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Regeneration of Miniature Potted Rose (*Rosa hybrida* L.) via Somatic Embryogenesis

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Summary

Flowering plants were regenerated from *in vitro* grown leaves of miniature rose (*Rosa hybrida* L. 'Linda') via somatic embryogenesis. Sufficient amounts of callus were produced on *in vitro* leaves of 8 cultivars ('Leonie', 'Linda', 'Sonja', 'Toledo', 'Tiffany', 'Mette', 'Etna' and 'Andromeda') incubated on MS media fortified with either 45.2 or 90.5 μM 2,4- dichlorophenoxyacetic acid (2,4-D) for 6 weeks. Except for 'Tiffany' and 'Andromeda', embryogenic callus was induced on the callus of all cultivars incubated on MS media supplemented with different concentrations of zeatin or thidiazuron (TDZ). The highest frequency of primary somatic embryogenesis (30 %) was achieved with 'Sonja' on medium containing 22.7 μM TDZ, but the embryos from 'Sonja' failed to develop into plants. Only the somatic embryos of 'Linda' developed into flowering plants thus further work was carried out with this cultivar. The frequency of primary somatic embryogenesis in 'Linda' was 3.3 % on medium containing either 45.6 μM zeatin or 45.4 μM TDZ. The highest proliferation rate (3.8-fold) and the highest frequency of embryo maturation (76.6 %) were obtained on $^1/_2$ MS medium fortified with 7.57 μM abscisic acid. Germination and subsequent shoot development on mature somatic embryos occurred in a medium containing 1.42 μM 1-naphthaleneacetic acid (NAA), 17.76 μM 6-benzylaminopurine (BAP) and 1.44 μM gibberellic acid (GA_3). Shoots were rooted on $^1/_2$ MS medium supplemented with 5.7 μM 3-indole acetic acid (IAA) and 9.84 μM indole-3-butyric acid (IBA) and transferred to the greenhouse. The plants flowered after about 10 weeks and were morphologically indistinguishable from the original plant.

Key words. callus – plant growth regulator – plant regeneration – rose – somatic embryos

Introduction

Rose, the economically most important ornamental plant worldwide, is cultivated widely for use as garden plants, cut flowers, potted plants and for the perfume industry. Production of new cultivars is driven by grower and customer demands for new traits such as pest and disease resistance, longer vase life, new flower forms, color and scent. Conventional breeding methods for introducing new genes into rose cultivars are constrained by a limited gene pool and a high degree of inter-cultivar sterility due to different ploidy levels. The process is also time-consuming. Biotechnological methods, such as biolistic or *Agrobacterium*-mediated transformation, could provide an easy way to introduce a desired gene into established cultivars.

The main prerequisite for genetic transformation is whole plant regeneration from transformable explants. Although the frequency of somatic embryogenesis in rose is generally low and the maximum reported is 37 percent, several reports have concluded that somatic embryos are the best material for transformation of this plant species (Rout et al. 1991; Firoozabady et al. 1994; Van Der Salm et al. 1996; Condliffe et al. 2003).

There is not any universal, genotype-independent method for somatic embryogenesis in rose. Different explants including *in vitro* grown leaves (Visessuwan et al. 1997; Dohm et al. 2001), immature leaves and internodal stem segments (Rout et al. 1991; Hsia and Korban 1996; Li et al. 2002), greenhouse grown leaves (Kintzios et al. 1999), *in vitro* derived petioles and roots (Marchant et al. 1996) and adventitious roots (Derks et al. 1995; Van Der Salm et al. 1996), petals (Murali et al. 1996), filaments (Noriega and Söndahl 1991) and different flower parts (Arene et al. 1993) have been used for induction of somatic embryogenesis in different rose cultivars. Although embryogenic callus were derived in some rose cultivars, not all studies resulted in the successful recovery of flowering plants. Also, except for *R. chinensis minima* 'Red Sunblaze' and 'Baby Katie' (Hsia and Korban 1996; Li et al. 2002) which are miniature roses, the rest of the investigated cultivars were either garden roses or cut roses or used as both. Denmark is the largest producer of miniature potted roses in the world. Therefore, the present study investigated whole plant regeneration via somatic embryogenesis of eight miniature potted rose cultivars which are commercially grown in Denmark, in order to proceed with genetic transformation.

