

## I.5 Regeneration of Plants from Protoplasts of *Larix* Species (Larch)

P.J. CHAREST and K. KLIMASZEWSKA<sup>1</sup>

### 1 Introduction

#### 1.1 Distribution and Importance of *Larix*

*Larix* species (or larch) are widely distributed in the cold temperate climate of the Northern Hemisphere (Table 1). Ten species are generally recognized (Boyle et al. 1989) that grow in a number of drastically different environments (Burns and Honkala 1990). For instance, *Larix laricina* grows in a cold climate on soil with high moisture and acidity. *Larix lyallii* grows on high mountains in a snowy and cold climate on dry, rocky, and acidic soils. In contrast, *L. occidentalis* grows in a milder climate on good soils. Larches have a relatively good annual net carbon gain and high growth rate due to their more efficient use of nutrients (Gower and Richards 1990). They have good wood quality and rotting resistance, which makes them suitable for the lumber industry (New Brunswick Forest Research Advisory Committee 1986), but their use is limited by their availability. Biologically, *Larix* species are interesting because of their potential for interspecific hybridization and their deciduous habit as conifers. Natural hybrids such as *Larix leptolepis* × *Larix decidua* and its reciprocal have demonstrated hybrid vigor (Boyle et al. 1989).

#### 1.2 Review of Protoplast Isolation from *Picea* and *Larix* Embryogenic Cultures

The most suitable tissue culture system for protoplast isolation in conifers has been somatic embryogenesis. In spruce and larch, somatic embryogenic tissues are obtained from zygotic embryos, and cotyledons or needles of young seedling explants (Lelu et al. 1993). The embryogenic tissues can be used to produce somatic embryos that will subsequently germinate and regenerate into trees. Larch and spruce embryogenic tissues are easily grown in liquid medium to produce suspensions of embryonal mass. Protoplast isolation and regrowth of

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<sup>1</sup> Molecular Genetics and Tissue Culture Group  
Petawawa National Forestry Institute  
Chalk River, Ontario  
Canada, K0J 1J0

**Table 1.** Species of the genus *Larix* and distribution

Species	Common name	Geographic distribution
<i>Larix decidua</i> Mill.	European larch, Alpine larch	Europe
<i>Larix gmelini</i> (Rupr.) Kuzeneva <sup>a</sup>	Dahurian larch, Korean larch, East Siberian larch, Olga Bay larch, North China larch, Prince Rupprecht's larch, Kurile larch, Sakhalian larch	Eastern Asia, Siberia, Mongolia, Korea, China
<i>Larix griffithii</i> Hook	Himalayan larch, Sikkim larch	Eastern Asia
<i>Larix laricina</i> (Du Roi) K. Koch	Tamarack, Eastern larch	North America
<i>Larix leptolepis</i> (Sieb. and Zucc.) Gord. <sup>b</sup>	Japanese larch	Japan
<i>Larix lyallii</i> Parl.	Alpine larch, Lyall larch	Western North America
<i>Larix mastersiana</i> Rheder and Wilson	Master's larch	Eastern Asia
<i>Larix occidentalis</i> Nutt.	Western larch, Tamarack	Western North America
<i>Larix potaninii</i> Batal	Chinese larch	Eastern Asia
<i>Larix sibirica</i> Ledeb <sup>c</sup>	Siberian larch, West Siberian larch	Russia, Siberia, Mongolia

<sup>a</sup> Synonyms: *Larix koreensis*, *L. principis-rupprechtii*, *L. kurilensis*.

<sup>b</sup> Synonym: *Larix kaempferi*.

<sup>c</sup> Synonym: *Larix sukacewii*.

protoplasts from somatic embryogenic tissues in spruce and larch have been carried out successfully with cell suspensions only. In general, the isolation procedure comprises four steps: (1) pretreatment of the embryogenic suspension, (2) enzymatic digestion of cell walls, (3) separation of the protoplasts from the debris, and (4) regrowth of the embryonal mass from protoplasts.

