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ORIGINAL PAPER

Somatic embryogenesis from zygotic embryos and shoot-tips of the Chilean tree *Gomortega keule*

Diego Muñoz-Concha · Sean Mayes · Gracia Ribas · Michael R. Davey

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Abstract The endangered Chilean tree *Gomortega keule* (Mol.) Baillon produces edible fruit, making it a potential crop. However, its cultivation from seed or cuttings is extremely difficult. This paper reports the induction of somatic embryogenesis and the initiation of liquid cultures in this species. Callus was induced from zygotic embryos and field-collected shoots. Somatic embryogenesis on zygotic embryos occurred at a low frequency. Induction of somatic embryogenesis was accomplished on micropropagated shoots after 6.5 months on semi-solid Murashige and Skoog (MS) medium with 30 g/l sucrose, 1.0 mg/l 2,4-dichlorophenoxyacetic acid and 1.0 mg/l 6-(γ,γ-dimethylallylamino) purine (2iP). Liquid cultures of compact callus and small aggregates were obtained and showed optimum proliferation in MS medium with 20 g/l sucrose, 0.01 mg/l α-naphthaleneacetic acid and 0.1 mg/l 2iP. The proliferation of friable embryogenic callus was observed in liquid medium and will allow the propagation of selected genotypes of this tree on a large scale. Genetic variation in two embryogenic genotypes cultured in vitro was not detected in an assessment using microsatellites; this approach is suitable for tracing genotypes.

Keywords Callus · In vitro culture · Plant growth regulators · Gomortegaceae · Laurales

D. Muñoz-Concha (✉)
Departamento de Ciencas Agrarias, Universidad Católica del Maule, Casilla 7-D, Curicó, Chile
e-mail: dmunoz@ucm.cl

S. Mayes · G. Ribas · M. R. Davey
Plant and Crop Sciences Division, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	6-(γ,γ-Dimethylallylamino) purine
AC	Activated charcoal
BA	6-Benzyladenine
GA_3	Gibberellic acid
FDA	Fluorescein diacetate
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthaleneacetic acid
PGRs	Plant growth regulators
PVP	Polyvinylpyrrolidone
SE	Somatic embryogenesis
WP	Woody plant medium (Lloyd and McCown 1981)

Introduction

The evergreen tree *Gomortega keule* (Mol.) Baillon belongs to the monotypic family Gomortegaceae and is endemic to a small area near the coast of central-south Chile (Muñoz 1986; Rodríguez and Quezada 2001; Hechenleitner et al. 2005), within one of the 25 biodiversity hot spots with urgent priority for conservation on a global scale (Myers et al. 2000). The species is considered endangered (Walter and Gillett 1998), with recommendation for the initiation of ex situ conservation programmes (Le Quesne and Stark 2006). The edible fruits of *G. keule* have been used since the mid-sixteenth century (Torrejón and Cisternas 2003), while its wood is of excellent quality (Rodríguez et al. 1983). The tree also has possible medicinal properties (Albert 1924). Despite its potential, *G. keule* has not been cultivated, possibly because its propagation from seed and cuttings is extremely difficult. Even

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the conditions for establishment from seed in habitat are not understood. Consequently, there is extreme urgency to multiply *G. keule* in order to re-establish plants in habitat and to provide adequate material for eventual introduction of this species as a commercial crop in Chile, or other areas with a Mediterranean-type climate.

In vitro approaches have been explored in attempts to obtain shoot proliferation (Calderón-Baltierra et al. 1993; Jordan et al. 2005). Recently, success has been achieved in regenerating shoots from explants of zygotic embryos via organogenesis (Muñoz-Concha and Davey 2011). Somatic embryogenesis (SE) is also a principal route for micropropagation, with the potential to maximize, in a short time, the number of genetically uniform plants, because organized meristems from which the somatic embryos arise are generally considered highly stable (Rani and Raina 2000). Propagation by SE has been developed for *Aralia elata* (Dai et al. 2011) and *Desmodium motorium* (Devi and Narmathabai 2011), this pathway of plant regeneration also being important in *Olea europaea* because of its recalcitrance to regeneration in vitro (Capelo et al. 2010; Cerezo et al. 2011). In addition, embryogenic cultures are excellent material for cryopreservation of some woody species (Wilhelm 2000; Efendi and Litz 2003; Krishna and Singh 2007; Gonzalez-Arnao et al. 2008), and have been used for genetic transformation of woody plants (Gómez-Lim and Litz 2004; Krishna and Singh 2007; Kumar et al. 2006).

As somatic embryos can be induced in liquid cultures, the latter are attractive to mass propagate material using bioreactors (Yoeup and Chakrabarty 2003). In *Colocasia esculenta*, an efficient protocol was developed by Deo et al. (2010) for plant regeneration from SE cultures in liquid medium. Liquid cultures are also the preferred material with which to study the biosynthesis of secondary metabolites under controlled conditions (Zhou and Wu 2006). This may be applicable in realizing the potential medicinal properties of *G. keule*. Since somaclonal variation may occur during cell culture, it is essential to assess the genetic fidelity of regenerated plants. DNA microsatellite markers have been employed already to study somaclonal variation in trees such as *Olea* spp. (Lopes et al. 2009), *Picea abies* (Harvengt et al. 2001), *Pinus pinaster* (Marum et al. 2009) and *Quercus suber* (Santos et al. 2007).

