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In vitro Propagation of *Maytenus canariensis* (Loes.) Kand. & Sund. from Apical Meristem Culture

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Abstract

Mature plants of *Maytenus canariensis* (Loes.) Kund. & Sund. were established for apical meristem cultures using branches (two-month-old) collected during the first seven weeks of spring. Most satisfactory results were obtained using a modified Woody Plant Macronutrients solution (WPM.2) enriched with 15 mM of KNO_3 and supplemented with MS micronutrients plus 2.96 µM thiamine-HCl, 4.86 µM pyridoxine-HCl, 2 mM L-cystenie HCl, 1.44 µM GA_3, 2.2 µM BAP, 1.1 µM IAA, 0.56 mM myo-inositol, 2.2 mM glucose and 5 g/l agarose. The effects of WPM.2 and plant growth regulators were strongly dependent on the physiological state of the mother plant. Whole shoots were used for *in vitro* regeneration of multiple shoots and best results were achieved with WPM.2 in combination with 4.44 µM BAP and 2.85 µM IAA or 2.69 µM NAA. Shoots rooted with different combinations and concentrations of auxins without any symptom of chlorosis or necrosis in quarter-strength WPM.2 salts combined with 1.71 µM IAA plus 1.61 µM NAA. Shoots and roots grew well under low light intensity of 15 $\mu M/m^2xs$ for 13 days followed by 33 $\mu M/m^2xs$ for 33 days.

Introduction

Maytenus canariensis (Loes.) Kund. & Sund. is a species endemic to the Canary Islands, Spain and belongs to the Celastraceae family. This species is used as medicinal and ornamental plant, known as peralillo (Bramwell and Bramwell 1990, Pérez de Paz and Medina 1988). Different parts of this woody plant serve as an important source of pharmaceutical natural products (Pérez de Paz and Hernández 1999). The ability to produce seedlings in this species by traditional

techniques is quite limited compared to other Celastraceae's medicinal plants (Pereira et al. 1994, 2000). Although *M. canariensis* is a copious seed producer, seed germination and seedling survivality are very low, both in natural habitat and under laboratory conditions. Tissue culture technology provides an alternative method for solving these problems. So far there is only one report about *in vitro* regeneration of plantlet from callus of *M. canariensis* (Mederos-Molina 1995). The use of *in vitro* techniques has become important for their commercial propagation because of the potential of *M. canariensis* to be economically exploited as ornamental shrubs and/or arboreal material.

Materials and Methods

Juvenile branches of two-month-old *Maytenus canariensis* mature plants were collected during the first seven weeks of Spring. Branches were sterilized with a solution of 1.5 g/l mercuric chloride to which 2 ml of Tween-80 was added, with constant stirring for 13 min followed by four rinses in sterile distilled water. Apical meristems of about 0.5 - 0.7 mm containing two leaf primordia were carefully dissected from the shoot tips using sterile scalpel under stereo-microscope in the laminar flow cabinet. After dissection single apical meristems were placed into 24 ∇ 16 mm test tubes each containing 10 ml culture medium.

In the first experiment, the media used were White (1951), Heller (1953), Murashige and Skoog (1962), Gamborg (1984), Quoirin and Lepoivre (1977). Twelve apical meristems were cultured for each treatment. In the second experiment, the media used were Schenk and Hildebrandt (1972) basal macroelement and Woody Plant Medium (WPM) Lloyd and McCown (1981) basal macroelement. In the third experiment, the media used were the SH and WPM basal macroelements formula. Also, these normal culture media were used with various modifications involving the addtion of 1.2 mM $(NH_4)_2SO_4$ [SH.1], 2.4 mM $(NH_4)_2SO_4$ [SH.2], 3.6 mM $(NH_4)_2SO_4$ [SH.3], 4.2 mM $(NH_4)_2SO_4$ [SH.4], 10 mM KNO_3 [WPM.1], 15 mM KNO_3 [WPM.2], 20 mM KNO_3 [WPM.3] and 25 mM KNO_3 [WPM.4]. All macroelement media were supplemented with Fe-EDTA and the microelement formula described by Murashige and Skoog (1962) plus 2.96 µM thiamine-HCl, 4.86 µM pyridoxine-HCl, 2 mM L-cysteine HCl, 0.56 mM myo-inositol, 202 mM glucose and 5 g/l agarose. These culture media were supplemented with 1.44 µM GA_3 combined with BAP (control, 1.1, 2.2 and 3.3 µM) and IAA (control, 1.1 and 2.2 µM). Twenty four apical meristems were used for each treatment. In every experiment, all explants were transferred to fresh culture media at 13 days intervals. The first and second one were repeated twice and the third experiment was repeated three times.