Cell suspensions are initiated by taking pieces of embryonal masses grown on solid media and adding them to liquid media followed by shaking on a rotary shaker at 100–150 rpm. In most cases, the medium used for suspension cultures is the same as that used for the maintenance of embryogenic tissues on solid media; in some cases, there are slight changes in the growth regulator content. The subcultures are more frequent than those associated with cultures on solid media, ranging from 1 to 2 weeks.

Variations are found in the protoplast isolation procedure depending on the species and the laboratory. Sometimes, an osmotic pretreatment is included in the procedure which involves placing the cells in a mannitol solution for 1 h. The enzyme composition used in the digestion step varies from study to study, but all digestions take place at 25 °C in darkness. Regrowth of protoplasts is difficult because of their fragility and requires a gradual change in osmoticum to minimize stress. Trees have been regenerated from protoplasts of *Larix × eurolepis* (Klimaszewska 1989a) and *Picea glauca* (Attree et al. 1989).

The embryonal mass is generally composed of two types of cells: meristematic and suspensor cells. These yield protoplasts of different sizes after enzymatic digestion. The meristematic cells are smaller and less vacuolated than the suspensor cells. For this reason, some more refined gradients (Percoll or Ficoll, Pharmacia LKB, Quebec, Canada) have been used to separate different types of protoplasts. It is believed that only meristematic cells regrow better after protoplast isolation.

### 1.2.1 Protoplast Isolation and Culture with *Larix*

With *Larix × eurolepis* (Klimaszewska 1989a), it was possible to isolate protoplasts from embryonal mass grown on solid medium and in liquid medium; however, the protoplast yield was doubled when cultures in liquid medium were used ( $6 \times 10^5/g$  for line L2 and  $1.3 \times 10^6/g$  fresh weight for line L1). Digestion of the embryonal cells from an embryogenic cell suspension resulted in the release of different types of protoplasts that required application of a discontinuous Percoll gradient to fractionate the different subpopulations. Large vacuolated protoplasts with a single nucleus and small, densely cytoplasmic protoplasts with one to three nuclei were observed. Typical results of fractionated protoplasts from an embryogenic cell line are presented in Fig. 1. Of the three fractions, only the fraction containing the small, densely cytoplasmic, uninucleate protoplasts yielded embryonal mass. In this subpopulation, cell walls were detected after 1 day and the first cell division after 2 days. Unequally divided cells comprised of embryonal and suspensor initials were observed after 5 to 6 days of culture. Early somatic embryos were detected after 23–28 days. Somatic plantlets were regenerated and planted in the field. The regenerated trees were 6

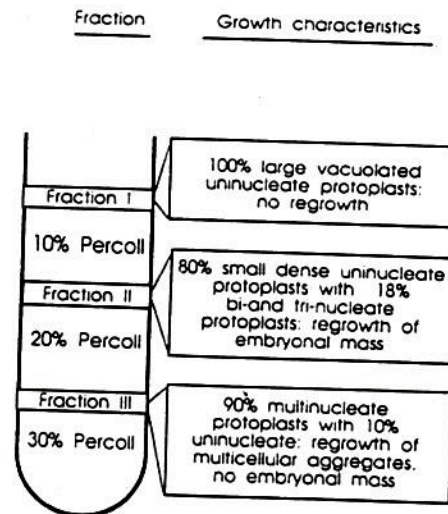


Fig. 1. Fractionation of *Larix* protoplasts on a discontinuous Percoll density gradient and growth characteristics in culture



years old in 1993 and were showing normal phenotype. Representative results from protoplast isolation of *Larix* embryogenic cell lines are shown in Figs. 2 and 3.

