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In Vitro Regeneration of *Gomortega keule* (*Gomortegaceae*), a Chilean Endemic Tree in Danger of Extinction

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Summary

Axillary buds of nodal sections from 2-year old trees of *Gomortega keule* (Mol.) Baillon, 'Queule', 'Keule', (Family: *Gomortegaceae*) were induced to sprout and developed many shoots in presence of 0.54 µM naphthaleneacetic acid (NAA) and 4.44 µM benzylaminopurine (BA) in a liquid WP-medium (LLOYD and MCCOWN 1980) within a period of 2–3 weeks. After transfer to a medium containing 22.2 µM BA, each new single node section of about 0.5 cm initiated numerous adventitious shoots; alternatively, roots were formed when these explants were transferred to a medium containing 2.95, 12.3 or 24.6 µM indolebutyric acid (IBA). Root formation took place in presence of 24.6 µM IBA in approx. 46 % of the sub-cultured nodal segments after a period of 3 months, leading to plantlets. Regeneration response was restricted to the sprouts of axillary buds; other explant-types tested, i.e. petioles, leaf, and internodal sections showed callus formation only and no other responses.

Zusammenfassung

***In vitro* Regeneration von *Gomortega keule* (*Gomortegaceae*), einer in Chile heimischen, vom Aussterben bedrohten Baumart.** In Achselknospen von Nodienexplantaten 2 Jahre alter Bäumen von *Gomortega keule* (Mol.) Baillon, 'Queule', 'Keule', (Familie: *Gomortegaceae*) wurde die Sprossbildung induziert. In einem flüssigen WP-Medium (LLOYD and MCCOWN 1980) mit 54 µM Naphthylessigsäure (NAA) und 4.44 µM Benzylaminopurin (BA) wurden innerhalb von 2–3 Wochen zahlreiche Sprosse gebildet. Nach dem Umsetzen auf ein Medium mit 22.2 µM BA entwickelte jedes neue 0.5 cm große Nodienexplantat zahlreiche Sprosse. Wurzeln wurden gebildet, wenn es auf einem Medium mit 2.95, 12.3 oder 24.6 µM Indolbuttersäure (IBA) kultiviert wurde. Annähernd 46 % der Nodienexplantate aus der Unterkultur bildeten nach 3 Monaten auf Medium mit 24.6 µM IBA Wurzeln und entwickelten sich zu Pflänzchen. Eine Regeneration fand nur bei Sprossen aus Achselknospen statt, die anderen untersuchten Explantate wie Blattstiele, Blätter und Internodien reagierten nur mit Kallusbildung.

Key words. *Gomortega keule* – endangered species – organogenesis – *in vitro* regeneration

Introduction

Gomortega keule (Mol.) Baillon, 'Keule', 'Queule', a monotypic species of the Family *Gomortegaceae* (RODRIGUEZ et al. 1983), related to the Laurales (VILEGAS et al. 1991; RENNER 1998, 1999; HEO et al. 2004) is a Chilean endemic valuable tree that is registered as one of the eleven most endangered species on the "Red List of Chilean Terrestrial Flora" (BENOIT 1989). *G. keule* is only represented in some specific locations between 50–300 m.a.s.l in South-Central Chile (S.L. 35–37 ° approx.) with a few discontinuous and dispersed stands remaining (SAN MARTIN and SANCHEZ 2000). Today, it is restricted to a couple of hectares with not more than 1000 adult individuals (ECHEVERRÍA and MOREY 2004; HERRERA et al. 2004). This evergreen tree has been appreciated by its qualities: durable wood with decorative veins, large trunk, aromatic leaves, and edible fruit, as well as its use in traditional medicine as hallucinogen, and fuel (MUÑOZ 1965; HOFFMANN 1982; RODRIGUEZ et al. 1983). It has recently been reported that the essential oils of this species have insecticide properties, inhibiting larval development (TORRES et al. 2003).