In all the experiments, 27-day-old shoots (43 - 49 mm) were collected from the establishment stage and inoculated onto WPM.2 + 15 mM KNO_3. All macroelement media were supplemented with Fe-EDTA and the microelement formula described by Murashige and Skoog (1962) plus 2.96 µM thiamine-HCl, 4.86 µM pyridine-HCl, 2 mM L-cysteine HCl, 0.56 mM myo-inositol, 202 mM glucose and 5 g/l agarose. The effect of plant growth regulators was studied on *in vitro* multiplication and rooting of shoots. The medium was supplemented with cytokinins (Kn and BAP) and auxins (IAA, NAA and IBA) at various concentrations (Tables 3 and 4). To evaluate the influence of salts solutions to induce multiple shoots, whole roots were subcultured using the basal WPM.2, half-strength and the quarter-strength WPM.2 media. The pH was adjusted to 5.6 in all the culture media which were sterilized under 0.5 atmosphere at 115_C for 15 min. The cultures were incubated in a growth chamber at 25 ± 1_C under a 16 h photoperiod provided by Sylvania Gro-Lux fluorescent lights between 10 and 40 µM/m^2xs and 77 % relative humidity. Each treatment had 24 replicates and all the experiments were repeated twice.

To study the organogenic ability of the meristems in each of the above macroelement solutions, the following parameters such as apical meristem with developed shoot, the elongation of shoots and their quality (vigour stems = 3, slightly vigour stems = 2 and not vigour stems = 1) was evaluated.

Results were analysed by the Duncan Multiple Range Test (DMRT) using the statiscal graphic system of the Statistical Graphics Corporation computer program and $p < 0.05$ constituted a significant difference.

Results and Discussion

Preliminary study with $AgNO_3$ at optimal concentrations used by Mederos and Trujillo (1999 a, b) stopped secretion of the browning exudate from the apical meristem cultures of *Maytenus canariensis* (Loes.) Kund. & Sund. but did not stimulate any development. To stop the production of the browning exudate, an efficient method was developed with L-cysteine HCl and low light intensity during the first ten days of initial cultures (Mederos-Molina 2002).

In the first attempt to grow *Maytenus canariensis* apical meristems, the culture media used in the first and second experiments resulted in poor formation of shoots (33 - 42 %) with rossetted leaves. All the shoots showed signs of localized leaf chlorosis. Shoot death as well as inhibition of shoot elongation were observed in all treatments except in the basal SH and WPM media (data not shown). Table 1 shows that the best results were obtained in

the SH.1 and SH.2 solutions where the ApMdS were 71 and 79 %, respectively. In SH.3 and SH.4 media most shoots had malformed leaves (data not shown). The longest shoots developed in SH, SH.1 and SH.2 treatments, however, only a few quality of shoots (slightly vigour stems) were obtained in SH macro-

Table 1. *In vitro* establishment of apical meristems of *Maytenus canariensis* on diferent SH macroelement solutions supplemented with 2 mM L-cysteine, 1.44 µM GA_3, 2.2 µM BAP and 1.1 µM IAA. Cultures were incubated under 16 h photoperiod with a light intensity of 33 µE/m^2xs.