The present work evaluated the use of different explants for the induction of SE in *G. keule* and the use of liquid media of different formulations, especially in relation to plant growth regulators (PGRs). The genetic fidelity of embryogenic cultures was assessed using DNA microsatellite markers, and their potential confirmed for genotype tracing in tissue culture-derived material.

Materials and methods

Plant material

Plant material (fruits, shoots) was collected from the national protected area 'Reserva Nacional Los Queules' (Corporación Nacional Forestal, 500 m above sea level; genotype B3-2853) and from 'Predio Ralbún' (Forestal Celco S.A., 540 m above sea level; genotypes S3-104, S3-112, S3-172, S4-A3 and S5-230) in the Region of Maule, Chile.

Callus induction

Zygotic embryos were excised from seeds and surface-sterilized by immersion in 4% (v/v) Domestos® bleach solution (Johnson Diversey Ltd., Northampton, UK) for 10 min, followed by thorough washing with autoclaved, reverse osmosis water. The concentration of Domestos® was increased to 10% (v/v) for 60 min for shoots.

Zygotic embryos and shoots were cultured on medium prepared by dissolving Woody Plant Medium (WP; Lloyd and McCown 1981) or Murashige and Skoog (1962) medium (MS) salts (Duchefa) in reverse osmosis water with the addition of 20 g/l sucrose. The pH was adjusted to 5.7 before addition of 8 g/l agar (Sigma-Aldrich A7002) and autoclaving (20 min, 121°C, 1.03 kg/cm^2). Filter-sterilized solutions of α-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) were added when the medium was <60°C. Medium (30 ml aliquots) was poured into autoclaved 175 ml screw-capped Powder-Round glass jars (Beatson Clark and Co. Ltd., Rotherham, UK). Cultures were incubated at 25°C with a 16 h photoperiod ("Daylight" fluorescent tubes; 60 μmol/m^2/s).

Induction of SE

Zygotic embryos were cultured with illumination or in the dark, with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.1–1 mg/l) or NAA (0.01 mg/l) and 0.1 mg/l 6-(γ,γ-dimethylallylamino) purine (2iP) or 1.0 mg/l BA, and with 0–2 g/l activated charcoal (AC).

In order to evaluate the proliferation and development of somatic embryos, material was used from genotypes S3-172 and S3-112, considering a genotype as all the cultured material derived from a single individual (zygotic embryo or tree). Embryogenic friable callus of genotype S3-172 was placed on semi-solid MS medium (8 g/l agar) at 18 and 25°C, with 0–10 g/l of polyvinylpyrrolidone (PVP) molecular weight 360,000 and 0.1 mg/l gibberellic acid (GA_3), 0.01 mg/l NAA and 1.0 mg/l BA. Cultures were evaluated after 40 days. Additionally, compact callus of genotype S3-112 was placed on semi-solid MS medium

with 0.01 mg/l NAA, 1.0 mg/l BA and 0–0.1 mg/l GA_3. The response was evaluated after 3 months of culture.

SE induction was evaluated on 10 mm shoot-tips of genotypes S3-104 and S4-A3 obtained in previous experiments by the authors from zygotic embryos cultured on semi-solid WP medium with 0.1 mg/l NAA and 1.0 mg/l BA. For induction of SE, shoot-tips were incubated on semi-solid MS medium with combinations of 0.1–1.0 mg/l 2,4-D and 0.1–1.0 mg/l 2iP for 6.5 months.

Cultures in liquid media

Callus obtained from 32 zygotic embryos and from buds of 10 wild trees cultured on semi-solid media, was incubated in liquid MS or WP media, with the auxins indole-3-acetic acid, indole-3-butyric acid, NAA and 2,4-D at 0.0–2.0 mg/l, the cytokinins BA and 2iP at 0.1–2.0 mg/l, 0.0–0.1 mg/l GA_3, 20–30 g/l sucrose and 10 g/l PVP. Three replicates (250 ml Erlenmeyer flasks; 80 ml aliquots of medium) were incubated on an orbital shaker at 80 rpm, at 25°C with a 16 h photoperiod and a light intensity of 20 μmol/m^2/s.

The growth of compact callus in liquid media was evaluated by inoculating 2 g of callus (genotypes S3-172 and S3-112) into 80 ml of MS or WP-based medium supplemented with 0–0.1 mg/l auxins (NAA, 2,4-D), 0–5.0 mg/l 2iP, 20–30 g/l sucrose and 0–10 g/l PVP. Six replicates (250 ml flasks) were maintained under the same conditions as for the initiation of cultures, or in the dark, the media being changed every 14 days. The increase in fresh weight (percentage of initial weight) was measured. When cultures consisted of small embryogenic aggregates (genotype S3-112), 50% of the medium was changed every 7 days. Increase in tissue volume of these small aggregates was evaluated using 250 ml Erlenmeyer flasks with graduated side arms (8 replicates). The viability of cells in liquid medium was observed by staining with fluorescein diacetate (FDA) and ultra-violet microscopy (Saunders et al. 1986).

Statistical procedures included ANOVA or *t* tests when homogeneous variances and normal distribution were present. Mann-Whitney test and Chi-square test were also employed using the software Minitab 15.1© 2006 and GenStat 11© 2008.

