Europ.J.Hort.Sci., **69** (2). S. 73–78, 2004, ISSN 1611-4426. © Verlag Eugen Ulmer GmbH & Co. Stuttgart

In vitro Culture of *Quillaja saponaria* Mol. (Soap-bark Tree), Rosaceae

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Summary

Nodal sections of *Quillaja saponaria,* 'Quillay', including the proximal part of leaf laminae from 2-year old trees, as well as polynodal sections (3–4 axillary buds), were cultivated in MS-medium (MURASHIGE and SKOOG (1962) in the presence of various cytokinins, especially high levels of benzyladenine (BA) or thidiazuron (TDZ). This initiated profuse branching of the axillary bud and the induction of many new shoots and also callus formation in both explant types. From a single polynodal explant, more than 200 new multiple shoots developed in a period of 3–4 months. Proliferation of new shoots occurred mostly under the preexisting axillary bud(s) of the section and at the proximal end of the petiole with its intact leaf attached. Shoot clusters or isolated shoots elongated in subculture in the presence of MS-medium without growth regulators or formed roots when supplemented with indolebutyric acid (IBA) within a period of 30–60 days, leading to plantlets. Root formation occurred in ½ strength MS-medium with added 1 % sucrose and 0.6, 1.2 and 5.0 mg l^{-1} IBA, as single hormone. Roots did not form in lower IBA levels (0.1 mg l^{-1}) while the addition of phloroglucinol or thiamine did not enhance rhizogenesis besides the IBA. Despite the strong shoot initiation occurring in both nodal and polynodal explants, morphogenesis was not observed in leaf explant sections, and callus derived from nodal sections, formed green globular structures only without further differentiation.

Zusammenfassung

***In vitro*-Kultur von *Quillaja saponaria* Mol. (Seifenrindenbaum), Rosaceae.** Nodale Segmente einschließlich der proximalen Blattenden von zweijährigen *Quillaja saponaria* Pflanzen als auch polynodal-Segmente mit 3–4 axillären Sprossen wurden in MURASHIGE und SKOOG (1962) -Medium (MS) mit einer hohen Konzentration von Benzyladenin (BA) und Thidiazuron (TDZ) kultiviert. Beide Explantate zeigten eine Verzweigung der vorhandenen axillären Sprosse und Bildung von neuen Sprossen sowie Kallus. Aus einem einzigen polynodalen Explantat bildeten sich innerhalb von 3–4 Monaten mehr als 200 neue Sprosse. Die Bildung von neuen Sprossen zeigte sich unter den axillären Sprossen und am proximalen Ende des Blattstiels. In Subkultur verlängerten sich die neuen Sprosse oder konnten auf MS-Medium (Makroelemente ½-Konzentration) mit 1 % Saccharose und 0.6, 1.2 oder 5.0 mg l^{-1} IBA innerhalb von zwei Monaten zur Wurzelbildung induziert werden. Bei niedriger Konzentration von IBA (0.1 mg l^{-1}) oder bei Zugabe von Phloroglucinol oder Thiamin zeigten sich keine fördernden Effekte auf die Wurzelbildung verglichen mit IBA-Behandlungen ohne Addendazugabe. Trotz der intensiven Sprossbildung der nodalen bzw. polynodalen Explantate konnte bei nodalem Kallus sowie bei Blättern keine Morphogenese nachgewiesen werden und die Differenzierung erfolgte nur bis zur Bildung von grünen globulären Strukturen.

Key words. *Quillaja saponaria* – soap-bark tree – *in vitro* propagation – organogenesis

Introduction

Quillaja saponaria Mol. (Soap-bark tree) Rosaceae, 'Quillay' is a native chilean slow-growing evergreen tree, that develops in poor soils of semiarid regions from seacoast up to 2000 m.a.s.l. Quillay is an economically important species that has been exploited for many years. The bark, and other parts of the plant contain saponins that are widely used as detergents. Saponins are important as natural foaming agents in foods and beverages, photographic emulsions and in cosmetic applications due to their emollient effects, and are also used in the treatments of fungal, bacterial and protozoal diseases of livestock (SAN MARTIN and BRIONES 1999, 2000). Currently, *Q. saponaria* saponin is winning interest as adjuvants on the immune response increasing the effectiveness of both injected and oral vaccines, as the association of the viral protein with saponin facilitates its transport across cell membranes (KENSIL et al. 1991; HANCOCK, 1995, RÖNNBERG et al. 1997; SO et al. 1997). Saponins are also been reported as of value in lowering human cholesterol (MALINOW et al. 1979) and tumor treatments (GEBARA et al. 1995). A product with nematicidal properties has been developed recently [QL Agri´35 TM, BASF] (SAN MARTIN, pers. comm). NORD and KENNE (1999) indicate that there is a great variation of saponin content between trees. Due to extremely uncontrolled exploitation in recent years natural stands have been seriously affected; therefore, new plantations are necessary and should be based on selected and/or more productive germplasm and the use of asexually propagated material. So far, little informa-

tion on propagation is available for this species. Rooting of cuttings seems seasonally restricted and affected by various factors i.e. the physiological condition of the donor plants, among others (SANTELICES and BOBADILLA 1997). In this report we describe an alternative in vitro multiplication technique based on root formation on the de novo induced shoots, making it possible to obtain plantlets during the whole year.