Materials and Methods

Establishment of shoot cultures

Proliferating shoot cultures of 8 miniature rose cultivars ('Leonie', 'Linda', 'Sonja', 'Toledo', 'Tiffany', 'Mette', 'Etna' and 'Andromeda') were established *in vitro* by surface sterilized nodal stem segments of greenhouse grown plants. Sterilization was carried out using 2 % sodium hypochlorite and 0.1 % tween-20 in screw-capped bottles for 20 min with vigorous shaking every 5 min followed by rinsing 3–5 times with sterile deionized water. For 'Etna', 3 % sodium hypochlorite was necessary for sterilization due to its compact growth habit. Sterilized nodal stem segments were cultured in plastic containers (99 mm × 91 mm, Danefeld, Research and Breeding Station, Denmark) containing full strength MS (Murashige and Skoog 1962) salts supplemented with 0.021 μM NAA, 4.44 μM BAP, 0.28 μM GA_3 and solidified with 4 g l^{-1} agar (Bacto Agar, Becton Dickinson, USA) and 1.5 g l^{-1} Gelrite (Duchefa, The Netherlands). The shoots were sub-cultured in the above mentioned medium, but without GA_3 every 6 weeks.

All media used in the study consisted of half- or full-strength MS salts, Staba vitamins (Staba 1969), 100 mg l^{-1} myoinositol, 20 g l^{-1} sucrose and 10 g l^{-1} glucose. However, for shoot proliferation and elongation medium, FeEDTA (which is part of the standard MS salts) was replaced by 96 mg l^{-1} FeEDDHA (Duchefa, The Netherlands), a more stable iron chelate. The pH of all media were adjusted to 5.7 using 1 N NaOH and either 8 g l^{-1} agar or 3 g l^{-1} Gelrite or a combination of both gelling agents (4 g l^{-1} agar and 1.5 g l^{-1} Gelrite) was added prior to autoclaving at 121 °C for 20 min.

Production of callus on in vitro *grown leaves*

Various growth regulators selected on the basis of previous reports (Rout et al. 1991; Hsia and Korban 1996; Kintzios et al. 1999; Dohm et al. 2001; Li et al. 2002) were used primarily to produce sufficient callus on *in vitro* grown leaves of these cultivars for further experiments. Five different concentrations of 2,4-D (Table 1) were selected after obtaining promising results from preliminary experiments. Whole leaves of each cultivar were excised from shoot proliferation cultures and were wounded transversely on the adaxial surface at 2–4 mm intervals using a scalpel. Leaves were placed on the medium in 90 ×15 mm Petri dishes with abaxial side down. Media consisted of MS salts and different concentrations of 2,4-D (4.52, 13.5, 45.2, 90.5 and 135.7 μM) and solidified with 3 g l^{-1} Gelrite. All treatments were repeated three times and each replication consisted of a single Petri dish containing 10 leaf explants. All cultures were kept in the dark at 23±2°C for about six weeks and then data on numbers of explants with non-embryogenic and embryogenic callus were recorded.

Induction of primary somatic embryos

Callus produced on media containing either 45.2 or 90.5 μM 2,4-D (Table 1) was transferred to new media for induction of somatic embryos. Explants with few callus and the ones with excessive callus (produced on 135.7 μM 2,4-D) were also transferred to embryo induction media separately, but the results were not used for data analysis. At this stage three sub experiments were carried out: In the first, explants of all cultivars (Table 1) with sufficient callus were incubated on MS medium, supplemented with various concentration of zeatin (18.2, 27.4, 45.6 and 91.2 μM) or thidiazuron (TDZ, 4.5, 13.6, 22.7 and 45.4 μM) and solidified with 8 g l^{-1} agar. Explants were subcultured on fresh medium once every month for five to seven months (depending on the cultivar). In the second sub experiment, calluses from 'Linda' and 'Toledo' were placed on the same media as above. Following two consecutive passages on fresh media, callus was transferred to the plant growth regulator- (PGR) free media. In the third sub experiment, callus from 'Linda' and 'Leonie' was incubated on $^1/_2$ MS medium with the same concentration of zeatin and TDZ as above. Following three consecutive passages, explants were transferred to PGR-free media.

All treatments were repeated three times and each replication consisted of a single Petri dish containing 10 callus explants. The number of explants that produced embryogenic callus was recorded.