Macroelement solutions	Percentage ApMdS	Length of shoot (cm)	Quality of shoots
Control	0	0.0 ± 0.0^a	0.0 ± 0.0^a
SH	67	2.2 ± 0.42^c	1.5 ± 0.52^b
SH.1	71	3.1 ± 0.47^d	2.5 ± 0.49^c
SH.2	79	2.4 ± 0.45^c	2.5 ± 0.47^c
SH.3	58	1.6 ± 0.59^b	1.5 ± 0.55^b
SH.4	62	1.4 ± 0.57^b	1.5 ± 0.58^b

Data were collected at 39 days of culture. Results were obtained from 27-day-old shoots. Each value represents the mean of developed shoots ± S.E. Means in columns followed by the same letter are not significantly different at 0.05 level of confidence (Duncan's test).

element solution. The SH.1 and SH.2 showed significant difference in terms of shoot length. Table 2 shows that the most satisfactory results were obtained in the WPM.2 and WPM.3 where the ApMdS were 87 and 79 %, respectively. Moreover, the length of shoots and quality parameters were discernibly different ($p < 0.05$). In case of ApMdS length and quality parameters were concerned, the WPM.2 was significantly different ($p < 0.05$) with respect to the other macroelement media, that is, control, WPM, WPM.1, WPM.3 and WPM.4.

The effect of plant growth regulators on growth and development of shoots was also observed. The best percentage of developing shoots was achieved with combination of 1.44 µM GA_3, 2.2 µM BAP and 1.1 µM IAA (Tables 1 and 2) and these results are in agreement with other results previously reported (Mederos-Molina 2001, 2002; Mederos et al. 1997/1998).

The results of comparison (Tables 1 and 2) established that the WPM.2 gave better results than any other macroelement solution. WPM.2 has proved to be a vital factor in the *in vitro* organogenesis of *Maytenus canariensis* as shown by the fact that during the *in vitro* establishment of different types of explants, significant differences ($p < 0.05$) have been observed on similar culture media (Mederos-Molina 2001, 2002). These observations are in accordance with the results of other woody plants or medicinal species

(Mederos-Molina 2002; Pereira et al. 1994, 1995, 2000; Mederos et al. 1997/1998). On the other hand, optimal composition of macronutrients strongly depended on explants's age and genotype of *M. canariensis* (Mederos-Molina 1995). The above study has shown how important it is to test a wide range of macronutrient solutions in an attempt to optimize the *in vitro* growth and development of shoots.

Table 2. *In vitro* establishment of apical meristems of *Maytenus canariensis* on different WPM supplemented with 2 mM L-cysteine, 1.44 µM GA_3, 2.2 µM BAP and 1.1 µM IAA. Cultures were incubated under 16 h photoperiod with a light intensity of 33 µE/m^2xs.

Macroelement solutions	Percentage ApMdS	Length of shoot (cm)	Quality of shoots
Control	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
WPM	46	2.4 ± 0.26^{c}	1.5 ± 0.17^{b}
WPM.1	62	$3.5 \pm 0.32^{d,c}$	2.5 ± 0.29^{c}
WPM.2	87	4.6 ± 0.24^{f}	3.0 ± 0.17^{d}
WPM.3	79	$4.1 \pm 0.29^{e,d}$	2.5 ± 0.25^{c}
WPM.4	58	$1.8 \pm 0.33^{b,c}$	1.5 ± 0.17^{b}

Data were collected at 39 days of culture. Results were obtained from 27-day-old shoots. Each value represents the mean of developed shoots ± S.E. Means in columns followed by the same letter are not significantly different at 0.05 level of confidence (Duncan's test).

One of the difficulties eventually needs to be overcome in initiating the multiplication and rooting of *Maytenus canariensis* is the brown exudation. This causes darkening of the explant and leads the shoot to death. This problem has been previously solved by numerous workers (Mederos-Molina 2002). However, the exudation of phenolic compounds was more severe in *Maytenus canariensis* after excision from the parent plant and in the initial stage of apical meristem establishment. The absence of browning in basal or modified SH and WPM.2 media was observed in presence of L-cysteine HCl and low intensity light (15 µM/m^2xs) during the first 13 days of multiplication and at the rooting stages. Similar results was also obtained previously in other woody plant species (Mederos-Molina 2001, 2002, Mederos et al. 1999 a, b). Moreover, no significant differences could be seen for the parameters of quality of shoots (not vigorous) and morphological appearance (some primordial leaflets or some malformed leaves). On the other hand, less than 42 % of the shoots were developed normally (data not given). These results seem to be related to the vegetative stage of parent plants, type and the age of explants and more so the genotype of Celastraceae's medicinal plant (Pereira et al. 1993, 1994, 1995, 2000; Mederos-Molina 1995).