Material and Methods

List of abbreviations

BA: benzyladenine; IBA: indolebutyric acid; MS: MURASHIGE and SKOOG (1962) medium; NAA: naphthaleneacetic acid; TDZ: thidiazuron; Z: zeatin; 2,4-D: dichlorophenoxiacetic acid

Two-year old plants grown in a nursery close to Santiago were used as explant sources. Nodal sections of 0.5 cm attached to the half proximal part of the leaf blade, polynodal segments with 4–5 axillary buds from the apical region of the stem and leaf explants (entire leaf or 1 cm^2 sections from the proximal region), were cultivated *in vitro* as a source for production of new shoots. All explants were washed and surface disinfected with Captan and Benlate® (0.02 % each) for 30 min under constant agitation, later disinfected with 3 % Na hypoclorite for 5 min and washed in sterile water. The culture of nodal and leaf sections was performed in tubes (25 x 130 mm) containing 12.5 ml MS-salts, supplemented by 3 % sucrose, jelled with 0.6 % Agar (Midesa ®) and various plant growth regulators. Polynodal segments were cultured floating on liquid nutrient solution in 125 ml Erlenmeyer flasks. New shoots or small shoot clusters forming in both type of explants were isolated and subcultured during 60 days for elongation and rooting. Rooting was attempted in a ½ strength MS solid media either devoid of growth regulators or having various IBA levels respectively, alone or in combination with phloroglucinol or thiamine. All explants were maintained in a 14 hours light regime of 48 $\mu mol\ m^{-2}\ s^{-1}$ provided by daylight fluorescent lamps (Philips TLT 40W/54 R.S.) and at a temperature of 22±1 °C. Rooted plantlets approx. 3.0–5.0 cm high were transferred to non-sterile conditions in a substrate composed of equal volumes of sand/vermiculite/organic soil (OS®), derived from leaves of *Lithraea caustica*. The composition of OS is (data by ANASAC, Inc.): N-total, 1.0 $g\ kg^{-1}$; P-total, 1.1 $g\ kg^{-1}$; P_2O_5-total,

Table 1. Summary of morphogenic responses of subcultured nodal and polynodal sections of *Q. saponaria,* after 3 months [1].

Growth regulators ($mg\ l^{-1}$)		Callus formation (% explants)	Diameter explant (mm)[2]	Average new shoots/explant	Axillary bud sprouting (pre-existing)	Remarks
Nodal sections						
No growth regulators		0.0 a	2.0 a	0.0 a	No	Yellow green explant
2,4-D	0.1					
TDZ	0.1	100.0 c	5.5 c	0.0 a	No	Green callus
NAA	0.5					
TDZ	1.0	100.0 c	5.9 c	21.4 b	Yes	Green callus
NAA	0.1					
BA	0.1	78.9 c	3.5[a]	0.0 a	Yes	Green callus
NAA	0.1					
BA	0.5	100.0 c	5.9 c	0.0 a	Yes	Explant and callus green
NAA	0.1					
BA	2.0	100.0 c	4.2 b	0.0 a	Yes	Explant and callus green
NAA	0.5					
BA	2.0	89.5 c	6.2 c	7.2 b	Yes	Callus with green clusters
BA	5.0	50.0 b	4.7 b	> 50 c[3]	Yes	Explant and callus intensively green
Polynodal sections						
BA	2.5	100.0	10.6	>50 c[3]	Yes	Explant and callus intensively green
BA	5.0	100.0	7.5	>100 c[3]	Yes	Explant and callus intensively green

[1]Results from 20 initial explants/treatment.
[2]Diameter of callus originating at the axillary bud level (including diam. of nodal section), where new shoots arose.
[3]Numerous new shoots after 3 months. Shoot number estimated due to small size and close packing of shoot clusters.

2.5 g kg^{-1}; K-total, 17.4 g kg^{-1}; K_2O, 21.0 g kg^{-1}; Organic matter, 235.6 g kg^{-1}. After that the plantlets were kept in growth chambers for 1-month; followed later by acclimatization on transfer to the greenhouse.