Proliferation and maturation of primary somatic embryos

The most promising cultivars, 'Linda' and 'Sonja', were chosen for the subsequent experiments. Hard and compact embryogenic callus of 'Linda' and friable embryogenic callus of 'Sonja' from the first experiments were transferred to a new medium for proliferation and maturation of somatic embryos. This medium consisted of $^1/_2$ MS salts, solidified with 8 g l^{-1} agar and supplemented with four levels of ABA (3.78, 7.57, 15.13 and

Table 1. Effect of different concentrations of 2,4-D on callus production in leaf explants of 8 miniature rose cultivars (*Rosa hybrida*).

2,4-D (μM)	No. of leaf explants	Percent of leaf explants forming callus							
		'Linda'	'Leonie'	'Sonja'	'Toledo'	'Tiffany'	'Mette'	'Andromeda'	'Etna'
4.52	30	2.0 d	5.3 c	1.7 c	2.7 b	4.0 c	7.7 c	1.0 c	16.0 c
13.5	30	36.0 c	36.0 b	53.0 b	18.7 b	60.0 b	62.0 b	51.6 b	61.0 b
45.2	30	88.6 b	88.3 a	100.0 a	72.0 a	91.0 a	88.7 ab	96.6 a	86.3 a
90.5	30	97.0 ab	97.0 a	90.0 a	82.3 a	100.0 a	94.7 ab	98.3 a	87.3 a
135.7	30	99.0 a	100.0 a	98.0 a	85.3 a	99.0 a	99.3 a	100.0 a	93.3 a

Means with different letter in each column are significantly different at P<0.05.

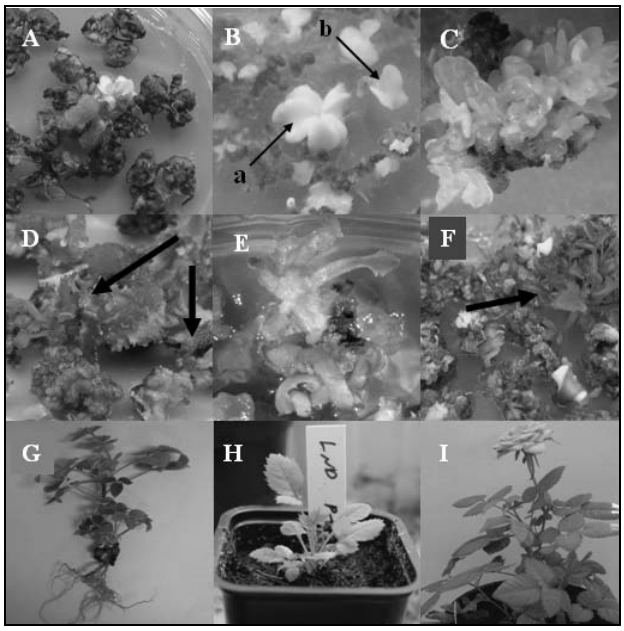


Fig. 1. Plant regeneration from somatic embryos derived from *in vitro* leaves of miniature rose, *Rosa hybrida* 'Linda'. (A) Embryogenic callus together with somatic embryos induced on callus produced on 2,4-D (×1). (B) Friable embryogenic callus with malformed somatic embryos (×9). a) One somatic embryo with several cotyledons b) One somatic embryo with one cotyledon. (C) Cluster of mature somatic embryos with cotyledonary leaves (×9). (D) Greening and germination (arrows) of mature somatic embryos one month after transferring to regeneration media (×4). (E) Abnormal shoot regenerated from germinated embryos and remained undeveloped (×4). (F) Regeneration and elongation of normal shoot (arrow) from germinated somatic embryos (×1). (G) *In vitro* adventitious root production on multiplied shoots (×0.5). (H) Plantlet two weeks after transferring to soil (×0.5) and (I) flowering plant 2 months after transferring to 9 cm plastic pot (×0.3).

18.92 μM). These concentrations were selected according to results of previous publications (Li et al. 2002; Sharma et al. 2004; Ogata et al. 2005) and our preliminary experiments. Each treatment was replicated three times and each replication consisted of a single Petri dish containing 1 g embryogenic callus (10 callus clumps, 100 mg each). Data on increased weight of embryogenic callus (proliferation rate) and also number of explants with mature embryos (embryos with developed cotyledons) were collected after 6 weeks.