On the WPM.2 proliferation of new shoots was obtained in absence of callus formation. Proliferation was dependent on plant growth regulators added into this medium. The highest rate of multiplication (number of shoots

Table 3. Effect of various concentrations of plant growth regulators supplemented in WPM.2 on shoot proliferation and shoot length of *Maytenus canariensis*.

Kn	BAP	IAA (μM)	NAA	IBA	% shoots[1] proliferation	No. of shoot per culture	Average of length of shoots (mm)
0	0	0	0	0	29	0	0
2.32	0	0	0	0	54	$1.0^{b} \pm 0.2$	$8^{b} \pm 0.0$
4.64	0	0	0	0	50	$1.0^{b} \pm 0.2$	$8^{b} \pm 0.0$
0	2.22	0	0	0	58	$1.4^{c} \pm 0.4$	$10^{c} \pm 0.4$
0	4.44	0	0	0	54	$1.9^{d} \pm 0.5$	$10^{c} \pm 0.3$
2.32	0	1.71	0	0	68	$1.0^{b} \pm 0.2$	$17^{d} \pm 0.4$
2.32	0	2.85	0	0	67	$1.4^{c} \pm 0.3$	$20^{c,d} \pm 0.5$
2.32	0	0	1.61	0	71	$1.4^{c} \pm 0.5$	$22^{e,f} \pm 0.8$
2.32	0	0	2.69	0	68	$1.4^{c} \pm 0.4$	$20^{c,d} \pm 0.8$
2.32	0	0	0	1.48	42	$1.0^{b} \pm 0.0$	$17^{d} \pm 0.5$
2.32	0	0	0	2.46	37	$1.0^{b} \pm 0.0$	$17^{d} \pm 0.7$
4.64	0	1.71	0	0	50	$2.0^{d} \pm 0.4$	$10^{c} \pm 0.6$
4.64	0	2.85	0	0	54	$2.0^{d} \pm 0.6$	$10^{c} \pm 0.4$
4.64	0	0	1.61	0	50	$2.0^{d} \pm 0.7$	$17^{c} \pm 0.6$
4.64	0	0	2.69	0	46	$2.0^{d} \pm 0.6$	$20^{c,d} \pm 0.6$
4.64	0	0	0	1.48	37	$1.4^{c} \pm 0.6$	$8^{b} \pm 0.1$
4.64	0	0	0	2.64	33	$1.4^{c} \pm 0.7$	$8^{b} \pm 0.1$
0	2.22	1.71	0	0	79	$2.5^{c,d} \pm 0.5$	$30^{g} \pm 0.5$
0	2.22	2.85	0	0	75	$2.5^{c,d} \pm 0.6$	$33^{b,c} \pm 0.4$
0	2.22	0	1.61	0	69	$2.5^{c,d} \pm 0.7$	$36^{h,i} \pm 0.3$
0	2.22	0	2.69	0	71	$2.5^{c,d} \pm 0.8$	$30^{g} \pm 0.4$
0	2.22	0	0	1.48	67	$2.0^{d} \pm 0.6$	$22^{e,f} \pm 0.8$
0	2.22	0	0	2.46	67	$2.0^{d} \pm 0.8$	$20^{c,d} \pm 0.8$
0	4.44	1.71	0	0	83	$3.0^{f} \pm 0.7$	$36^{h,i} \pm 0.3$
0	4.44	2.85	0	0	87	$4.4^{i} \pm 0.5$	$40^{i,j} \pm 0.3$
0	4.44	0	1.61	0	75	$3.3^{f,g} \pm 0.2$	$36^{i} \pm 0.2$
0	4.44	0	2.69	0	92	$3.7^{g,h} \pm 0.7$	$33^{g,h} \pm 0.3$
0	4.44	0	0	1.48	58	$3.0^{f} \pm 1.0$	$22^{e,f} \pm 0.8$
0	4.44	0	0	2.46	62	$3.0^{f} \pm 0.9$	$22^{e,f} \pm 0.7$

