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Somatic Embryogenesis from *In Vitro* Grown Leaf Explants of *Rosa hybrida* L.

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Abstract

Somatic embryogenesis was initiated from *in vitro* grown leaf explants of rose following an induction period of four weeks on MS basal medium supplemented with auxin and several subcultures on MS medium with cytokinin. '4th of July' showed the highest regeneration frequencies on 1 mg/L NAA followed by culture on medium with 4 mg/L zeatin. The embryogenic callus was propagated on MS medium with NAA, zeatin and GA_3. Germination of somatic embryos was achieved on MS medium with 1 mg/L BA. Somatic embryo derived plantlets were hardened and successfully transferred to the greenhouse.

Key words: Regeneration, embryogenic callus, germination, plantlet.

Introduction

Rose is one of the most economically important flowers worldwide. Genetic improvement of rose through conventional breeding is limited by several factors such as polyploidy and the highly heterozygous nature of existing cultivars. Genetic engineering, based on tissue culture technology, provides an option for overcoming these restrictions. Although the establishment of somatic embryogenesis can provide target material for genetic manipulations and also for the clonal propagation of plants *in vitro*, the mechanisms involved in embryogenic callus induction and differentiation are still poorly understood.

In roses, somatic embryogenesis has been obtained from variety of explants such as calli derived from leaf (De Wit et al. 1990), immature leaf and stem segments (Rout et al. 1991), immature seeds (Kunitake et al. 1993), petioles and roots (Marchant et al. 1996; Roberts et al. 1995), and anther filaments (Noriega and Sondahl 1991). According to the published material, the callus initiation from rose tissues required the presence of an auxin, (mainly 2,4-dichorophenoxy acetic acid (2,4-D) and α-naphthalene acetic acid (NAA),) in the culture medium. Other strong auxins such as dicamba (Murali et al. 1996) and picloram (Kintzios et al. 1999) have been used as alternatives. Preincubation in a high 2,4-D concentration increased the frequency of embryogenesis (Hsia and Korban 1996; Marchant et al. 1996). The transfer of embryogenic callus to a medium with reduced auxin concentration or without auxin resulted in the maturation of somatic embryos and regeneration of plants.

One critical point in the plant regeneration via somatic embryogenesis is the further development and germination of the embryo in rose (Dohm et al. 2001). Omission or lowering the concentration of 2,4-D from the culture medium helped the embryo development and germination in many cultivars of *Rosa* spp. (Roberts et al. 1990; Mathews et al. 1991). Roberts et al. (1995) also reported that chilling at 4°C for 2 weeks improved the germination rates from 12 to 24%. The combination of BA with methyl laurate also stimulates conversion of somatic embryos of a hybrid cultivar Vague (Sarasan et al. 2001). It has been demonstrated that periods of stress during tissue culture, such as desiccation (Lee at al. 2001) and cold (Marchant et al. 1996), may increase the number of embryos converted to plants. In this report, the efficient protocol for regeneration via somatic embryogenesis was described. This protocol proved to be applicable to a broad range of different genotypes using *in vitro* grown the rose tissue.

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Materials and Methods

Plant materials

Four commercial rose hybrid cultivars and one rose species were used in this study. They were *Rosa hybrida* cvs. 4th of July, Tournament of Roses, Graham Thomas, Sequoia Ruby, and *R. chinensis* cv. Old Blush. Shoot cultures were raised through axillary bud proliferation on a medium containing MS basal medium (Murashige and Skoog 1962), 0.5 mg/L BA, 30 g/L sucrose and 7 g/L agar. Every four weeks the shoot cluster was subdivided into single shoots and transferred onto fresh medium. For the regeneration experiment, fully developed leaf explants were harvested from four-week-old cultures. The shoot cultures were incubated in a 16/8 h photoperiod by using cool white fluorescent lamps (90 μmol/m^{-2}/s^{-1}) at a temperature of 21°C.

Culture medium and condition

Leaf segments of 0.5 cm long were excised and inoculated with the adaxial side down on MS basal medium supplemented with different plant growth regulators (PGR; 2,4-D, NAA) at the concentration of 0.1, 0.5, 1 and 2 mg/L and solidified with 0.24% gelrite for initiating of callus. The pH of media was adjusted to 5.8 using 1N NaOH or 1N HCl, autoclaved at 121°C for 20 min and poured into polystyrene 100 × 15 mm Petri dishes (25 mL of medium per dish, nine explants per dish). A minimum of 45 explants from each cultivar was inoculated onto each medium and grown for 4 weeks at 25°C either under 16/8 h photoperiod provided by cool white fluorescent lamps (40-50 μmol/m^{-2}/s^{-1}) or under dark conditions.