Genetic study using microsatellites

DNA was extracted from 1.5 year-old cultures (embryogenic callus) of genotypes S3-112 and S3-172. For each genotype, DNA extraction from 10 samples of independent source material followed the method of Lander et al. (2007). Forward primers for 9 microsatellite loci (Lander et al. 2007; Muñoz-Concha 2010), were labelled with a blue, green or black fluorescent dye (WellRED Primers; Sigma). Polymerase chain reactions (20 μl reaction volume) were performed using 2 μl template DNA (10 ng/μl), 2 μl buffer (Standard Taq Reaction Buffer, New England Biolabs), 0.2 μl dNTP (10 mM, Sigma), 0.05 μl acetylated bovine serum albumin (10 μg/μl), 0.02 μl (100 μM) of each forward and reverse primers, 15 μl pure autoclaved H_2O and 0.2 μl *Taq* polymerase (GoTaq® Flexi DNA Polymerase; Promega). The reaction conditions were 94°C for 3 min, 30 cycles of 94°C 1 min, 1 min at the specific annealing temperature of each primer, followed by 72°C 1 min, and a final extension at 72°C for 20 min. The annealing temperatures were 60.9 (Gk-1), 55.4 (Gk-6), 65.0 (Gk-30, Gk-31 and Gk-35), 56.8 (Gk-39) and 52.7°C (CS2 and CS8). Fragment size determination for the amplified products was performed by capillary electrophoresis in a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, USA). The presence of alleles for all samples were compared within each embryogenic genotype.

Results

Induction of callus

Callus developed from the hypocotyls of zygotic embryos when these were cultured on semi-solid MS-based medium. Generally, the growth of callus was not sustained for more than 2–3 months, except callus that developed and which showed constant proliferation from three zygotic embryos (genotypes S3-112, S3-172 and S5-230) and from a bud of a field-collected shoot (genotype B3-2853). Callus of genotype S3-112 developed on semi-solid MS medium with 0.01 mg/l NAA, 1.0 mg/l BA and 0.1 mg/l GA_3. Medium used for genotype S3-172 was also MS with 0.01 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l GA_3, while for genotype S5-230 incubation was in the dark on MS medium with 0.1 mg/l 2iP and 0.01 mg/l NAA. Callus of genotype B3-2853 was initiated on semi-solid WP medium with 0.1 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l GA_3. Although callus induction occurred on both MS and WP media, MS-based medium induced a more frequent response.

Induction of somatic embryos on semi-solid medium

Of ca. 560 embryos cultured, SE on zygotic embryos of genotypes S3-172 and S5-230 occurred at the very limited rate of 0.35% of the embryos with no observable influence of the type or concentration of PGRs and the presence of light and AC. Direct SE was observed on the radicle (genotype S3-172) after 96 days of culture (Fig. 1a). A zygotic embryo (genotype S5-230) produced embryogenic callus, as well as somatic embryos, from its radicle after 7 months of culture.

Proliferation of friable embryogenic callus in genotype S3-112 was observed on semi-solid MS medium with 0.01 mg/l NAA and 1.0 mg/l BA (Fig. 1b); generally embryos had morphological abnormalities. In genotype S3-172, cultures at 18 or 25°C and in the presence (10 g/l) or absence of PVP and PGRs (0.1 mg/l GA_3, 0.01 mg/l NAA and 1.0 mg/l BA) exhibited growth of embryogenic friable callus and chlorophyll production regardless of the temperature or the concentration of PVP. In some cases, callus became necrotic, this necrosis being more frequent at 25°C on medium without PGRs (Chi square test, $P = 0.0417$), and at 18°C on medium lacking PVP (Chi square test, $P = 0.003$). Recurrent proliferation of somatic embryos of genotype S3-172 was observed on non-differentiated tissue, as well as the production of friable white embryogenic callus on the same medium for more than 12 months with subculture every 4–8 weeks.

Compact callus of genotype S3-112 developed somatic embryos after 74 days of culture on semi-solid MS medium with 0.01 mg/l NAA, 1.0 mg/l BA and 10 g/l PVP. GA_3 was not essential to induce SE. However, this response was variable, since in independent experiments, some compact callus of genotype S3-112 failed to develop somatic embryos under the same conditions (with GA_3). However, it did exhibit SE in the absence of GA_3.

Maturation of somatic embryos was observed in genotype S3-172, somatic embryos having a radicle and cotyledons, the latter containing chlorophyll when somatic embryos were >5 mm in length (Fig. 1c). Although the cotyledons were often abnormal in morphology, the development of shoots followed the transfer of somatic embryos to semi-solid WP medium with 0.1 mg/l NAA, 1.0 mg/l BA and 0.5 mg/l GA_3. Rooting of mature embryos of genotype S3-172 occurred after 6 months on WP medium containing 2 g/l AC, but lacking PGRs, with subsequent transfer of somatic embryo-derived plants to the glasshouse. Similarly, somatic embryos of genotype S5-230 rooted after 2 months on WP medium with 2 g/l AC without PGRs.

Somatic embryos developed when shoot-tips of genotype S4-A3 where cultured on semi-solid MS medium with 2,4-D (0.1–1.0 mg/l) and 2iP (0.1–1.0 mg/l) for 196 days (Fig. 1d). Only compact yellow callus was observed on shoot-tips of genotype S3-104 cultured under the same conditions. No significant differences were found (Chi-square tests) in the initiation of SE in response to 2,4-D and 2iP over the concentration ranges tested of these growth regulators.

