PLANTLET REGENERATION VIA SOMATIC EMBRYOGENESIS FROM SUBCULTURED CALLUS OF MATURE EMBRYOS OF PICEA ABIES (NORWAY SPRUCE)

PRAMOD K. GUPTA AND DON J. DURZAN

Department of Pomology, University of California, Davis, California 95616

(Accepted 8 September; editor Jennie Mather)

SUMMARY

Embryogenic callus was initiated from radicles of mature embryos removed from imbibed seeds (24 h). Embryogenic and other nonembryogenic types of callus proliferated on a modified half-strength Murashige-Skoog medium (MS) basal medium (BM) supplemented with myo-inositol, casein hydrolysate (CH), L-glutamine (gln) and growth regulators kinetin (KN), N\(^6\)-benzyladenine (BAP) each (20 \( \times \) 10\(^{-6} \) M), 2,4-dichlorophenoxyacetic acid (2,4-D) (50 \( \times \) 10\(^{-4} \) M). Embryogenic callus bearing suspensor-like cells in a mucilaginous gel matrix was isolated and maintained by subculture every 10 to 12 days on BM with KN, BAP each (2 \( \times \) 10\(^{-6} \) M) and 2,4-D (5 \( \times \) 10\(^{-4} \) M). Somatic embryos developed spontaneously from the callus on this medium at 23 ± 1°C. Closer examination revealed that numerous polyembryonic clusters, comprised of elongated cells (suspensors) and small dense cells with large nuclei (somatic embryos), occurred in the viscous gel. When this enriched embryonal-suspensor mass was subcultured to low 2,4-D (1 \( \times \) 10\(^{-4} \) M), globular embryos developed by 40 to 60 days. Upon transfer to a liquid medium without growth regulators, the embryos elongated and developed cotyledons and shoots with needles. Plantlet development was completed by 30 days in a basal medium without CH, gln and growth regulators. The total culture time was 150 days. Approximately 40 ± 10 embryos were formed from 500 mg of initial callus. Somatic embryogenesis became aberrant if embryos remained attached to the callus mass and were not subcultured within 10 to 12 days according to the described protocol. Somatic embryos were encapsulated in an alginate gel and stored at 4°C for nearly two months without visible adverse effects on viability.

Key words: Picea abies; somatic polyembryogenesis; mature embryos.

INTRODUCTION

The regeneration of plantlets via somatic embryogenesis has been considered as a method of producing a large number of woody plants efficiently (1). Recently, somatic embryogenesis and plantlet regeneration has been reported in conifers (2,5-8,12,13). Much of the success for this process is based on the selection and proliferation of embryogenic tissues from immature embryos. Early evidence for somatic embryogenesis in Picea abies comes from the formation of embryo-like structures in callus from explants of cotyledons taken from immature (10) and mature embryos (9) of P. abies.

This paper extends earlier observations on mature embryos of conifers (5,9) by using seeds of P. abies stored for 2 years at 4°C. Individual seeds were surface-sterilized (4) and imbibed for 24 h in sterile water. Embryos were excised and inoculated directly onto a basal cultured medium (BM-2, see below).

CULTURE MEDIA AND CONDITIONS. A Murashige-Skoog medium (MS), basal medium (BM) comprised of salts, vitamins and glycine (11) with modified levels of NH\(_4\)NO\(_3\) (550 mg/l), KNO\(_3\) (4676 mg/l) and thiamine HC\(_1\) (0.1 mg/l) was formulated for somatic embryogenesis. For embryogenesis, half-strength, modified-MS, basal medium was supplemented with casein hydrolysate (CH) (500 mg/l), myo-inositol (1000 mg/l), gln (450 mg/l) and sucrose (3%). This modification is identified as BM-1. Other BM modifications are:

BM-2. BM-1 + KN, BAP each (2 \( \times \) 10\(^{-4} \) M) + 2,4-D (5 \( \times \) 10\(^{-4} \) M).