In spite of the natural regeneration of this species by shoot regrowth in cut trees, long-term, continuous and uncontrolled multipurpose exploitation of natural stands has seriously affected the remaining populations (RODRIGUEZ et al. 1983; GREZ et al. 1997, ARMESTO et al. 2001). The natural regeneration pattern of *G. keule* is poorly understood; pollination and seed dispersal biology are unknown (RENNER 1998); problems in natural regeneration result in a widespread lack of seedlings in established populations. One reason might be that sexual regeneration requires considerable canopy opening. The species appears to be shade-intolerant this develops into strongly discontinuous regeneration patterns (VILLEGAS et al. 2003). Moreover, while germination under greenhouse conditions reaches approx. 25 %; the seeds remaining at least one winter on the forest floor, undergoing natural scarification, require at least 2 years to complete germination (DONOSO and ESCOBAR 1985), resulting in additional seedling losses (ESPEJO et al. 2000). According to

DOHLEN (1999), i*n vivo* speed of germination was slow and the seedlings produced were not strong. Asexual propagation has been attempted, but only a few successes are reported. So far, root formation in cuttings has been very low (3.3 %) (PEÑA 1995). The use of mature zygotic embryos was successful to obtain plantlets under *in vitro* conditions (CALDERON-BALTIERRA et al. 1993). In this report, several explant types were explored as to their capacity to perform morphogenesis.

Material and Methods

List of abbreviations

BA: 6-benzylaminopurine; IBA: indole-3-butyric acid;, GA_3: gibberellic acid; NAA: α-naphthaleneacetic acid; TDZ: 1-phenyl-3-(1,2,3,-thidiazol-5-yl)urea (thidiazuron); WP: LLOYD and MCCOWN-medium (1980).

Table 1. Regenerative responses of various explant-types of *G. keule* after a culture period of 2 months *in vitro*[1].

Explant	Growth regulators (μM)		% Ax. bud sprouting	Aver. shoots explant	% Root primordia	% Callus formation	Remarks
Nodal sections	NAA	0.00	0.0 c	0.0	0.0 b	0.0 c	Explants yellow
	BA	0.00					
	NAA	0.05	12.5 b	1.7	0.0 b	0.0 c	Explants green [2a]
	BA	0.44					
	NAA	0.05	0.0 c	0.0	0.0 b	0.0 c	Explants yellow
	BA	2.22					
	GA_3	0.12					
	NAA	0.54	100.0 a	4.5	100.0 a	16.7 b	Explants green[2b]
	BA	4.44					
	NAA	1.61	0.0 c	0.0	0.0 b	0.0 c	Explants yellow
	BA	0.44					
	GA_3	0.03					
	NAA	2.69	0.0 c	0.0	0.0 b	42.9 a	Explants
	BA	0.44					yellow/brown
			% Shoot formation	% Root formation	% Callus formation	Diam. callus (mm)	Remarks
Internodal sections	NAA	0.54	0.0	0.0	40.1 a	5.0	Explants green
	BA	4.44					
	NAA	0.54	0.0	0.0	16.7 b	5.0	Green
	BA	2.2					
	NAA	5.37	0.0	0.0	12.5 b	2.0	Green
	BA	0.44					
	NAA	26.90	0.0	0.0	65.6 a	2.0	Green
	BA	0.44					
Petioles with leaf section	NAA	0.54	40.0 a	0.0	40.1 a	2.0	Explants green
	BA	4.44					
	NAA	26.90	0.0 b	0.0	0.0 c	0.0	Green
	BA	0.44					
	IBA	0.005	0.0 b	0.0	25.0 b	2.0	Brown
	TDZ	0.45					
	IBA	0.01	0.0 b	0.0	40.1 a	2.0	Brown
	TDZ	2.27					
	TDZ	0.45	0.0 b	0.0	0.0 c	0.0	Brown
	TDZ	2.27	0.0 b	0.0	12.5 c	5.0	Green

[1]Results from aver. initial 25 explants/treatment. Leaf explants did not result in any response and data are not shown in the table.
[2]Treatment resulting in the formation of numerous new shoots after subculture (Table 2). Differences by comparison of proportions (Stats™).
[2a]Mult. shoots in subcult. [2b]Mult. shoots or roots in subcult.