Regeneration of embryonal mass from small protoplasts with dense cytoplasm has been observed in loblolly pine and Douglas fir (Gupta and Durzan 1987; Gupta et al. 1988). In addition, the single-cell origin of somatic embryos

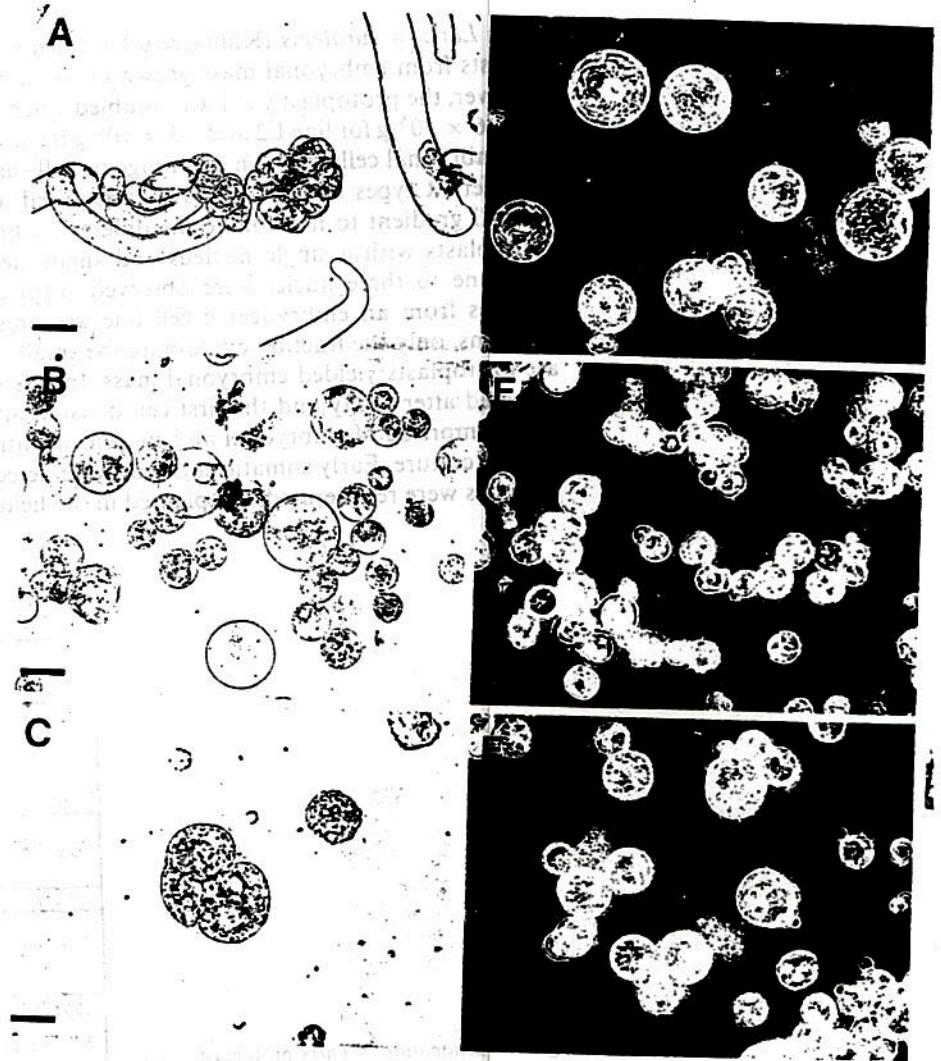


Fig. 2A-F. Isolation of *Larix* protoplasts. A Embryonic suspension of *Larix × eurlepis*, bar = 30  $\mu$ m; B freshly isolated protoplasts, bar = 36  $\mu$ m; C equal division after 6 days, bar = 21  $\mu$ m; D fraction I on 10% Percoll, bar = 45  $\mu$ m; E fraction II on 20% Percoll, bar = 26  $\mu$ m; F fraction III on 30% Percoll, bar = 26  $\mu$ m

