Maturation and Conversion of Somatic Embryos of Three Genetically Diverse Rose Cultivars

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Abstract. Embryogenic callus cultures of three genetically diverse cultivars of rose (Rosa hybrida L.), the floribunda 'Trumpeter', the multiflora 'Dr. Huey', and the hybrid tea 'Tineké', were used to study the effect of various carbohydrates and osmotically active compounds on somatic embryo maturation and conversion. Cotyledonary-stage embryos were produced by dispersing callus in liquid medium followed by filtration to isolate globular-stage embryos. Quantitative experiments were conducted to determine maturation and conversion of the three rose cultivars in response to medium with sucrose, glucose, fructose, or mannitol as the primary carbon source and also in response to various concentrations of either myo-inositol, polyethylene glycol, or mannitol in combination with 3% sucrose. Conversion of 27% was achieved for 'Trumpeter' embryos following their maturation on 3% fructose. 'Dr. Huey' embryos required maturation on medium containing 3% sucrose supplemented with either 2.5% or 5% mannitol for 36% and 61% conversion, respectively. Maturation of 'Tineké' embryos on either 3% sucrose, 3% glucose, or 3% fructose resulted in a maximum 12% conversion.

Roses are one of the most important floral crops in the world. However, most of the modern cultivars are susceptible to various microbial and insect pests. This necessitates the regular use of fungicides and insecticides by rose growers and substantially adds to their production expenses. Molecular biology techniques are now available for genetic modification of rose in order to obtain better resistance to pathogens. While several researchers report successful transformation of rose and was therefore also selected as an osmoticum in this study.

Materials and Methods

Establishment and maintenance of embryogenic cultures. Embryogenic callus cultures were established using the procedure of Marchant et al. (1996) for 'Trumpeter' and the procedure of Derks et al. (1995) for 'Tineké' and 'Dr. Huey'. Cultures of 'Tineké' were maintained on MS medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose, 18.1 μM dicamba (3,6-dichloro-o-anisic acid), 0.46 μM kinetin, 0.25% (w/v) Phytagar (Sigma Chemical Co., St. Louis), and the following in mg·L⁻¹: glycine, 2.0; thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5; myo-inositol, 100.0. Cultures of 'Trumpeter' and 'Dr. Huey' were maintained on SH medium (Schenk and Hildebrandt, 1972) with 3% (w/v) sucrose, 13.6 μM 2,4-D (2,4-dichlorophenoxyacetic acid), 0.25% (w/v) Phytagar, and the following in mg·L⁻¹: thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5; L-proline, 300. Both media were adjusted to pH 5.8 prior to autoclaving 20 min at 18 psi, 121 °C, and then 25 mL of medium were placed in each 100 × 20 mm Petri plate. All cultures were maintained in the dark at 25 °C and subcultured onto fresh medium every 5–6 weeks.

Preparation of inoculum for use in embryo maturation experiments. About 20 g of embryogenic callus were placed in a baffled flask with 50 mL of medium and placed on an orbital shaker set at 124 rpm for 2–3 h to break up the callus. Medium used for 'Trumpeter' and 'Dr. Huey' consisted of SH salts and vitamins and 1.5% sucrose. Medium used for 'Tineké' consisted of MS salts and vitamins, 100 mg·L⁻¹ myo-inositol, 2.0 mg·L⁻¹ glycine, and 1.5% sucrose. The suspension produced by shaking was sequentially filtered through stainless steel sieves with mesh sizes of 200, 118, and 85 μm. Washed cotyledonary-stage embryos and a mixture of globular-stage embryos, proembryogenic masses, and nonembryogenic callus cells. Additional medium was used during this process as needed to wash cells through the sieves. Cells that passed through the 850-μm mesh were washed three times with the same medium used for filtering, but without sucrose, and resuspended in 150–200 mL of this medium. Plates (100 × 20 mm) were inoculated using 2 mL of the suspension (three plates per treatment) and then placed in the dark at 25 °C.