BM-3. BM-1 + KN, BAP each (2 \( \times \) 10\(^{-4} \) M) + 2,4-D (5 \( \times \) 10\(^{-4} \) M).

BM-4. BM-1 + KN, BAP each (2 \( \times \) 10\(^{-4} \) M) + 2,4-D (1 \( \times \) 10\(^{-4} \) M).

685
All media were adjusted to pH 5.7 at 24°C with KOH and HCl and solidified with Bacto-agar (Difco) 0.6%. Test-tubes containing liquid medium were provided with filter paper support for explants and callus. All media with growth regulators were autoclaved (1.1 kg cm⁻² at 121°C for 20 min). Cultures were incubated in darkness at 23 ± 1°C, ca. 60% relative humidity and transferred to light after 30 days for late embryogenic development.

All experiments leading to the demonstration of somatic polyembryogenesis were carried out with five replications and these were repeated at least three times.

**Encapsulation and Storage.** Individual somatic embryos at the stage shown in Fig. 9 were encapsulated in a gel (0.1% sodium alginate with 100 mM Ca(NO₃)₂). The encapsulation procedure was used to store several hundred embryos at 4°C ± 1°C in darkness for two months.

**Microscopy.** Somatic embryos were embedded in paraplast for histological examination as described by Feder and O'Brien, 1968 (3).

**RESULTS**

Within 30 days on BM-2, 5-6% of the excised embryos developed two types of visually distinct callus (Fig. 1). Callus developing from cotyledons was green and compact. Callus from radicals was white, translucent, and embedded in a viscous mucilaginous matrix. This callus contained a proliferating embryonal-suspensor mass. Mucilaginous calli were also reported with other conifer species (5,7,9,10).

**FIG. 1.** Growth of two types of calli from mature embryos on BM-2 after 30 days. e) green compact callus developed from cotyledon, ne white translucent callus developed from radicle. The white callus contains a proliferating embryonal-suspensor mass with embryonal initials under the conditions of this study. ×8

**FIG. 2.** Growth of the subcultured embryonal-suspensor mass on BM-3 after 12 days. This type of mucilaginous cellular mass yields true-to-type polyembryonic development. ×10

The formation of the embryonal-suspensor mass is not dependent upon having an intact embryo as the initial explant (10,12). The nonembryogenic callus and proliferating embryonal-suspensor masses were subcultured with low cytokinin and 2,4-D (BM-3; Fig. 2). Within 10-15 days, numerous somatic embryos emerged from the white mucilaginous embryonal-suspensor mass derived from the radical. Embryos in this mass developed in networks of polyembryonic clusters. Each embryo consisted of a linear array of elongated cells at one end (like suspensors) and a small highly dense cluster of cells with large nuclei typical of developing embryos at the other end (Fig. 3). These polyembryonic somatic structures were strikingly similar to those seen during the early stages of zygotic embryogenesis. Somatic embryos were not evident among calli originating from cotyledons under our experimental conditions.

Aberrant development, involving the amplification of shoot and root development, was observed at longer subculture rates when embryogenic cells developed more as a callus and polyembryonic clusters were not separated and dispersed from one another (Figs. 5,6,7).

Embryogenic cell masses were maintained at 10- to 12-day intervals by subculture on the same medium. Embryonic growth beyond the globular stage was always arrested in this medium. For this reason, proliferating embryonal-suspensor masses with their polyembryonic clusters were transferred to a low concentration of 2,4-D (1 × 10⁻⁴ M) medium (BM-4). Within 15 days, enlarged globular stages of development were observed (Fig. 4). By 14 days, some (ca. >25%) of these numerous globular structures produced chlorophyll even when maintained in darkness. Morphogenesis of embryos continued on the same medium up to the torpedo stage of late embryonic development.

We have frequently observed that polyembryonic somatic embryos are sensitive to subculture rate and