Plant material and cultivation conditions

Nodal sections, each with two axillary buds, petioles with $^1/_4$ leaf blade attached, leaf sections of approx. 0.75 cm^2 and 1.0 cm long stem internodes from 1–2 year-old juvenile plants, were assayed as primary explants. These plants, all of approx. 50 cm in height, were obtained from a nursery close to Santiago (Vivero y Jardín Pumahuida Ltda.) then maintained for two weeks under greenhouse conditions for acclimatization. All explants were established in WP media containing various growth regulator combinations. Explants were washed, surface disinfected with Captan 80 % WP (Micro Flo Co, USA) and Benlate® (du Pont & Co. Inc., France) (each 0.02 %) for 30 min under constant agitation and disinfected with 5 % commercial Na hypochlorite for 5 min. The explants were cultured in Pyrex tubes (25 x 130 mm) containing approx. 13.0 ml of nutrient solution on Whatman Nº 1 paper-bridges. Media were supplemented with 2 % sucrose and 1.0 % PVP-360, adjusted to pH 5.7 prior to autoclaving at 121 ºC during 20 min. Gelled media were used for rooting or induction of multiple shoots (0.6 % Agar, Midesa ®). Growth conditions were a 16-h light regime (57 μmol m^{-2} s^{-1}) using Philips fluorescent lamps TLT40W/54RS-Day light at 24±1 °C.

Results and Discussion

Axillary-bud development was observed in nodal sections within 3 weeks in culture. Several shoots arose from each of the two preformed axillary buds in presence of 4.44 μM BA and 0.54 μM NAA within 2–3 weeks (Fig. 1). Sprouting was observed in 100 % of the explants, other growth regulator combinations were less effective (Table 1). Shoot growth and shoot branching continued after transfer of these explants (same growth regulator combinations); some shoots reached approx. 3–5 cm within 2 months. Callus formation and root primordia also appeared at the base of these explants (Fig. 2). The callus turned brown.

Multiple shoot formation, or alternatively roots, were formed by sub-culturing 0.5 cm nodal segments (with internodal portions) originating from the primary cultures. In presence of 22.2 μM BA, multiple shoots (over 40 new green shoots/tube of approx. 2–4 mm) as compact clusters could be observed after 90 days (Fig. 3). Alternatively, when the former explants were sub-cultured in presence of IBA, root formation took place (Fig. 4). In presence of 24.6 μM IBA approx. 46 % of the explants formed 3–5 roots after 3 months, leading to plantlets. Rooting was also induced by 2.95 and 12.3 μM IBA but at a lower percentage (Table 2).



Fig. 1. Axillary bud-sprouting, shoot development and shoot branching from a nodal explant cultivated in presence of 0.54 μM NAA and 4.44 μM BA after approx. 2–3 weeks.

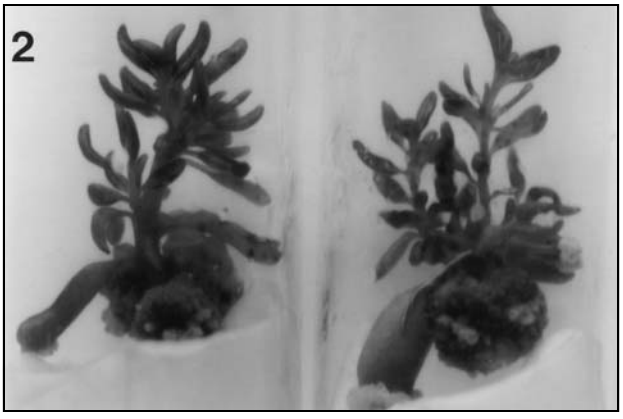


Fig. 2. Development of shoots, callus, and a root primordium in subculture, same growth regulator combinations, after 2–3 months.