Induction of somatic embryogenesis

Four weeks later, explants were transferred into MS medium with BA, kinetin, zeatin and Thidiazuron (TDZ) at various concentrations (0.5-5 mg/L) with 3% sucrose, and were transferred to fresh medium every 4 weeks. In order to permit further proliferation of embryogenesis, embryogenic clusters (5 mm in diameter) were transferred on MS medium with 0.25 mg/L NAA, 1.5 mg/L zeatin and 1 mg/L GA at 25°C under 16/8 h photoperiod by using cool white fluorescent lamps (40-50 μmol/m^{-2}/s^{-1}).

Embryo germination and recovery of plantlets

After 4 weeks, 50 randomly selected embryos per treatment were transferred to MS medium supplemented with 3% sucrose and the growth regulators BA at 0-1.0 mg/L. Rooting of these shoots was induced by subculturing on MS medium with 0.1 mg/L NAA. The regenerating somatic embryos as well as the shoots were incubated at 25°C under 16/8 h photoperiod as mentioned previously. For greenhouse adaptation the rooted plantlets were grown in pots containing autoclaved Sunshine Mix 4. The plantlets were kept in polyethylene bags for 2-3 weeks, which were progressively opened to adapt the plantlets to a lower humidity. The number of callus-forming explants, callus producing embryos and germinating embryos were recorded at the end of each subculture phase.

Results and Discussion

Induction of embryogenic callus

Leaf explants enlarged and developed callus at the cut surfaces within 7-10 days of inoculation. There was a little callus formation when leaf explants were cultured on media with 0.1 mg/L (the lowest concentration) of either 2,4-D or NAA whereas 55% to 100% of the leaf explants produced callus when cultured on media with 1 mg/L 2,4-D or NAA. Callus morphology differed with the various PGR treatments. Callus produced with 2,4-D tended to be grayish-white with various degrees of friability. A higher concentration (2 mg/L) of 2,4-D resulted in extremely friable and watery callus. However callus obtained with NAA was less friable than that of 2,4-D treatments. On the media with NAA leaf explants produced callus with primarily small, single cells and very small cell clumps. Root formation occurred on media with all concentrations of NAA and less frequently on media with 2,4-D. 'Tournament of Roses' and '4th of July' showed the best response for callus formation over all PGR treatments. Callus cultured under 16/8 h photoperiod using cool white fluorescent lamps (40-50 μmol/m^{-2}/s^{-1}) was initially hard and compact in appearance with pale yellow-white or green coloration and delayed the callus growth. Callus cultured under dark condition was less compact and showed reddish-brown.

Induction and proliferation of somatic embryogenesis

After 4 weeks on callus induction medium, the explants were subcultured on MS medium with zeatin or TDZ for induction of somatic embryogenesis. About 3 weeks after transfer onto medium with 2 or 4 mg/L zeatin, the regeneration of somatic embryos started and continued for approximately 3 subculture cycles (12 weeks). The secondary somatic embryogenesis, regenerating embryo and embryogenic calli were observed during subsequent subcultures with fresh medium. The cultivar '4th of July' showed the highest regeneration frequencies on MS basal medium containing 1 mg/L NAA followed by culture

on Ms basal medium containing 4 mg/L zeatin. Somatic embryogenesis was observed on only one ('4th of July') of the 5 genotypes tested (Table 1). After transfer of an embryogenic cluster (a mixture of globular embryos and regenerating embryos) onto MS medium with 0.25 mg/L NAA, 1.5 mg/L zeatin and 1 mg/L GA (Noriega and Sondahl 1991), the clusters developed into a mixture of smooth embryogenic callus tissue, more developed embryos and hard green callus tissue. The embryogenic callus tissue could be separated to a pure embryogenic callus line by 2-3 subcultures (8-12 weeks). Although, in a previous study, somatic embryogenic callus was initiated from adventitious roots of *R. chinensis* (data not shown), it was not initiated from *in vitro* grown leaf explant in any of the treatments.

As was previously reported (Hsia and Korban 1996; Dohm et al. 2001), callus induction from leaf explants took place with auxin/cytokinin combination or auxin alone, but somatic embryogenesis progressed only when cultured on MS medium with auxins alone for first 4 weeks of the callus induction periods. Noriega and Sondahl (1991) and Kintzios et al. (1999), however, reported that an auxin/cytokinin combination was essential for the induction of embryogenic callus in rose tissue.

Genotype was a significant factor influencing explant embryogenic differentiation in culture, since only two of five investigated cultivars responded positively to somatic embryogenesis treatment. A similar genotypic response to somatic embryogenesis among *R. hybrida* cultivars has been reported (De Wit et al. 1990; Hsia and Korban 1996).