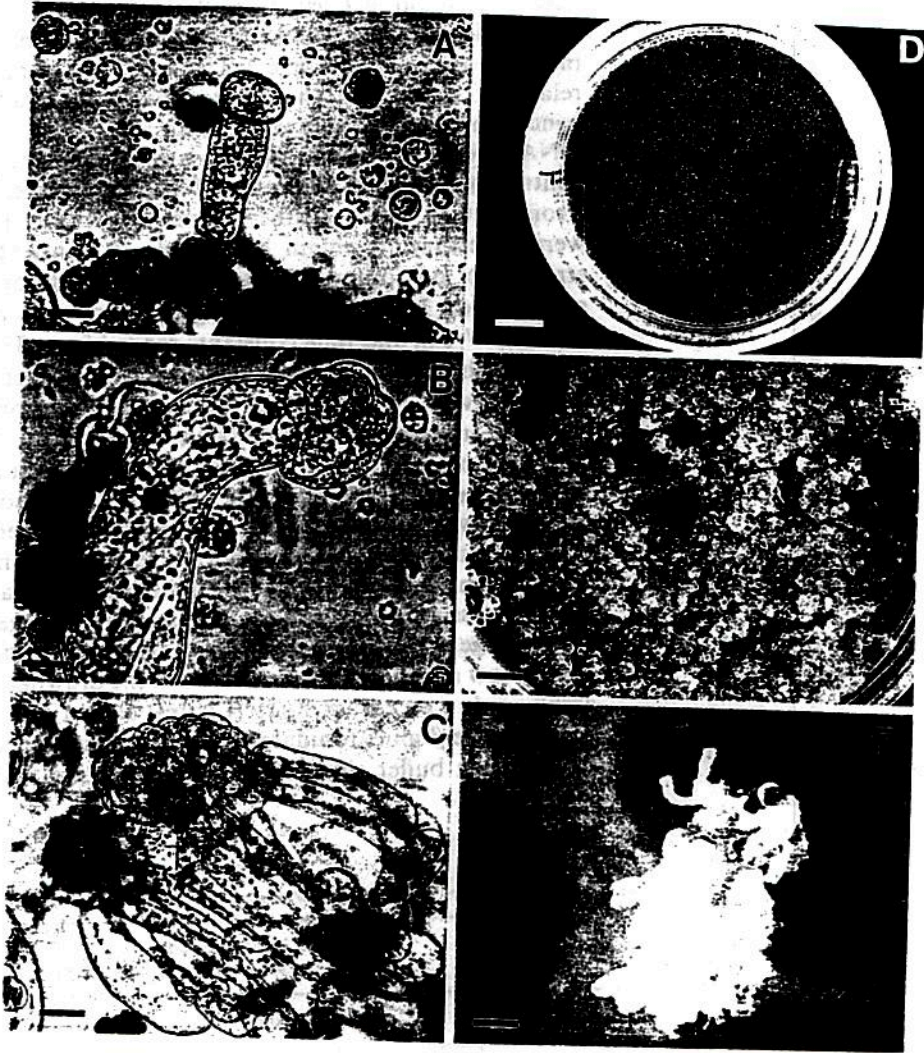


Fig. 3A-F. Growth of embryonal mass from isolated protoplasts of fraction II. A Unequal division after 11 days, *bar* = 29  $\mu$ m; B meristematic cells with suspensor initials after 23 days of culture, *bar* = 28  $\mu$ m; C embryonal mass after 5 weeks, *bar* = 57  $\mu$ m; D colonies growing in liquid medium after 6-8 weeks of culture, *bar* = 8 mm; E embryonal mass after 4 weeks on solid medium, *bar* = 10 mm; F typical embryonal mass showing developing somatic embryo, *bar* = 2 mm

followed by an unequal division has been demonstrated for *Picea abies* (Nagmani et al. 1987) and *Picea glauca* (Hakman and Fowke 1987). These results indicate that it is possible to regenerate embryonal mass from *Larix* protoplasts and that trees can subsequently be grown from these cultures.