Results and Discussion

Initiation of new shoots from nodal and polynodal explants

Results of morphogenic responses are shown in Table 1. Various combinations of growth regulators provoked various effects simultaneously: increase of the nodal explant diameter with callus formation; initiation of many new shoots and axillary bud elongation with branching (Figs. 1, 2, 3). The effect occurred in various growth regulator combinations but normally at higher levels of BA or TDZ. When BA was used in a level of 5.0 mg l^{-1} as a single hormone the effect was very strong; after 4 months more than 200 compact shoots formed on a single polynodal explant. In these treatments the new shoots that initiated were compressed in clusters along the whole explant (Fig. 4). Treatments including TDZ combined with NAA also lead to shoot formation but to a lesser extend. On the other hand, the axillary buds sprouted and also formed new shoots suitable for subculture.

Callus formation normally occurred along the whole explant but it was more abundant under the axillary bud and at the proximal end of the petiole. However, the origin of the shoot was not the surrounding callus but the tissue of the original node segment. In cultures supplemented with 5.0 mg l^{-1} BA or 1.0 mg l^{-1} TDZ, the callus turned green and formed dense embryonic-like clusters, but never developed organs.

Leaf explants

Results are shown in Table 2. Responses were found only in whole leaf explants; not in leaf sections, consisting in callus formation in all combinations of growth regulators assayed. The callus was compact and green when elicited by relatively high levels of TDZ or BA. The callus was commonly originated on the veins at the adaxial surface on the proximal portion of the leaf, never from its distal end. Depending on the growth regulators used, the leaf explants and callus retained their



Fig. 1. Development of new shoots along a nodal section in presence of BA



Fig. 2. Nodal section showing development of many shoots at the base of the leaf petiole surrounded by callus in presence of BA 5.0 mg l^{-1}.





Fig. 3. and Fig 4. Close-up of two shoot clusters forming at the base of the petiole that had first enlarged and formed callus tissue. The shoots do not derive from the callus.

Table 2. Summary of the results of entire *Q. saponaria* leaf explants culture after 3 months [(1)].

	First culture[(1)]				Subculture	
Growth regulators (mg l^{-1})	Callus formation	Diameter. callus (%)	Shoots (%) (mm)	Remarks	Growth regulators and Addenda (mg l^{-1})	Remarks
NAA 0.1					No regulators	No growth
BA 0.1						
NAA 0.1	100.0	4.2	0.0	Callus yellow	No regulators	No growth
BA 2.0					NAA 0.1; BA 2.0; CH, 200	Callus growth
					NAA 0.1; BA 2.0; Glu, 200	Callus brown
					NAA 0.1; BA 2.0; Cyst, 20	Callus brown
NAA 1.0	100.0	5.9	0.0	Callus yellow	NAA 0.2; TDZ 1.0	Callus brown
BA 0.5					NAA 0.5; TDZ 1.0	Callus brown
NAA 0.2	100.0	n.m	0.0	Callus yellow	NAA 0.0; BA 0.1	Callus green
Z 5.0					NAA 0.1; BA 0.5	Callus green
NAA 0.5	93.7	6.1	0.0	Callus green	No regulators	Callus brown
TDZ 2.0						
TDZ 2.0	100.0	2.3	0.0	Callus green	No regulators	Callus brown

[(1)]Results from 20 initial leaf explants/treatment. N.m: not measured; CH: casein hydrolysate; Glu: glutamine; Cyst: cysteine.

green colour throughout the culture (3 months). However, no morphogenic responses leading to shoots or roots occurred in these explants, in any of the growth conditions assayed, or despite the addition of various organic substances added for this purpose and widely cited as enhancers (casein hydrolysate, glutamine and cysteine).

Rooting and regeneration of plantlets

The new formed shoots developed in nodal and polynodal sections in presence of high BA or TDZ concentrations (5.0 mg l^{-1} and 1.0 mg l^{-1}, respectively) were short (approx. 5 mm long) and compressed. The shoots when directly transferred to a medium without growth regulators elongated only and did not form roots; besides, the clusters continued forming new shoots (Fig. 5). This effect is attributed to the high cytokinin level still remaining in these explants; i.e. the TDZ has been reported to have a strong effect on shoot formation and/or somatic embryogenesis in a large number of woody species (HUETTEMAN and PREECE 1993).



Fig. 5. Elongation of a subcultured shoot in media without growth regulators after 2 months. Axillary buds develop at the proximal end probably due to the initial high content of cytokinin-like regulators in the first medium (arrow).