Initiation of liquid cultures and embryogenic cell suspensions

Experiments to establish liquid cultures involved material derived from 32 zygotic embryos and buds from shoots of 10 individual trees in habitat. Different concentrations of 2,4-D, AC and PVP had no influence on the initiation of cultures in liquid media. Tissues from only 3 genotypes

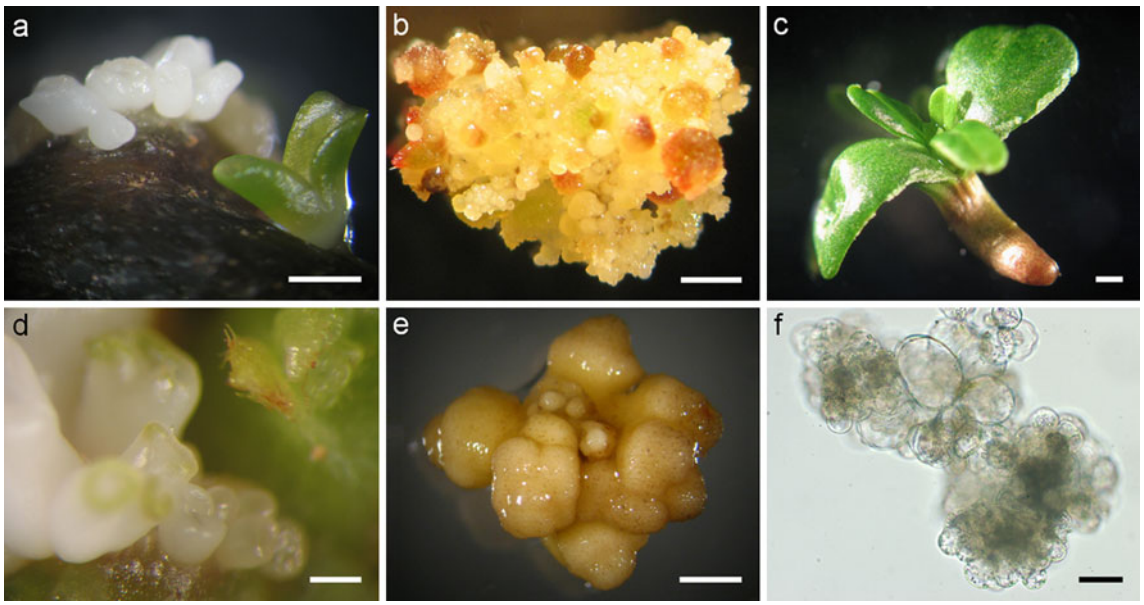

Fig. 1 Callus cultures in liquid media and somatic embryos of *Gomortega keule*. **a** Direct somatic embryogenesis on a zygotic embryo cultured on semi-solid MS medium with 0.01 mg/l NAA and 1.0 mg/l BA, of genotype S3-172. **b** Embryogenic friable callus of genotype S3-112 cultured as in **a**. **c** Conversion of a somatic embryo to a shoot of genotype S3-172. **d** Somatic embryos developed on shoots of genotype S4-A3 cultured on MS medium with 0.1 mg/l 2,4-D and 0.1 mg/l 2iP for 196 d. **e** Compact callus of genotype S3-112 cultured in MS liquid medium with 0.01 mg/l NAA and 0.1 mg/l 2iP. **f** A small aggregate of living cells of genotype S3-112 cultured as in **e**, and observed with bright field illumination. *Bars* 1 mm (**a**, **b**, **c**, **e**), 0.3 mm (**d**) and 20 μm (**f**)

(S3-112, S3-172 and B3-2853) proliferated in culture. Zygotic embryos died when extracted from seeds and incubated immediately in liquid medium.

Callus from genotype S3-112 proliferated for more than 19 months in liquid MS medium, growing as both compact, nodular aggregates (Fig. 1e) and smaller clusters of cells (Fig. 1f). Sucrose at 20 g/l in MS medium induced more growth than sucrose at 30 g/l (t test, $P = 0.024$). Tissue growth was similar in both liquid WP and MS-based media with or without PVP when assessed after 42 days of culture. Growth was influenced by NAA concentration after 14 days of culture with two concentrations of 2iP (0.1, 1.0 mg/l) and NAA (0.01, 0.1 mg/l; two-way ANOVA, $P = 0.008$ for NAA). In other experiments, involving combinations of NAA (0, 0.01 mg/l) and 2iP (0, 0.1 mg/l) ($n = 5$ or 6), there was a significant effect of 2iP ($P < 0.001$) after 44 days of culture (two-way ANOVA). In agreement with this, in independent experiments, concentrations of 2iP from 0.1 to 5.0 mg/l influenced the growth of liquid cultures (ANOVA, $P < 0.001$) with the greatest growth at the minimum concentration (Fig. 2a). Tissue growth was affected by 2,4-D and 2iP concentration (ANOVA, $P < 0.001$); 0.1 mg/l 2,4-D and 0.1 mg/l 2iP reduced growth. Liquid MS medium with 0.01 mg/l NAA and 0.1 mg/l 2iP was optimum, allowing sustained proliferation of small aggregates.

Proliferation of compact tissue in liquid medium was observed when somatic embryos of genotype S3-172, induced on semi-solid medium, were transferred to liquid medium. The calli showed constant proliferation in liquid medium over more than 12 months, and a very similar morphology to those of genotype S3-112, but without the production of small aggregates. Small aggregates originated from compact callus of genotype S3-112, also proliferated during more than 18 months in MS liquid medium with 0.01 mg/l NAA and 0.1 mg/l 2iP; tissue volume in the culture increased at a rate of 0.1 ml/day (Fig. 2b).

