and that of ABs elongated to shoot was 75.6 to 93.3%. And the number of regenerated shoots per explant was 1.5 to 1.8. To inoculate 7 explants per petri plate was most effective for regeneration of ABs as well as shoot elongation (Table 3). Lee et al. (1999) reported that to inoculate 8 explants per petri plate was effective for shoot regeneration in chrysanthemum.

In this study, there was no difference in initial darkness duration. However, Famiani et al. (1994) reported that darkness was important for regeneration. They reported that no organogenesis was obtained without darkness and darkness applied not only at the beginning of organogenesis but also during the last proliferation subculture was effective for shoot regeneration. Ibrahim and Debergh (2001), also, reported that dark treatment of 7 days resulted in higher ABs regeneration than that of 4 days. In further study, it needs to extend initial darkness to raise ABs regeneration efficiency.

Regeneration from *in vitro* root

To induce embryogenic callus, *in vitro* roots [a part of root including root tip (PRIRT) and a part of root without root tip (PRWRT)] were incubated in petri plates containing MS or SH medium supplemented with 11 mg·L^{-1} 2,4-D. The results were in figure 2 and 3. Van der Salm et al. (1996) reported that

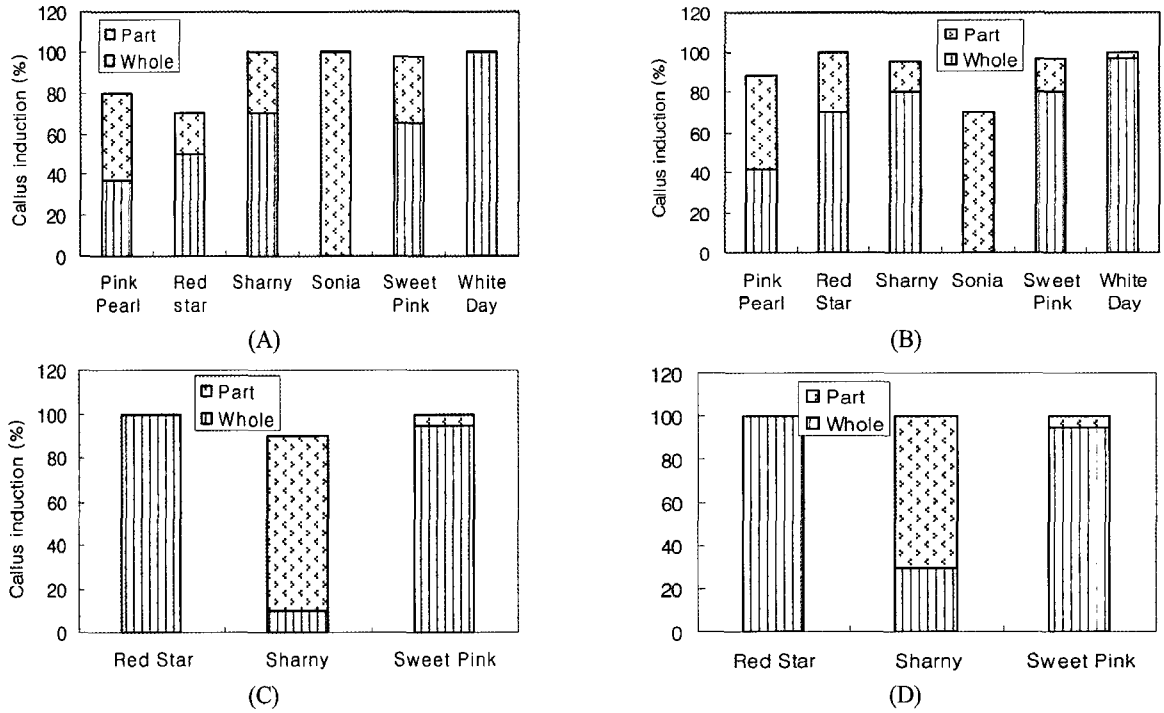
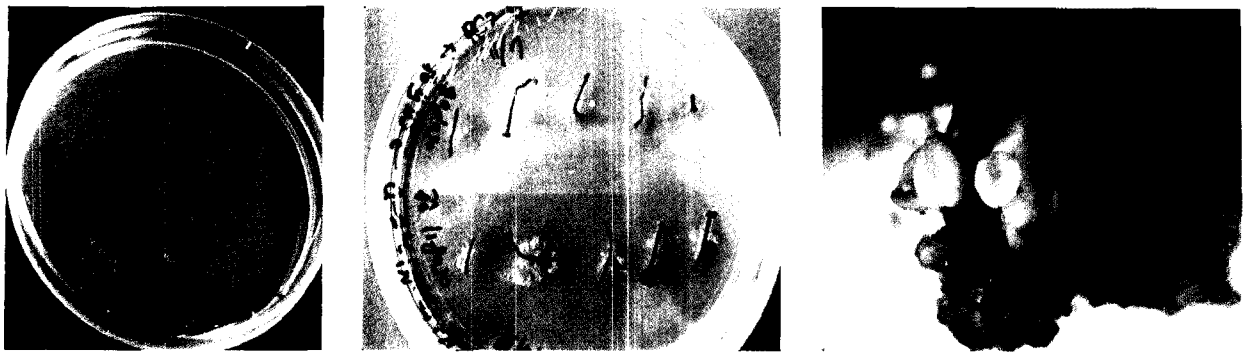