The shoots were transferred for rooting to a medium with different IBA concentrations, used alone or in combination with supplements. Rooting occurred within 2–3 months in $^1/_2$ strength MS-medium containing 2 % sucrose in presence of 0.6, 1.2 or 5.0 mg l^{-1} IBA, but not at 0.1 mg l^{-1} IBA. The best results were the formation of approx. 4–5 roots per explant, but root number varied according to conditions (Fig. 6, Table 3).

Previous studies have described phloroglucinol as an enhancer of root primordium formation or rhizogenesis in juvenile or adult explants of various herbaceous/woody species, – or in shoots still multiplying in response to high cytokinin levels (CHANCEL et al. 1980; JAMES and THURBON 1981; WELANDER and HUNTRIESER 1981; COMPTON and PREECE 1988). Thiamine has also been described as an enhancer (INOUE and MAEDA 1982; JORDAN et al. 2002). However, these enhancers added besides IBA resulted in a lower number of rooted shoots as compared to IBA (1.2 mg l^{-1}) used alone.

Rooted plantlets that grew to 5.0 cm approx. (Fig. 7), were first placed to non-sterile conditions and after acclimatization in growth chambers transferred to greenhouse conditions.

Acknowledgements

The author thanks Ms. A. Goreux for revision of this manuscript.

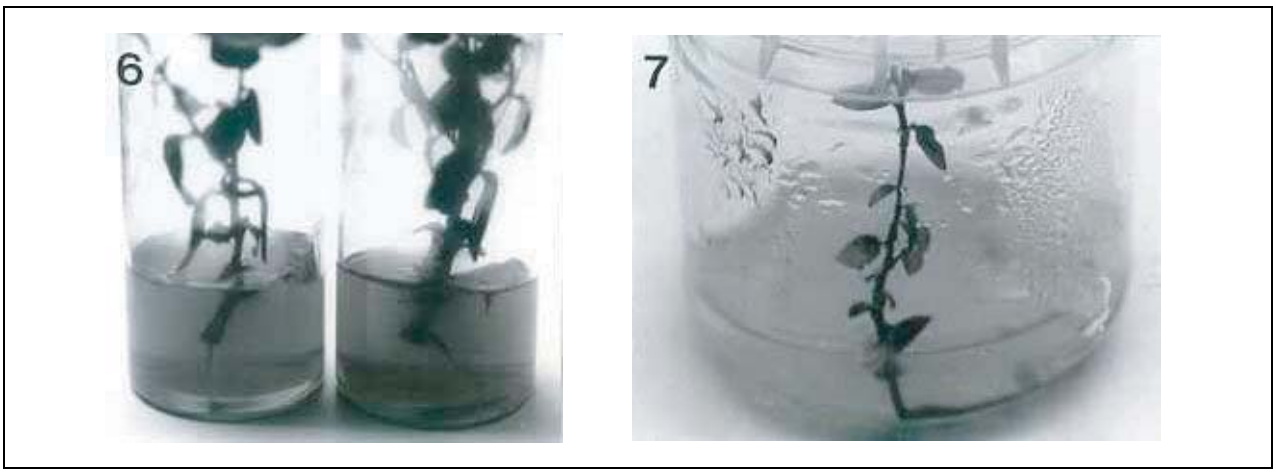


Fig. 6. and Fig. 7. Rooted shoots in presence of IBA 1.2 mg l^{-1} with 1 or 2 roots.

Table 3. Effect of IBA and addenda on root formation of subcultured shoots initiated on nodal or polynodal segments in presence of 5.0 mg l^{-1} BA, after 2 months in culture(1).

IBA (mg l^{-1})	Addenda (mg l^{-1})(2)		% Root formation(3)	Roots/shoot explant	Remarks
	P	T			
0.0	0.0	0.0	0.0a	0.0	Shoots green, elongating
0.1	0.0	0.0	0.0a	0.0	Shoots green, elongating
0.1	100	0.0	0.0a	0.0	Shoots green, some yellowish
0.6	0.0	0.0	50.0 b	1.5±0.7	Shoots green
0.6	100	0.0	50.0 b	1.7±0.9	Few shoots forming, most turned yellow
1.2	0.0	0.0	100.0 c	5.0±3.8	Best rooting conditions and for shoot development. Clusters with numerous green shoots; plantlets.
1.2	100	0.0	60.0 b	2.0±1.6	Shoots green, clusters with numerous shoots, platelets
5.0	0.0	100	60.0 b	1.0±0.9	Few shoots, most turned yellow
5.0	0.0	200	65.0 b	2.3±0.6	Shoots de-differentiating in callus, turning yellow.

(1)Results from 20 Explants/treatment.
(2)Supplemented with phloroglucinol (P) or thiamine (T).
(3)Rooting (%) differences by "comparison of proportions" (Stats™). Values followed by different letters are significantly different.

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Received November 27, 2003 / Accepted March 4, 2004

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