Small aggregates were also obtained from callus that originated from a bud of a field-collected shoot of the genotype B3-2853. Tissue proliferation became stable after 18 months in MS liquid medium with 0.01 mg/l NAA and 0.1 mg/l 2iP, the cells being highly vacuolated, irregular and elongate in shape.

Genetic study of embryogenic cultures

Ten samples, each one from independent cultures, assessed for genotypes S3-112 and S3-172, showed the presence of the same microsatellite alleles. This full matching of the genetic profile amongst the samples of each genotype confirmed the genetic identity for all the samples within each group of in vitro material (Table 1; Fig. 3).

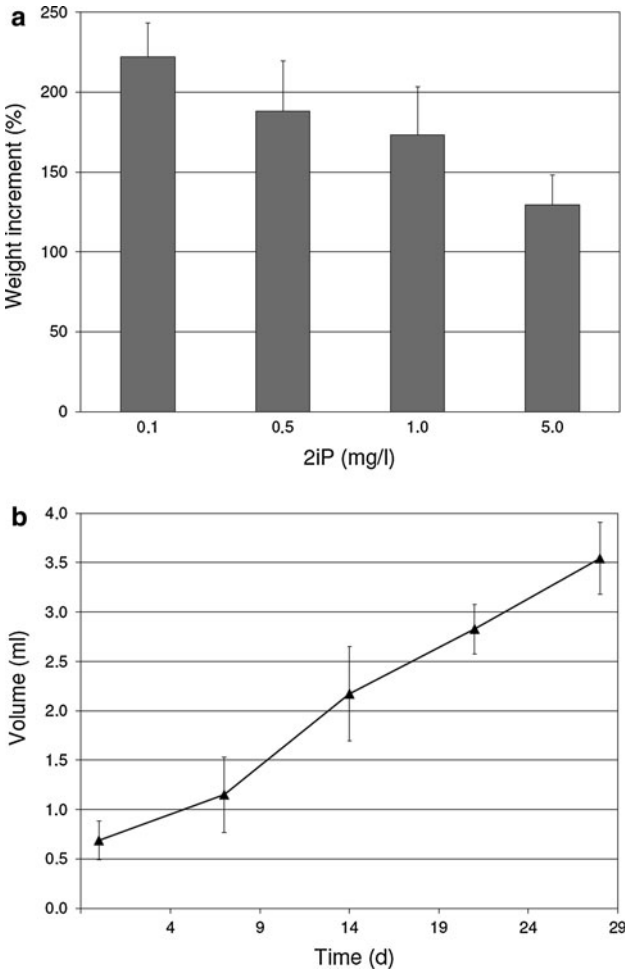

Fig. 2 Growth of callus of *Gomortega keule* genotype S3-112 in liquid medium. **a** Fresh weight increment (%) of compact callus at different concentrations of 2iP. **b** Volume increment (ml) of small aggregates over time

Table 1 Alleles (fragment size in bp) detected by microsatellites in two embryogenic genotypes of *Gomortega keule*

Microsatellite locus	Genotype S3-172	Genotype S3-112
Gk-6-1	98	98, 108
Gk-6-2	114	104, 120
Gk-30	182, 190	188
Gk-39	133, 201	133
Gk-35	226	224, 228
Gk-31	226	226, 228
Gk-1	224	226, 240
CS2	104	104
CS8	218	214

Discussion

Callus development was influenced more by the plant genotype rather than the culture conditions. In agreement