### 1.2.2 Electroporation of *Larix* Protoplast

Electroporation of *Larix* protoplasts was effective with both square and decay wave pulses (Charest et al. 1991). The factors tested with both electroporation methods are listed in Table 2. The decay wave apparatus was used because it is relatively easier and cheaper to construct (Bradshaw et al. 1987). Optimal conditions for the decay wave pulse were 300 V, 150  $\mu$ F, and 300  $\mu$ g/ml vector DNA as evaluated with the chloramphenicol acetyltransferase gene under the control of the 35S promoter of the cauliflower mosaic virus (pCaMVCN, Fromm et al. 1985). Neither carrier DNA nor chemical membrane disrupter were necessary to obtain high levels of transient gene expression. The field strength of our optimal electroporation condition was 600 V/cm with a time constant of 3.8 ms. Field strengths used with other conifers ranged from 200–700 V/cm; and time constants ranged from 35 to 170 ms which were 10 to 20 times higher than under our conditions. Vector DNA concentration was optimum with 300  $\mu$ g/ml, but higher concentrations could have yielded higher levels of transient gene expression. Three promoters were tested (35S; NOS promoter of the nopaline synthase gene, Fromm et al. 1985; and WIN promoter of the proteinase inhibitor gene IIK of potato, Thornburg et al. 1987). The 35S promoter yielded the highest level of transient gene expression and the WIN promoter the lowest. The reporter genes chloramphenicol acetyltransferase, neomycin phosphotransferase II, and  $\beta$ -glucuronidase were all used for transient gene expression with *Larix* protoplasts. The CAT gene was the most suitable for transient gene assay; however, the enzyme-linked immunosorbent NPT II assay that is now available could circumvent the drawbacks of the radioactive NPT II assay used in an earlier study (Charest et al. 1991). In addition, the fluorescent assay for the  $\beta$ -glucuronidase gene could be used because the use of methanol in the reaction buffer eliminated the background fluorescence problem (Charest et al. 1993).

**Table 2.** Factors tested with *Larix x eurolepis* protoplast electroporation

Factors	Values tested	
	With square wave pulse	With decay wave pulse
Voltage	300*, 400 V	100, 200, 300*, 400 V
Duration of pulse	10 ms	
Capacitance		50, 150*, 200, 350 $\mu$ F
Vector DNA concentration	100, 300* $\mu$ g/ml	0, 50, 100, 200, 300* $\mu$ g/ml
Membrane disrupter	Polyethylene glycol, none*	Polyethylene glycol, none*
Carrier DNA concentration	Not tested	0, 50, 150 $\mu$ g/ml
Optimal conditions within evaluated parameters	300 V, 10 ms, 300 $\mu$ g/ml vector DNA, no membrane disrupter	300 V, 150 $\mu$ F, 300 $\mu$ g/ml vector DNA, no membrane disrupter, no carrier DNA

\*Condition under which optimum transient CAT gene activity was detected. Electroporation volume was 1 ml with  $1 \times 10^6$  protoplasts using a circular electrode with a 0.5-cm gap. The resistance of the protoplast mixture was 22  $\Omega$ .

## 2 Establishing an Embryogenic Suspension

The following protocol has been used successfully with *Larix × eurolepis* embryogenic tissues produced from immature zygotic embryos in MSG culture medium (Klimaszewska 1989b). It is essential for protoplast isolation to obtain a healthy and actively growing embryogenic suspension. A great deal of variability has been observed in our laboratory and regular reinitiation of the embryogenic suspension is required.