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Maturation medium. The base medium for testing various carbohydrates and osmoticum consisted of MS salts and vitamins, 100 mg L\(^{-1}\) myo-inositol, 2 mg L\(^{-1}\) glycine, 0.25% (w/v) Phytagel, 0.5% (w/v) activated charcoal (AC), and 500 mg L\(^{-1}\) MES (2-[4-(2-hydroxyethyl)iminomethyl]morpholinol)ethanesulfonic acid). The carbohydrates tested included 3% (w/v) of either sucrose, glucose, fructose, or maltose. Media were prepared without either charcoal or Phytagel in 60% of the medium’s final volume and filter-sterilized to avoid degradation of the carbohydrates during autoclaving. The medium was adjusted to pH 5.8 prior to filter-sterilization through 0.2-μm filters. A solution containing AC and Phytagel in 40% of the final volume was autoclaved and added to filter-sterilized medium to give the final volume and concentration of all components. Cotyledonary-stage embryos in each plate were counted after 4–5 weeks on maturation medium. Only embryos with expanded cotyledons that were 2 mm or greater in diameter were scored as mature.

The osmotically active compounds were either 2.5%, 5.0%, or 7.5% (w/v) myo-inositol, PEG (m.w. 3350) or mannitol, and each was added to the base medium containing 3% (w/v) sucrose. Base medium containing 3% sucrose was used as a 0% osmoticum. Carbohydrates tested included 3% (w/v) of either fructose, glucose, or maltose. Media osmotica consisted of MS salts and vitamins, 3% sucrose, glucose, fructose, or maltose. Media were prepared without either charcoal or Phytagel in 60% of the medium and autoclaved in 94% of the total volume, cooled to 60\(^\circ\)C, and the remaining components added using sterile 50X stock solutions (Murashige and Skoog Complete Medium, 50X Concentrate; Gibco BRL/Life Technologies, Grand Island, N.Y.). The osmotic potential of each medium was measured in three replications using a 5500 Wescor Vapor Pressure Osmometer (Wescor, Logan, Utah).

Conversion of cotyledonary-stage embryos. Cotyledonary-stage embryos were cultured 10–11 weeks, without subculture, on each maturation medium containing various carbohydrates and osmoticum and then transferred to conversion medium (MS salts and vitamins, 3% sucrose, 100 mg L\(^{-1}\) myo-inositol, 2 mg L\(^{-1}\) glycine, 0.5% AC, 0.25% Phytagel). Cotyledonary-stage embryos, 150–200 taken from three plates, were transferred to conversion medium and kept in the dark for 1 week and then placed under cool-white fluorescent lights (10–20 μmol m\(^{-2}\)s\(^{-1}\)) with a 12-h photoperiod. The percentage of embryos that converted was determined by counting the number of embryos with both shoots and roots after 6 weeks on conversion medium.

Statistical analysis. An analysis of variance using Duncan’s new multiple range test with a 95% confidence interval (\(P < 0.05\)) was used for comparing the means of the osmotic and carbohydrate treatments for each cultivar (Figs. 2–5). A total of 150–200 embryos was isolated by filtration, and either three or four plates of 50 embryos each were prepared for each treatment. Each plate of 50 embryos was treated as a replicate.

Results and Discussion

Maturation of somatic embryos on various carbon sources. Mature, cotyledonary-stage embryos of the three cultivars, 'Trumper', 'Dr. Huey', and 'Tineké', developed following isolation of globular-stage embryos from embryogenic callus (Fig. 1 A and B). There were two obvious similarities in the way the cultivars responded to the four carbohydrates tested. First, no other carbohydrate induced more embryo maturation than sucrose (Fig. 2). These results were in contrast to those obtained with Rosa rugosa embryogenic cultures in which medium with glucose, fructose, or maltose all produced more somatic embryos than sucrose (Kunitake et al., 1993). Second, 3% maltose produced the fewest embryos in all three cultivars (Fig. 2). Marchant et al. (1996) used maltose in medium for germination of somatic embryos of 'Trumper' following their maturation on a medium containing sucrose. In loblolly pine, 6% maltose enhanced embryo maturation ≈10-fold.
compared to 3% sucrose. This effect may have been partly due to increased osmotic pressure in the medium by high maltose concentration (Li et al., 1998).