Development of somatic embryos into plants

Since somatic embryos from 'Sonja' did not develop into normal plants in preliminary experiments, further work was continued with 'Linda'. Mature somatic embryos of 'Linda' (Fig. 1C) were transferred to media with MS salts and FeEDDHA iron chelate, supplemented with different concentrations of NAA (0, 1.42 μM), BAP (0, 4.4, 8.9, 17.7, 26.6 μM) and GA_3 (0, 0.29, 1.44 μM) in various combinations (Table 4) for germination and shoot development. All media were solidified with 4 g l^{-1} agar and 1.5 g l^{-1} Gelrite. Specific hormonal concentrations used in these media were selected according to previous publications (Rout et al. 1991; Marchant et al. 1996; Visessuwan et al. 1997; Kintzios et al. 1999) and the results from preliminary experiments of the present study. Mature somatic embryos were subcultured in fresh medium after one month and then regenerated shoots were transferred to the shoot multiplication medium containing 0.021 μM NAA and 4.44 μM BAP. Each treatment was repeated three times and each replication consisted of a single Petri dish (90 × 18 mm) containing 10 explants (Table 4). Data on the number of explants with normal and abnormal regenerated shoots were collected.

Root production and acclimatization of plants

Sixty elongated shoots with normal appearance were transferred to a rooting medium consisting of $^1/_2$ MS salts, 5.7 μM IAA, 9.84 μM IBA and 8 g l^{-1} agar. Rooted plantlets were then transferred to 6 cm plastic pots containing peat (Pindstrup Mosebrug A/S Denmark) and covered with transparent polyethylene bags in the greenhouse. Plants were watered with 1 g l^{-1} Octave (a fungicide supplied by BASF, AGRO BV, Arnhem, The Netherlands) and 3 ml l^{-1} Bactimos (a larvicide from Abbott, North Chicago) to protect them from fungal and larvae attack. Plants were maintained under greenhouse conditions at 19±1°C and 16 h photoperiod (provided by SON-T high pressure sodium lamps with 600 μmol m^{-2} s^{-1} at plant surface). The cover was removed gradually over three weeks, and after one month the established plants were transferred to 9 cm plastic pots and grown until flowering.

Statistical analyses

All data were analysed using SAS statistical analysis package (Version 8.02). Statistical significances among mean values were assessed using Duncan's multiple range tests at $P<0.05$.

Results and Discussion

Callus production

Varying amounts of callus were produced on adaxial side of the leaves 6 weeks after incubation on different 2,4-D concentrations (Table 1). Both genotype and 2,4-D concentrations influenced the explants' ability to form callus. 2,4-D at 4.52 μM produced very little callus on all cultivars tested in the present study, but this concentration produced sufficient callus in garden roses, *R. hybrida* 'Pariser Charme' and 'Heckenzauber' (Dohm et al. 2001). Leaves of miniature roses became necrotic and died after two weeks of incubation on this 2,4-D concentration. Although 13.5 μM 2,4-D produced callus in all cultivars insufficient callus was produced for subsequent experiments. 45.2 μM 2,4-D produced sufficient callus (ca. 400 mg $explant^{-1}$) on 'Sonja', 'Tiffany', and 'Andromeda' and this concentration was used for further experiments. Li et al. (2002) also obtained similar results in miniature rose, *R. chinensis minima* 'Red Sunblaze'. Although no significant difference was observed between 45.2 and 90.5 μM 2,4-D in percentage of callus production on 'Lin-

Table 2. Effect of cytokinins (Zeatin and TDZ) on induction of embryogenic callus in callus explants of 8 miniature rose cultivars.