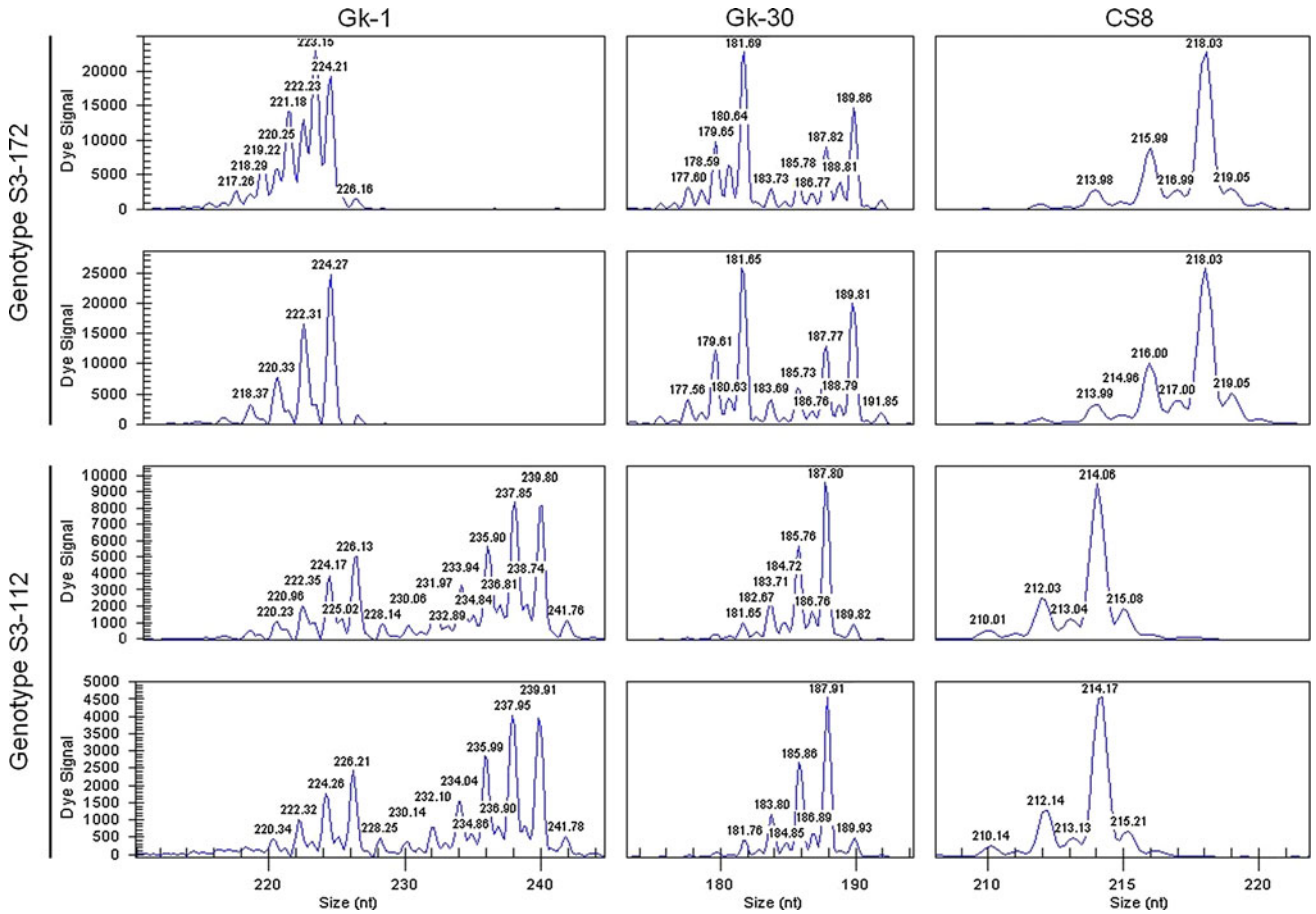

Fig. 3 Representative capillary electrophoresis profiles in 4 samples of two embryogenic genotypes of *Gomortega keule* at 3 microsatellite loci

with previous work, MS-medium was preferable for callus development (Muñoz-Concha 2010).

Spontaneous SE observed directly on zygotic embryos of *G. keule* cultured on semi-solid medium occurred at low frequency and gave the best embryogenic material in terms of proliferation. SE has also been reported in cultured mature zygotic embryos of the endangered tree *Ocotea catharinensis* (Moura-Costa et al. 1993), of the medicinal tree *Murraya koenigii* (Paul et al. 2011) and of the vine *Passiflora edulis* (Pinto et al. 2011). Although somatic embryos of *G. keule* had morphological abnormalities, such abnormal development is not uncommon in woody species (Yoeup and Chakrabarty 2003). The optimum conditions for somatic embryo development were on semi-solid MS-based medium.

SE was observed after prolonged exposure (6.5 months) of micropropagated shoot-tips to 2,4-D. An induction period of exposure to 2,4-D for 7 days did not induce SE. Thus, the induction of SE on shoot-tips of *G. keule*, may require the permanent presence of both 2,4-D and 2iP in the culture medium. Further research is needed to improve induction conditions, especially using shoot-tips of selected genotypes.

SE in the present experiments was restricted to five genotypes of *G. keule*, using zygotic embryos and shoot-tips as source material. SE is known to be highly dependent on genotype and species (Kumar et al. 2006; Pollastri 2008; Samson et al. 2006; Singh et al. 2003), which was the case for *G. keule*. Nevertheless, SE in *G. keule* represents a base for further work on mass propagation and longer term maintenance by cryopreservation. More reproducible induction of SE from shoot-tips and importantly, on material derived from adult elite trees, along with the maturation and conversion of somatic embryos to plants, are future priority research targets.

Cells cultured in suspension were generally highly vacuolated, and elongated or irregularly shaped, which are typical characteristics of non-embryogenic cells (Jayasankar et al. 1999). The fact that only 3 of the 42 genotypes assessed proliferated consistently, supports the notion that only a restricted number of genotypes of *G. keule* can be established in liquid medium.

Compact callus masses (>5 mm) cultured in liquid medium exhibited browning and limited production of light coloured actively growing new tissues. Such tissues needed to be dissected into smaller pieces at sub-culture to re-activate the cultures. A similar situation was reported in cultures of the tree *Eucommia ulmoides* by Miyanaga et al. (2004). Proliferation of embryogenic tissues was optimum in liquid medium, with the fragmentation by cutting of compact callus and subsequent transfer to new medium of the smaller aggregates being essential. This procedure, although time-consuming, stimulated the production of totipotent tissues.

Callus derived from a bud of a field-collected shoot proliferated in liquid medium, and produced small cell aggregates after 18 months of culture. However, cells showed typical non-embryogenic characteristics when examined microscopically. Although the aggregates grew on semi-solid medium, organogenesis was not observed. This example in the present work represented the only sustained proliferation of tissues from field-collected shoots. Consequently, future experiments to induce SE in *G. keule*, should focus on the use of zygotic embryos as the starting material.