Maturation and germination of somatic embryos

Cotyledonary-stage somatic embryos appeared 1-2 weeks after globular (Figure 1A) and heart-shaped embryos (Figure 1B) which were subcultured on MS basal medium with 0 to 1 mg/L BA and 3% sucrose. Most embryos obtained from this experiment were morphologically normal. Morphologically abnormal embryos failed to germinate. Germination was preceded by greening of the embryo within 2-3 days of transfer to light and accompanied by further elongation and expansion of the cotyledons (Figure 1C, D). The inclusion of 1 mg/L BA in the germination media increased and optimized germination (Table 2). Roots and shoots developed further when somatic embryos were transferred to MS medium containing a low concentration of NAA (0.1 mg/L). Whole regenerated plantlets bearing with three of four leaves were potted and acclimatized to greenhouse conditions with greater than 90% survival. All these plant were phenotypically normal (Figure 1F).

The beneficial effects of BA (alone or in combination with IAA or IBA) on somatic embryo maturation from rose explants (Marchant et al. 1996) and of kinetin on rose embryogenesis (Kunitake et al. 1993) have been previously documented with other rose genotypes. One critical step in the plant regeneration via somatic embryogenesis is the further development and

Table 1. Summary of plant growth regulator for inducting callus and somatic embryogenesis from leaf explant of roses.

Treatments[a] (mg/L)	Responding cultivar	Callus induction[b] (%)	Somatic embryogenesis[c] (%)
2,4-D 0.5	4th of July	100	6.6
/zeatin 2.0	Tournament of Roses	100	0
2,4-D 1.0	4th of July	100	24.4
/zeatin 4.0	Tournament of Roses	100	0
	Graham Thomas	88.8	0
	Sequoia Ruby	93.3	0
	R. chinensis	55.5	0
NAA 1.0	4th of July	100	17.7
/zeatin 4.0	Tournament of Roses	100	0

[a]Auxin/cytokinin, leaf explants were precultured on MS medium supplemented with auxin for 4 weeks and followed by several culture periods on MS medium with cytokinin.
[b]Fresh weight of callus per explant > 50 mg was recorded.
[c]Data were collected 14 weeks after culture initiation.

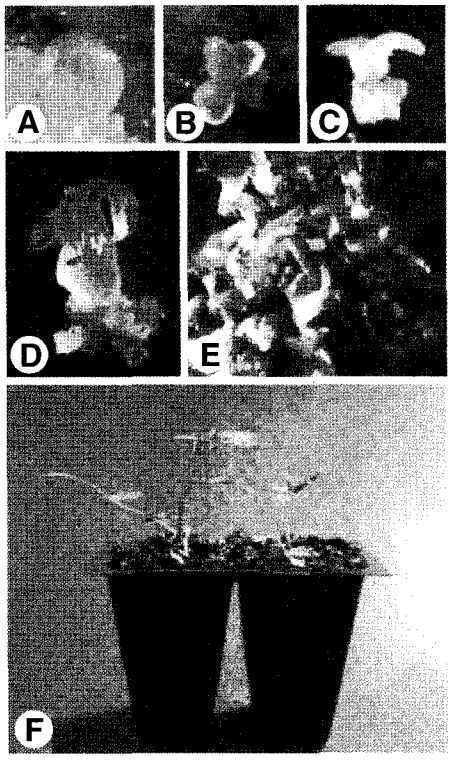

Figure 1. Plant regeneration from rose cv. 4th of July via somatic embryogenesis. Globular- (A) and heart-stage embryos (B) on embryogenic callus, C: Well developed cotyledonary-stage somatic embryo. Elongation (D) and whole germination (E) of embryos on MS medium with 0.1 mg/L BA and 3% sucrose, F: Phenotypically normal somatic embryo derived plants of rose cv. 4th of July.

Table 2. Effect of BA concentration on germination of somatic embryos of rose.

Germination medium (BA mg/L)	% Germination[a]	
	4 th of July	Tournament of Roses
0	0	0
0.5	5	3
1.0	11	8

[a]Percent germination was assessed from a minimum of 50 randomly selected embryos 4 weeks after transfer to germination medium.

germination of the embryo in rose (Dohm et al. 2001). In this study, a protocol for induction, maturation and germination of somatic embryos from *in vitro* grown leaf explant of *R. hybrida* was reported. The protocol reported in this paper for induction, maturation and germination of somatic embryos from *in vitro* grown leaf explants of *R. hybrida*, might help in the genetic improvement of roses for commercial use.

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