1. Small pieces of actively growing tissues (7-day-old if on 14-day subculture regime) are suspended in 25 ml of liquid medium (MSG) in a sterile 125-ml Erlenmeyer flask. One to 2 g of embryonal mass is required.
2. The flask is placed on a gyratory shaker at 115 rpm under low light intensity. After 7 days, 5–10 ml of fresh medium is added if the suspension has grown.
3. Step 2 is repeated if the suspension has grown further after 7 days. If there is no noticeable growth, the embryonal mass is allowed to settle and the supernatant is removed and replaced with an equal volume of fresh medium. If the suspension is very dense, it is divided into two flasks and an equal volume of fresh medium is added.
4. The procedure is repeated after 7 days. At the end of another 7 days, the cell suspensions are subcultured by transferring 7–10 ml of the suspension to 25 ml fresh medium in a new Erlenmeyer flask.
5. The cell suspension is established and subcultured every 7 days.

## 3 Protoplast Isolation from Embryogenic Suspensions

The success of protoplast isolation depends on the quality of the initial suspension cultures. Care should be taken to observe the suspension microscopically at every isolation to characterize its quality. Based on the following procedure, 10 g of embryonal mass should yield about  $1 \times 10^7$  protoplasts.

1. Embryonal cells from a 3- to 4-day-old suspension are collected by filtering through a 63- or 73- $\mu\text{m}$  nylon mesh filter.
2. In a large Petri dish (100  $\times$  25 mm), 1 g (fresh weight) of embryonal mass is suspended in 10 ml of pretreatment solution and left standing in darkness for 1 h at room temperature. Ten ml of the enzyme solution containing 0.5% (w/v) of both cellulase and macerozyme is added to the Petri dish, resulting in an enzyme concentration of 0.25%.
3. The Petri dish is sealed and placed on a gyratory shaker at 26 rpm overnight (about 17 h). For a 6-h digestion, a final concentration of 0.5% (w/v) of both cellulase and macerozyme is used.
4. The digestion mixture is gently filtered through two nylon mesh filters (a 63- $\mu\text{m}$  filter on top of a 44- $\mu\text{m}$  filter). The filtrate is centrifuged for 6 min at  $11.1 \times g$ .
5. The enzyme solution is removed and the pellet is resuspended in culture medium. The mixture is centrifuged again and the protoplast pellet is



resuspended at a density of  $2 \times 10^5$  protoplasts/ml as counted in an hemocytometer.

6. A Percoll gradient is prepared in a 15-ml centrifuge tube by layering, from the bottom up, three solutions of 30, 20 and 10% (v/v) Percoll, respectively, made with liquid MSG medium.
7. 1.5 to 2 ml of protoplast suspension is layered on top of the 10% Percoll fraction and centrifuged for 3 min at  $11.1 \times g$ . The middle band is collected and the density is counted.
8. To separate protoplasts from debris, a flotation method on 2% Ficoll and 0.4 M sucrose can be used. After step 4, the protoplasts are suspended in the Ficoll-sucrose solution and overlaid with 0.5 ml of culture medium.
9. The protoplasts are centrifuged for 6 min, collected from the interface, and washed twice in culture medium.

#### 4 Culture of Protoplasts

1. The density of protoplasts is adjusted to  $1 \times 10^5$  per ml with liquid culture medium. With a wide-mouth pipette, 1.5 to 2 ml of protoplast suspension is layered in a  $60 \times 15$  mm Petri dish (Falcon #3002, Becton Dickinson, CA, USA). The quality of the Petri dish is important because it will influence the layer thickness and the spreading of the protoplast suspension. An animal tissue culture grade Petri dish is required.
  2. The Petri dish is sealed and placed in the dark at  $25^\circ\text{C}$ . Growth is monitored every day.
  3. When 30–50% of the cells divide (between 7 and 12 days), 0.3 to 0.5 ml of culture medium with 0.2 M mannitol is added.
  4. After another 10 to 12 days, 0.5 ml of medium without mannitol is added to further lower the osmoticum. The total cell suspension is divided into other Petri dishes in such a way that a dish does not contain more than 3 ml. This is extremely important for oxygenation of the growing cells.
  5. The suspension is fed with fresh medium without mannitol until colonies of embryonal mass can be seen in about another 30 days.
  6. The visible colonies are then transferred to an MSG solid medium with 4 g/l gelrite and subcultured every 2 weeks.
- Alternatively, the protoplasts can be grown in solid medium right after isolation. In this case, the protoplast density is adjusted to  $2 \times 10^5$  per ml and 0.8 ml of protoplast suspension is added to 0.8 ml of melted medium with 1% SeaPlaque agarose (FMC Bioproducts, ME, USA). Then, steps 2 to 6 are followed. At the first addition of medium, the solid culture is sliced vertically to allow liquid medium to percolate through.