There were also differences among the cultivars in their response to the four carbohydrates. ‘Trumpeter’ produced more mature embryos (58–59/plate) on sucrose and fructose than on sucrose (35/plate) while more ‘Dr. Huey’ embryos (66–77/plate) matured on sucrose than on fructose (40/plate) (Fig. 2). ‘Tineké’ had no significant differences in the number of embryos maturing on sucrose, glucose, or fructose (35–47/plate) (Fig. 2).

Maturation of somatic embryos in response to osmotic treatments. The osmotic treatments produced varied results for the cultivars (Fig. 3). Increased osmotica stimulated maturation of embryos only for ‘Trumpeter’ by addition of either 2.5% or 7.5% PEG resulting in an osmotic potential of −0.54 or −0.62 MPa, respectively. All other osmotic treatments with myo-inositol, PEG, or mannitol either did not differ significantly from the control (3% sucrose) or inhibited embryo maturation.

PEG did not inhibit embryo maturation with increasing concentrations possibly because the osmotic potential at the highest PEG concentration tested was lower than for all concentrations of myo-inositol and mannitol tested (Fig. 3). Another difference between the osmotica is that both myo-inositol and mannitol are metabolized by plant cells (Nelson et al., 1998; Shen et al., 1999; Wright et al., 1989) whereas PEG is a nonpermeating and non-plasmolyzing osmoticum that is not metabolized (Attree et al., 1995; Nelson et al., 1998; Shen et al., 1999; Wright et al., 1989).

Conversion of cotyledonary-stage embryos following maturation on the base medium containing various carbohydrates. The carbohydrate in the maturation medium affected conversion of ‘Trumpeter’ and ‘Tineké’ embryos, but not ‘Dr. Huey’. Conversion for ‘Trumpeter’ was more than two times higher (27%) when cotyledonary-stage embryos had matured on 3% fructose rather than on 3% sucrose (Fig. 4). Conversion of ‘Tineké’ embryos was best (12%) after embryos had matured on 3% sucrose, 3% glucose, or 3% fructose (Fig. 4).

Conversion of embryos from ‘Dr. Huey’ responded quite differently from the other two cultivars (Fig. 4). Embryos of ‘Dr. Huey’ that matured on sucrose, glucose, or fructose did not convert, and only one of 200 embryos from the 3% maltose treatment converted.

Conversion of cotyledonary-stage embryos following maturation on the base medium containing 3% sucrose and various osmotica. All globular-stage embryos that developed to the cotyledonary-stage on maturation medium containing 3% sucrose and an osmoticum, either myo-inositol, PEG, or mannitol, were transferred to conversion medium.

Embryos of ‘Trumpeter’ and ‘Tineké’ that matured on the maturation media with various osmotic treatments did not show any improvement in conversion rates over sucrose alone (Fig. 5).

‘Dr. Huey’ embryos that matured on the osmotic treatments converted and responded positively to increasing concentrations of myo-inositol and mannitol up to 5%. The highest conversion rates were 39% with 2.5% mannitol and 61% with 5.0% mannitol. In comparison, conversion was significantly lower when embryos matured on myo-inositol rather than mannitol although the osmotic potential was similar. This indicated that the rate of conversion was affected by the
Fig. 4. Percentage of cotyledonary-stage embryos of three *Rosa hybrida* cvs. ('Trumpeter', 'Dr. Huey', and 'Tineke') by carbohydrate source (3% sucrose, glucose, fructose, or maltose) as shown on the X axis. Means (three replicates of 50 embryos each) followed by different letters are significantly different at $P \leq 0.05$ according to Duncan’s new multiple range test done separately for each cultivar.

Conclusions

High rates of somatic embryo maturation and conversion of multiple rose cultivars is required for development of efficient transformation systems of this horticulturally important crop. This study demonstrated that various carbohydrates or osmoticum could be used to improve maturation, and more importantly, conversion of somatic embryos from rose cultivars. Conversion above 60% was obtained for ‘Dr. Huey’ followed by ‘Trumpeter’ on 5% mannitol, 27% for ‘Trumpeter’, and 12% for ‘Tineke’ embryos on 3% sucrose. This study also demonstrated that treatment effects are highly cultivar-specific for roses and validates the need to optimize regeneration protocols for individual cultivars.

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