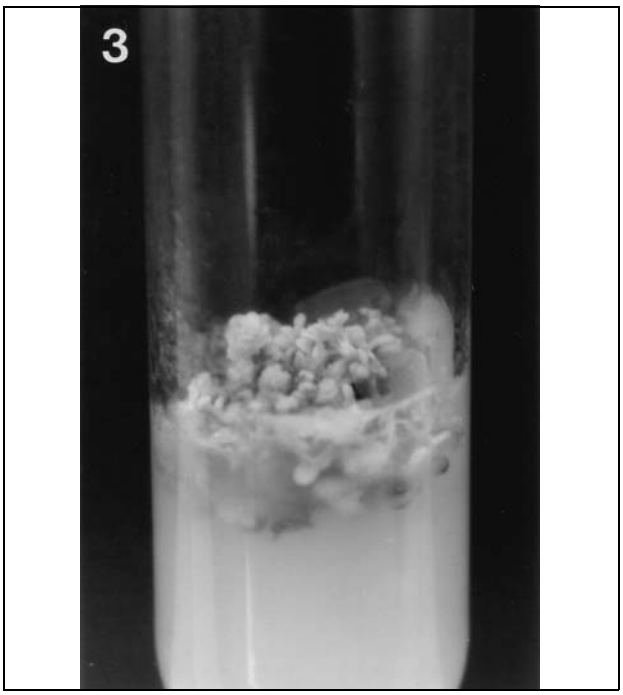


Fig. 3. Close-up showing shoot clusters with approx. 50–60 new shoots of approx 1–2 mm formed along the shoot axis of a sprout, in presence of 22.2 μM BA after approx. 3 months.

Table 2. Effect of growth regulators on nodal segments from axillary-bud sprouts in subculture[1,2].

Growth regulator	(μM)	New shoots (Nr)[3]	% Rooted explants[3]	Roots/ explant	% Callus formation[3]	Remarks
BA	0.44	0 b	0.0 b	0.0	0.0 c	no responses
	22.20	>40 a[4]	0.0 b	0.0	0.0 c	bright green shoots
IBA	0.00	0 b	0.0 b	0.0	0.0 c	explants green
	2.95	0 b	8.3 b	3.5	100.0 a	shoot elongation, green
	12.30	0 b	4.4 b	2.0	65.2 b	explants green
	24.60	0 b	45.8 a	4.3	54.2 b	explants green
	34.30	0 b	0.0 b	0.0	0.0 c	most explants brown

[1]Nodal sections excised from shoots first developed in presence of 0.54 μM NAA and 4.44 μM BA (Table 1).
[2]Results from 25 initial nodal explants/treatment in jelled medium after approx. 3 months.
[3]New shoots, rooting and callus formation differences (%) by comparison of proportions (Stats™).
[4]Shoot number estimated due to small size and packing of shoot clusters (Fig. 3).

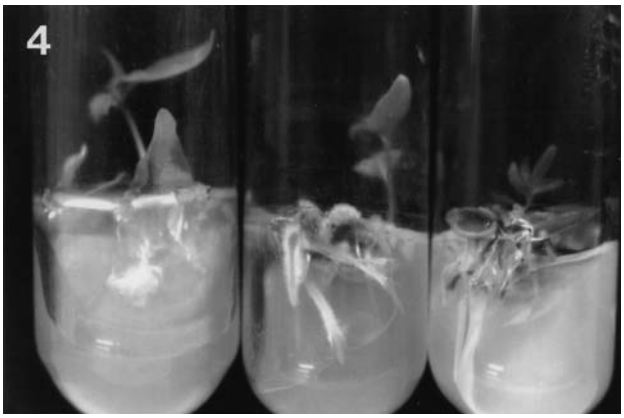


Fig. 4. Root formation of nodal segments in presence of 24.6 μM IBA within 3 months.

In terms of plantlet regeneration, among the different explant types assayed, only the axillary bud sprouts were successful. No response was observed in leaf explants and/or only callus formation was achieved (Table 1). Also no morphogenic response could be induced after subculture of callus (data not shown). Limited regeneration responses have been reported earlier for this species (CALDERÓN-BALTIERRA et al. 1993), also affecting rooting in cuttings (PEÑA 1995). Browning was common in many explants or their calli, attributed to phenolic oxidation (Table 1; Fig. 2), and is also linked to the scarce rooting responses found in cuttings (PEÑA 1995). In addition, several other compounds detected in this species such as coumarins, alkaloids (URZUA et al. 1982; VILEGAS et al. 1991), and probably the presence of i.e. lignans, neo-lignans, allyl- and propenylphenols, common to the Laurelean alliance (VILEGAS et al. 1989, 1991), may affect growth and regeneration responses.

Despite the restrictions found in the various explant types, axillary bud sprouts proved to be suitable for regeneration and as a satisfactory source of juvenile explants for further work. It is assumed that their use can represent an alternative for the regeneration of selected plant material of this species.

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