## 5 Regeneration of Trees from Protoplasts

The protocol to regenerate trees is essentially the same as that used to regenerate trees from embryonal mass (Klimaszewska 1989a; von Aderkas et al. 1990; Lelu et al. 1993).

1. Pieces of embryonal mass are transferred onto an MSG maturation medium with 1% (w/v) charcoal and 4 g/l gelrite and placed under a 16-h photoperiod at 25 °C for 7 days.
2. Pieces of embryonal mass are transferred onto MSG medium containing 0.2 M sucrose, 40 μM ABA, and 4 g/l gelrite in tall Petri dishes (100 × 25 mm).
3. After 14 days, somatic embryos will be formed and should be removed from the surrounding tissues. They are placed on a nylon mesh (200 μm) on MSG medium containing 0.1 M sucrose, 40 μM ABA, and 4 g/l gelrite in 100 × 15 mm Petri dishes.
4. After another 14 days, the somatic embryos will have matured and can be held at this stage for up to 2 months before they are germinated.
5. The somatic embryos are germinated on MSG medium with 0.06 M sucrose and 4 g/l gelrite.
6. When the radicle is well developed, the plantlet can be transferred to peat moss or another substrate in a high humidity environment.

## 6 Summary

Protoplasts can be isolated effectively from embryonal mass of *Larix × eurolepis*. The protoplast yield from an embryogenic liquid culture is around  $1 \times 10^6$ /g fresh weight of starting material. These protoplasts can be cultured to form cell colonies that differentiate into embryonal mass. This mass develops from the subpopulation of protoplasts that are small, densely cytoplasmic, and predominantly uninucleate. Subsequently, the cultures yield somatic embryos that germinate to produce somatic plantlets. In addition, the protoplasts isolated by the method described herein are amenable to gene delivery via electroporation.

## 7 Protocols

### a) Media for Protoplast Isolation, Culture, and Plant Regeneration

All solutions required filter sterilization. Once colonies are obtained, regular autoclaving of the tissue culture medium is adequate. The following solutions are required.

### b) Protoplast Isolation and Culture

The pretreatment solution is composed of 0.4 M mannitol, 10 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5 mM morpholin-oethanesulfonic acid (MES), and 0.1% (w/v) Macerase (Calbiochem, Hoechst). The pH is adjusted to 5.8.

The double-strength enzyme solution is made with 0.5% (w/v) cellulase Onozuka R10, 0.5% (w/v) macerozyme Onozuka R10, 0.4 M mannitol, 10 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 5 mM MES. The pH is adjusted to 5.8.

The culture medium is MSG supplemented with 0.4 M mannitol, 5 mM MES, 0.05 M sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid, and 0.5 mg/l benzylaminopurine (Klimaszewska 1989b). The pH is adjusted to 5.8.

The Percoll gradients are made of Percoll added to the culture medium at the desired concentrations (10, 20, and 30% v/v).

The 2% Ficoll-0.4 M sucrose solution is made by dissolving Ficoll and sucrose in culture medium devoid of mannitol and sucrose. The pH is adjusted to 5.8.

### c) Growth of Protoplasts and Plant Regeneration

The basic culture medium is MSG (Klimaszewska 1989a).

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