Embryogenic cultures of *G. keule* in liquid medium may facilitate the development of methods for mass propagation. This approach may enable plants to be regenerated from field-collected material for establishment in the field for conservation and commercial purposes. Liquid cultures of embryogenic tissues have been developed for the endangered medicinal woody plant *Eleutherococcus senticosus* (Choi et al. 2002) and this route may also constitute the best approach for *G. keule*. Additionally, cultures in liquid medium may be relevant for secondary metabolite production in *G. keule*, as it has been explored for the medicinal tree *Eucommia ulmoides* (Miyanaga et al. 2004), and given the possible medicinal uses of *G. keule* (Albert 1924).

Genetic differences were not detected with microsatellites among the samples within each of the two clones studied, suggesting that in *G. keule* there is no genetic variation or mutation detectable by microsatellites following culture involving SE. These markers are suitable for tracing clonal lines and could prove important in longer-term quality control of micropropagated material of *G. keule*. However, low levels of somaclonal variation are unlikely to be detected by microsatellites. Similar findings have been reported using microsatellites to assess genetic variation in plant material derived from embryogenic cultures, where no genetic variation was found in trees including *Olea* spp. (Lopes et al. 2009), *Picea abies* (Harvengt et al. 2001) or *Quercus suber* (Santos et al. 2007). In contrast, using microsatellites in plants regenerated from SE, Marum et al. (2009) observed around 10% genetic variation in *Pinus pinaster*, and Prado et al. (2010) obtained 10 mutant plants (32%) in one of the 6 cultivars of *Vitis vinifera* that they studied.

Conclusions

Overall, these results demonstrate that the response of *G. keule* in culture is extremely dependent upon source material, necessitating extensive screening of germplasm in order to recognise responsive genotypes. Nevertheless, micropropagation of *G. keule* is now possible through SE and with the use of liquid cultures, providing an additional pathway to organogenesis. Although at present SE is more complex, labour-intensive and results in less regenerated plants than organogenesis, SE represents an important potential pathway for the multiplication of adult trees of this endangered species, since the regeneration of plants by organogenesis has been achieved only from zygotic embryos as source material.

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References

Albert F (1924) Materias primas vegetales y animales. Sociedad Imprenta y Litografía Universo, Santiago

Calderon-Baltierra X, Perez F, Rotella A (1993) In vitro propagation of a Chilean endangered plant species: *Gomortega keule* (Mol.) Baillon (Magnoliopsidae, Gomortegaceae). Bosque 14:23–28

Capelo AM, Silva S, Brito G, Santos C (2010) Somatic embryogenesis induction in leaves and petioles of a mature wild olive. Plant Cell Tiss Org Cult 103:237–242

Cerezo S, Mercado JA, Pliego-Alfaro F (2011) An efficient regeneration system via somatic embryogenesis in olive. Plant Cell Tiss Org Cult 106:337–344

Choi YE, Lee KS, Kim EY, Kim YS, Han JY, Kim HS, Jeong JH, Ko SK (2002) Mass production of Siberian ginseng plantlets through large-scale tank culture of somatic embryos. Plant Cell Rep 21:24–28

Dai JL, Tan X, Zhan YG, Zhang YQ, Xiao S, Gao Y, Xu DW, Wang T, Wang XC, You XL (2011) Rapid and repetitive plant regeneration of *Aralia elata* Seem. via somatic embryogenesis. Plant Cell Tiss Org Cult. doi:10.1007/s11240-010-9801-x

Deo PC, Taylor M, Harding RM, Tyagi AP, Becker DK (2010) Initiation of embryogenic cell suspensions of taro (*Colocasia esculenta* var. *esculenta*) and plant regeneration. Plant Cell Tiss Org Cult 100:283–291

Devi BC, Narmathabai V (2011) Somatic embryogenesis in the medicinal legume *Desmodium motorium* (Houtt.) Merr. Plant Cell Tiss Org Cult 106:409–418

Efendi D, Litz RE (2003) Cryopreservation of avocado. Proc V World Avocado Congr 1:111–114

Gómez-Lim MA, Litz RE (2004) Genetic transformation of perennial tropical fruits. In Vitro Cell Dev Biol-Plant 40:442–449

Gonzalez-Arnao MT, Panta A, Roca WM, Escobar RH, Engelmann F (2008) Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell Tiss Org Cult 92:1–13

Harvengt L, Trontin JF, Reymond I, Canlet F, Paques M (2001) Molecular evidence of true-to-type propagation of a 3-year-old Norway spruce through somatic embryogenesis. Planta 213:828–832

Hechenleitner P, Gardner MF, Thomas PI, Echeverría C, Escobar B, Brownless P, Martínez C (2005) Plantas amenazadas del centro-sur de Chile. Distribución, conservación y propagación, 1st edn. Universidad Austral de Chile and Royal Botanic Garden of Edinburgh

Jayasankar S, Gray DJ, Litz RE (1999) High-efficiency somatic embryogenesis and plant regeneration from suspension cultures of grapevine. Plant Cell Rep 18:533–537

Jordan M, Gonzalez J, Roveraro C (2005) In vitro regeneration of *Gomortega keule* (Gomortegaceae), a Chilean endemic tree in danger of extinction. Eur J Hort Sci 70:202–206

Krishna H, Singh SK (2007) Biotechnological advances in mango (*Mangifera indica* L.) and their future implication in crop improvement—a review. Biotechnol Adv 25:223–243