Zeatin (μM)	TDZ (μM)	No. of explants	Percent of callus explants forming embryogenic callus							
			'Linda'	'Leonie'	'Sonja'	'Toledo'	'Tiffany'	'Mette'	'Andromeda'	'Etna'
18.2	0.0	30	0.0 a	0.0 a	13.3 ab	3.3 a	0.0 a	0.0 a	0.0 a	0.0 b
27.4	0.0	30	0.0 a	0.0 a	13.3 ab	0.0 a	0.0 a	0.0 a	0.0 a	0.0 b
45.6	0.0	30	3.3 a	0.0 a	10.0 ab	0.0 a	0.0 a	0.0 a	0.0 a	0.0 b
91.2	0.0	30	0.0 a	0.0 a	0.0 b	0.0 a	0.0 a	0.0 a	0.0 a	0.0 b
0.0	4.5	30	0.0 a	3.3 a	6.7 ab	0.0 a	0.0 a	3.3 a	0.0 a	0.0 b
0.0	13.6	30	0.0 a	3.3 a	6.7 ab	3.3 a	0.0 a	6.7 a	0.0 a	0.0 b
0.0	22.7	30	0.0 a	0.0 a	30.0 a	0.0 a	0.0 a	0.0 a	0.0 a	3.3 b
0.0	45.4	30	3.3 a	0.0 a	10.0 ab	3.3 a	0.0 a	0.0 a	0.0 a	16.7 a

Means with different letter in each column are significantly different at P<0.05.

da', 'Leonie', 'Toledo', 'Mette' and 'Etna', the amount of callus in each explant produced by 45.2 μM 2,4-D was less than 200 mg explant^{-1} in the follow-up experiment. Therefore 90.5 μM 2,4-D was used in further experiments in these cultivars. Although 135.7 μM 2,4-D also produced sufficient callus (more than 700 mg explant^{-1}) in all cultivars, the callus was too fragile to subculture and was not used in further experiments. In contrast to our results, increased concentrations of 2,4-D have reduced callus production in some rose cultivars (Li et al. 2002; Van Der Salm et al. 1996).

Low concentrations of auxin (2,4-D, NAA, IAA, IBA, dicamba or p-chlorophenoxyacetic acid), alone or together with a cytokinin (BAP, kinetin, zeatin or 2iP), have been used successfully for callus production in rose cultivars (Noriega and Söndahl 1991; Rout et al. 1991; Arene et al. 1993; Derks et al. 1995; Hsia and Korban 1996; Marchant et al. 1996; Murali et al. 1996; Van Der Salm et al. 1996; Kintzios et al. 1999; Dohm et al. 2001) but only high concentrations of 2,4-D produced sufficient callus in cultivars tested in the present study. However, most investigated cultivars, were garden roses, which have a higher growth rate than miniature roses. The slow growth rate of miniature roses might be due to low endogenous hormonal levels, and this nature may create a demand for a high exogenous auxin concentration for callus production. Callus production in miniature rose, *R. chinensis minima* 'Red Sunblaze', which is the ancestor for all miniature roses (Horn 1992), also required 45.2 μM 2,4-D, a concentration that is considered high (Li et al. 2002). However, by using high auxin concentrations in callus production stage, somatic embryogenesis was reduced in some (Hsia and Korban 1996), while increased in other rose cultivars (Marchant et al. 1996; Visessuwan et al. 1997).

Cultivars and treatments had an influence on the colour and friability of the callus. As the concentration of 2,4-D was raised, the friability of the callus markedly increased. Calluses from 'Linda', 'Leonie' and 'Toledo' were compact and pale-green in colour, while callus initiated from 'Sonja' was soft, friable and yellowish in colour. Calluses from 'Etna' and 'Mette' were compact and brown. Embryogenic callus was not observed at callus production stage in any of these cultivars, but rhizogenic calluses were produced with different frequencies in some cultivars (data not shown). 'Sonja', 'Etna', 'Mette' and 'Andromeda' produced rhizogenic callus and among them 'Mette' showed the highest frequency of rhizogenic callus, whereas rhizogenic callus was not observed on 'Linda', 'Leonie', 'Tiffany' and 'Toledo'.