Figure 2. Callus induction from *in vitro* roots in *Rosa hybrida* L. ⁝⁝, rate of explants which had callus partially; |||, rate of explants which had callus on the whole; A and C, MS medium supplemented with 11 mg·L^{-1} 2, 4-D was used B and D, SH medium supplemented with 11 mg·L^{-1} 2, 4-D was used A and B, a part of root including root tip (PRIRT); C and D, a part of root without root tip (PRWRT).



(A) (B) (C)

Figure 3. Embryogenesis from callus induced from roots in *Rosa hybrida* L. cv. Sweet Pink. A, Roots incubated on regeneration medium B, Calli induced at an end of roots and C, Embryos induced from callus.

inclusion of 11 mg·L^{-1} 2,4-D to regeneration medium resulted in yellow jelly-like callus induction. Our results were same with their results. That is to say, color of calli induced from explants was yellow, although that color was a little different depend upon basal medium. Whereas calli induced on MS medium were light yellow, ones induced on SH medium were deep yellow. Callus induction rate from PRIRT explants have no difference between basal media. However, the rate of PRIRT explants covered with callus on the whole was higher on MS medium than on SH one in five cultivars except *R. hybrida* cv. White Day. Callus was induced from only part of PRIRT explants on both media in *R. hybrida* cv. Sonia. A type or rate of calli induced from PRIRT explants was not different between both media in *R. hybrida* cv. Pink Pearl. Whereas PRIRT explants were covered with callus on the whole, PRWRT explants were covered with callus partially in *R. hybrida* cv. Sharny. All PRWRT explants in *R. hybrida* cv. Red Star and 94.7% of PRWRT explants in *R. hybrida* cv. Sweet Pink were covered with callus on the whole and there was no difference between MS and SH medium.

When calli induced from PRIRT or PMWRT explants of the above six cultivars were transferred to the SH medium with 3 mg·L^{-1} 2, 4-D and 300 mg·L^{-1} L-proline through being cultured on SH or MS hormone free medium for four weeks, embryogenesis from calli of only *R. hybrida* cv. Sweet Pink was shown. They were what were induced not on MS but on SH medium (Fig. 3). Marchant et al. (1996), also, reported that somatic embryogenic callus was initiated from both root and petiole explants of two Floribunda rose (*R. hybrida* L.) cultivars on SH medium with 2,4-D, but not on MS medium.

In conclusion, we identified that BAP should be supplemented to the medium for shoot proliferation and the effect was somewhat different as genotype or gelling agent. And shoots regenerated from leaflets and embryogenic calli induced from roots in *R. hybrid* cv. Sweet Pink (Korean cultivar) were obtained. It might be able to be applied to develop transgenic rose using the protocol and products described in this study.

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