Kumar V, Naidu MM, Ravishankar GA (2006) Developments in coffee biotechnology—in vitro plant propagation and crop improvement. Plant Cell Tiss Org Cult 87:49–65

Lander TA, Boshier DH, Harris SA (2007) Isolation and characterization of eight polymorphic microsatellite loci for the endangered, endemic Chilean tree Gomortega keule (Gomortegaceae). Mol Ecol Notes 7:1332–1334

Le Quesne C, Stark D (2006) *Gomortega keule* (Mol.) Baillon. In: Donoso C (ed) Las especies arbóreas de los bosques templados de Chile y Argentina. Autoecología. Marisa Cuneo Ediciones, Valdivia, pp 277–284

Lloyd G, McCown B (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by the use of shoot-tip culture. Comb Proc Intl Plant Prop Soc 30:421–427

Lopes T, Capelo A, Brito G, Loureiro J, Santos C (2009) Genetic variability analyses of the somatic embryogenesis induction process in *Olea* spp. using nuclear microsatellites. Trees 23: 29–36

Marum L, Rocheta M, Maroco J, Oliveira MM, Miguel C (2009) Analysis of genetic stability at SSR loci during somatic embryogenesis in maritime pine (*Pinus pinaster*). Plant Cell Rep 28:673–682

Miyanaga K, Aly UI, Tanji Y, Unno H (2004) Aggregate characteristics of callus derived from woody plant *Eucommia ulmoides*. Biochem Eng J 21:149–153

Moura-Costa PH, Viana AM, Mantell SH (1993) In vitro plantlet regeneration of *Ocotea catharinensis*, an endangered Brazilian hardwood forest tree. Plant Cell Tiss Org Cult 35:279–286

Muñoz R (1986) El queule, una especie en peligro de extinción. Chile Forestal 134:16–18

Muñoz-Concha D (2010) *Gomortega keule*: micropropagation and germplasm characterization. Ph.D. thesis, University of Nottingham, UK, 212 pp

Muñoz-Concha D, Davey MR (2011) Micropropagation of the endangered Chilean tree, *Gomortega keule*. In Vitro Cell Dev Biol -Plant 47:170–175

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858

Paul S, Dam A, Bhattacharyya A, Bandyopadhyay TK (2011) An efficient regeneration system via direct and indirect somatic embryogenesis for the medicinal tree *Murraya koenigii*. Plant Cell Tiss Org Cult 105:271–283

Pinto DLP, de Almeida AMR, Rêgo MM, da Silva ML, de Oliveira EJ, Otoni WC (2011) Somatic embryogenesis from mature zygotic embryos of commercial passionfruit (*Passiflora edulis* Sims) genotypes. Plant Cell Tiss Org Cult. doi:10.1007/s11240-011-0003-y

Pollastri S (2008) Biotechnological advances in olive (*Olea europaea* L.): a review. Adv Hortic Sci 22:123–128

Prado MJ, Rodriguez E, Rey L, González MV, Santos C, Rey M (2010) Detection of somaclonal variants in somatic embryogenesis-regenerated plants of *Vitis vinifera* by flow cytometry and microsatellite markers. Plant Cell Tiss Org Cult 103:49–59

Rani V, Raina SN (2000) Genetic fidelity of organized meristems-derived mircropropagated plants: a critical reappraisal. In Vitro Cell Dev Biol-Plant 36:319–330

Rodríguez R, Quezada M (2001) Gomortegaceae. In: Marticorena C, Rodríguez R (eds) Flora de Chile, vol 2. Editorial Universidad de Concepción, Concepción, pp 16–17

Rodríguez R, Matthei O, Quezada M (1983) Flora arbórea de Chile. Editorial Universidad de Concepción, Concepción

Samson NP, Campa C, Le Gal L, Noirot M, Thomas G, Lokeswari TS, Kochko A (2006) Effect of primary culture medium composition on high frequency somatic embryogenesis in different *Coffea* species. Plant Cell Tiss Org Cult 86:37–45

Santos C, Loureiro J, Lopes T, Pinto G (2007) Genetic fidelity analysis of in vitro propagated cork oak (*Quercus suber* L.). In: Jain SM, Häggman H (eds) Protocols for micropropagation of woody trees and fruits. Springer, Dordrecht, pp 67–83

Saunders JA, Roskos LA, Mischke S, Aly MAM, Owens LS (1986) Behavior and viability of tobacco protoplasts in response to electrofusion parameters. Plant Physiol 80:117–121

Singh ND, Sahoo L, Sarin NB, Jaiwal PK (2003) The effect of TDZ on organogenesis and somatic embryogenesis in pigeonpea (*Cajanus cajan* L. Millsp). Plant Sci 164:341–347

Torrejón F, Cisternas M (2003) Early environmental impact in the Araucania territory inferred from Spanish chronicles and historiographical studies. Bosque 24:45–55

Walter KS, Gillett HJ (1998) 1997 IUCN red list of threatened plants. IUCN—The World Conservation Union, Cambridge

Wilhelm E (2000) Somatic embryogenesis in oak (*Quercus* spp.). In Vitro Cell Dev Biol-Plant 36:349–357

Yoeup PK, Chakrabarty D (2003) Micropropagation of woody plants using bioreactor. In: Jain SM, Ishii K (eds) Micropropagation of woody trees and fruits. Forestry Sciences 75. Kluwer, London, pp 735–755

Zhou LG, Wu JY (2006) Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinals in China. Nat Prod Rep 23:789–810