Induction of primary somatic embryogenesis

Explants with sufficient callus produced on certain 2,4-D concentrations from each cultivar (according to above mentioned results) were transferred to MS medium containing zeatin or TDZ and sub-cultured once every month. In the first sub experiment, embryogenic callus (Fig. 1A) was observed after 2 months in 'Toledo' and 'Sonja', after 3 months in 'Linda' and 'Leonie' and after 4 months in 'Etna' and 'Mette'. Repeated subculture on fresh medium increased embryogenesis in 'Sonja' and 'Leonie' but not in other cultivars. No embryogenic callus was observed in 'Tiffany' and 'Andromeda' after 5 subcultures on induction medium. The percentage of embryogenic callus induction for most of the cultivars was about 3.3. However, 'Sonja' showed a significant response to reach 30 % embryogenic callus induction (Table 2). The highest percentages of embryogenic callus in 'Sonja' (30 %) and 'Etna' (16.7 %) were induced on media containing 22.7 and 45.4 μM TDZ, respectively. Except for these two cultivars, no significant difference was observed between various concentrations of zeatin and TDZ in induction of somatic embryogenesis. In general, zeatin induced embryogenesis in 3 cultivars, while TDZ did so in 6 cultivars. Also, the highest embryogenesis percentage was obtained by using 13.6 and 45.4 μM TDZ. The reason for better response by using TDZ might be due to the higher stability of TDZ in plant tissues compared with that of zeatin (Mok et al. 2000). Li et al. (2002) also obtained somatic embryogenesis in miniature rose, *R. chinensis minima* 'Carefree Beauty' by using TDZ concentrations ranging from 2.3 to 54.5 μM, with and without 2.9 μM GA_3.

Various PGRs have been used for inducing embryogenic callus in different species and cultivars (reviewed by Jimenez 2005). Different concentrations of NAA (0.27 to 26.8 μM) alone or together with a cytokinin (such as kinetin, Zeatin, BAP or TDZ) and/or GA_3 have been used successfully for somatic embryogenesis in some rose cultivars (Noriega and Söndahl 1991; Rout et al. 1991; Arene et al.

Table 3. Effect of different ABA concentrations on proliferation rate of embryogenic callus (increased weight of embryogenic callus after 6 weeks) and maturation of somatic embryos in 'Linda' and 'Sonja'.

ABA (μM)	Proliferation rate of embryogenic callus (fold)		Percent embryo maturation	
	'Linda'	'Sonja'	'Linda'	'Sonja'
3.78	3.76 a	2.10 ab	43.3 ab	10.0 ab
7.57	3.86 a	1.16 b	76.6 a	2.5 ab
15.13	3.30 a	3.97 a	36.6 ab	15.0 a
18.92	3.10 a	1.87 ab	26.6 b	0.0 b

Means with different letter in each column are significantly different at P<0.05.

Table 4. Effects of different media on development of somatic embryos in 'Linda' cultivar.

Media	Growth regulators			No. of callus clumps	Percent embryo regeneration	
	NAA (μM)	BAP (μM)	GA_3 (μM)		Normal shoots	Abnormal shoots
P	0.00	0.00	0.00	30	0.0 d	33.3 a
A	1.42	0.00	0.00	30	3.3 d	33.3 a
B	1.42	4.44	0.29	30	30.0 c	33.3 a
C	1.42	4.44	1.44	30	30.0 c	40.0 a
D	1.42	8.90	0.29	30	43.3 bc	33.3 a
E	1.42	8.90	1.44	30	46.3 bc	33.3 a
F	1.42	17.76	0.29	30	60.0 ab	20.0 ab
H	1.42	17.76	1.44	30	70.0 a	10.0 b
I	1.42	26.60	0.29	30	30.0 c	20.0 ab
J	1.42	26.60	1.44	30	40.0 c	33.3 a

Means with different letter in each column are significantly different at P<0.05.

1993; Hsia and Korban 1996; Visessuwan et al. 1997; Dohm et al. 2001). In general, induction of embryogenic callus from vegetative explants is driven by an auxin or by a combination of auxin and cytokinin (Raemakers et al. 1995). In contrast to Marchant et al. (1996), who obtained up to 36 % embryogenic callus in *R. hybrida* 'Trumpeter' and 'Glad Tidings' after 2–4 months of repeated subculture on 13.5 μM 2,4-D, we did not detect any visible embryogenic callus with different 2,4-D concentrations. However, we obtained embryogenic callus in media supplemented with only cytokinin. Since we used high concentrations of 2,4-D in callus production stage it is possible that the embryos were induced by 2,4-D, but the proembryogenic cell masses did not develop into an easy visible stage. In addition, a carry over effect of this hormone might have had a role on induction of embryogenic callus together with cytokinin. Nevertheless, the use of TDZ alone or together with GA_3 resulted in a high frequency of somatic embryogenesis in *R. hybrida* 'Carefree Beauty' (Hsia and Korban 1996; Li et al. 2002). In contrast to these results, low levels of TDZ inhibited somatic embryogenesis in *R. hybrida* 'Carl Red' (Visessuwan et al. 1997).

Three types of embryogenic callus were observed in the present study: hard and compact callus with a yellow colour, which was produced in 'Linda' and 'Toledo'; friable jelly-like yellowish callus in 'Sonja' and the same texture but white in 'Leonie', 'Etna' and 'Mette'. Friable white callus also initiated from compact callus of 'Linda' upon subculture on the embryogenic callus proliferation medium. Proliferation of friable embryogenic callus in preliminary experiments resulted in high numbers of abnormal somatic embryos with one or more than two cotyledons (Fig. 1B). Therefore, this type of embryogenic callus was not used for further experiments. As indicated by Visessuwan et al. (1997), high moisture content in friable embryogenic callus appears to be responsible for producing embryos with undeveloped apical meristems. High concentrations of cytokinins can also lead to formation of abnormal somatic embryos. In some species, use of 2,4-D resulted in a high frequency of morphologically abnormal embryos which failed to convert into plants (Jimenez 2005). However, abnormality of somatic embryos is common in many species (Dodeman et al. 1997) and it has also been reported in many rose cultivars.

Embryogenic callus was not obtained from the second and third sub experiments in the present study, whereas PGR-free media previously have been used successfully for somatic embryogenesis in some rose cultivars (Kunitake et al. 1993; Derks et al. 1995; Van Der Salm et al. 1996; Visessuwan et al. 1997).

Proliferation and maturation of somatic embryos

Compact embryogenic calluses of 'Linda' as well as friable embryogenic calluses of 'Sonja' were transferred to ABA containing media for proliferation of embryogenic callus.

For quantification, "proliferation rate" was used which represents the ratio of the fresh weight of the embryogenic callus together with somatic embryos generated at the end of 6 weeks of incubation on ABA, to the initial fresh weight of primary embryogenic callus inoculated. In 'Linda' 7.57 μM ABA produced the highest proliferation rate (3.8-fold, Table 3), although there was no statistically proven effect of ABA concentration. In 'Sonja', 15.13 μM ABA encouraged higher proliferation of embryogenic callus (3.9-fold) than other concentrations (Table 3). The number of callus clumps with mature embryos was similar in ABA concentrations ranging from 3.78 to 15.13 μM in both 'Linda' (Fig. 1C) and 'Sonja'. However, 18.92 μM had negative effect on these cultivars (Table 3). The number of mature embryos in 'Sonja' was much lower compared with 'Linda'. Similar results with ABA application have been obtained by other researchers (Noriega and Söndahl 1991; Marchant et al. 1996; Murali et al. 1996). Marchant et al. (1996) used 3.8 μM ABA in combination with 2,4-D and GA_3 for embryo maturation in *R. hybrida* 'Trumpeter' and 'Glad tidings'. In addition, Li et al. (2002) obtained both the highest germination rate of somatic embryos and proliferation via secondary somatic embryogenesis in *R. hybrida* 'Carefree beauty' with application of 3.8 μM ABA. Exogenous application of ABA has also been used in other species for proliferation and maturation of somatic embryos. Ogata et al. (2005) obtained secondary somatic embryos from carrot seed-coat-derived somatic embryos with application of 1 μM ABA. Mature somatic embryos have been acquired by using 1 μM ABA in *Quercus suber* (Garcia-Martin et al. 2005) and 18.9 μM ABA in *Camellia sinensis* (Sharma et al. 2004). It has been shown that different stresses induce somatic embryogenesis in many plant species (Feher 2003). It is also known that endogenous ABA increases in many species after various stresses (reviewed by Milborrow 2001). It can be concluded that increased levels of endogenous or applied ABA could improve differentiation and maturation of somatic embryos as an adaptation mechanism in response to stress. Molecular and biochemical events underlying this phenomenon are not entirely clear and are yet to be elucidated.

Development of somatic embryos into whole plants

Greening of mature somatic embryos started two weeks after transferring to regeneration media. Germination and subsequent shoot development of somatic embryos occurred within one month of transfer to the regeneration media (Fig. 1D, Table 4). Two different shoot types were observed after two months in these cultures. Morphologically normal shoots developed into proper plantlets (Fig. 1F) while morphologically abnormal shoots remained undeveloped (Fig. 1E). The highest number of normal shoots (70.0 %) was obtained in medium H containing 1.42 μM NAA, 17.7 μM BAP and 1.4 μM GA_3, however the results (60.0 %) were similar to that from medium F which had the same amount of NAA and BAP but lower concentration of GA_3 (Table 4).

Different media including various concentrations of PGRs have been used by researchers for germination and shoot regeneration of somatic embryos. BAP, GA_3 and an auxin (NAA, IAA or IBA) have been utilized more than other PGRs in the stage of somatic embryo development. Rout et al. (1991) obtained 12 % normal shoot regeneration from somatic embryos of *R. hybrida* 'Landora' grown on $^1/_2$ MS medium supplemented with 2.2 μM BAP, 0.3 μM GA_3 and 24.7 μM adenine sulphate. Likewise, 4.4 μM BAP was used by Marchant et al. (1996) for obtaining 17 and 9 % shoot regeneration from mature somatic embryos of *R. hybrida* 'Trumpeter' and 'Glad Tidings', respectively. Kintzios et al. (1999) used 5.2 μM BAP together with 5.7 μM IAA for maturation and germination of somatic embryos in *R. hybrida* 'Soraya' and achieved normal shoot regeneration. Visessuwan et al. (1997) applied 8.8 μM BAP for shoot regeneration from somatic embryos of *R. hybrida* 'Carl Red' and *R. canina*. In the present study, the lowest number of normal shoots were obtained in PGR-free medium (medium P) and also in medium with only NAA (medium A), and their results were significantly different when compared to that from rest of the media containing BAP and GA_3. Li et al. (2002) also obtained poor germination of somatic embryos in PGR-free medium. Therefore, it seems that GA_3 and BAP are very important for germination and shoot elongation from somatic embryos. GA_3 is known for its effect on promoting seed germination by overcoming dormancy in zygotic seeds of some species and also for shoot elongation in some species (Davies 1995). Since somatic embryos do not have dormancy (Dodeman et al. 1997; Feher et al. 2003), GA_3 does not seem to be necessary for their germination. However, application of GA_3 lowers the endogenous levels of ABA and IAA (Davies 1995). Therefore, in the present study, it appears that positive effects of GA_3 might be caused by reducing negative effects of ABA which was used for proliferation of somatic embryos. However, no significant differences were observed between 0.29 μM and 1.44 μM of GA_3. BAP influences cell division and enlargement, lateral bud growth, leaf expansion and promotion of seed germination in many plant species (Mok et al. 2000). Hence, application of BAP can enhance shoot regeneration and multiplication. Nevertheless, a balance between hormones is required for normal germination and development. It appears that high concentrations of BAP could reduce the germination rate and/or development of somatic embryos. This agrees with the results of Marchant et al. (1996) where shoot development was achieved on lower concentrations of BAP compared to the concentrations used for germination of somatic embryos. The results from media B, C, D, E, I and J with BAP concentrations higher and lower than those in media F and H were not significantly different (Table 4). However, in terms of abnormal shoots, except for medium H which produced the lowest numbers, the results from the rest of the media were not significantly different. A low concentration of an auxin is usually used together with a cytokinin to improve the result.

Secondary somatic embryos were also observed in this stage of development. Different numbers of them produced on developed cotyledons which were mostly abnormal with fused transparent cotyledons. These embryos were not used for further experiments. Bipolar plantlets were not detected in the present study. Therefore, regenerated shoots were multiplied in MS medium fortified with 0.021 μM NAA and 4.44 μM BAP and adventitious roots were produced on them.

Root production and acclimatization of plants

Close to 100 % of shoots produced adventitious roots on $^1/_2$ MS media containing IAA and IBA. After transferring

to soil, 34 plants (57 %) survived and they flowered after 8 to 12 weeks. Regenerated plants were morphologically indistinguishable from the original plants (Fig. 1G–I).

In summary, a protocol for regeneration of miniature rose cultivar via somatic embryogenesis was developed in the present study. This protocol is used currently for genetic transformation work. Moreover, embryogenic callus obtained in the present study has been proliferated and maintained for over three years by sub-culturing every month. Further investigations to achieve higher rates of proliferation, better maturation and to extend life span of somatic embryos would be useful for rose breeding programs.

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