[1]There were 24 whole shoots for each treatment. Data were collected at 49 days of culture. Each value represents the mean of developed shoots ± S.E. Means in columns followed by the same letter are not significantly different at 0.05 level of confidence (Duncan's test).

developed in two weeks) for each treatment was observed on WPM.2 medium containing BAP combined with IAA or NAA. There was less response on WPM.2 supplemented with IBA (Table 3). The combination of BAP with IAA or NAA

is necessary to induce elongation *in vitro* shoot proliferation of *Maytenus canariensis*. The required growth regulators and their concentrations (Table 3) seem to be related to the type of explants cultured *in vitro* and in other experiments have been realized and reported earlier (Mederos-Molina 1995, 2001, 2002; Pereira et al. 1995; Mederos et al. 1996, 1998). There was no root development until three weeks after transfer to rooting media. The percentage of rooting was markedly different ($p < 0.05$) in WPM.2 and half-strength WPM.2 (less than 46% plantlets obtained with an average rooting between 1.7 and 2.3) (data not shown), or in quarter-strength WPM.2 (Table 4). With regards to the rooting media, quarter-strength WPM.2 was better because lower

Table 4. Effect of various auxins at different concentrations added into the quarter-strength WPM.2 medium (wihout cytokinin) on roots proliferation of *Maytenus canariensis*.

IAA	NAA (µM)	IBA	% of shoots[1] with roots	No. of roots per shoot	Average length of roots (mm)
1.71	0	0	46	$2.2^{c,b} \pm 0.4$	$24^{c,b} \pm 0.5$
2.85	0	0	54	$2.4^{d,c} \pm 0.5$	$27^{d,c} \pm 0.7$
0	1.61	0	42	$3.3^{e} \pm 0.6$	$19^{b,a} \pm 0.7$
0	2.69	0	54	$2.4^{d,c} \pm 0.7$	$19^{b,a} \pm 0.5$
0	0	1.48	33	$1.2^{a} \pm 0.8$	$15^{a} \pm 0.6$
0	0	2.46	37	$1.2^{a} \pm 0.6$	$15^{a} \pm 0.7$
1.71	1.61	0	71	$3.8^{f,e} \pm 0.5$	$35^{f,e} \pm 0.5$
1.71	2.69	0	62	$3.3^{e} \pm 0.6$	$29^{e,d,c} \pm 0.7$
1.71	0	1.48	37	$2.2^{c,b} \pm 0.7$	$24^{c,b} \pm 0.7$
1.71	0	2.46	42	$2.2^{c,b} \pm 0.6$	$27^{d,c} \pm 0.8$
0	1.61	1.48	50	$1.7^{b,a} \pm 0.7$	$24^{c,b} \pm 0.6$
0	1.61	2.46	46	$1.7^{b,a} \pm 0.6$	$24^{c,b} \pm 0.4$
1.71	1.61	1.48	33	$1.2^{a} \pm 0.3$	$19^{b,a} \pm 0.7$
1.71	1.61	2.46	25	$1.2^{a} \pm 0.5$	$15^{a} \pm 0.6$

[1]There were 24 shoots for each treatment. Data were collected at 53 days of culture. Each value represents the mean of developed roots ± S.E. Means in columns followed by the same letter are not significantly different at 0.05 level of confidence (Duncan's test).

mineral content is thought to be more suitable for *in vitro* rooting of *Maytenus canariensis*. Similar behaviour was observed in other medicinal plants and/ or woody plant species (Herman 2000; Mederos-Molina 2002). Moreover, inhibition of shoot multiplication and callus proliferation were apparent in week two due to high dose of auxins [3.42 - 11.42 µM IAA, 3.22 - 10.74 µM NAA, 2.95 - 9.80 µM IBA] (data not shown). BAP combined with IAA and NAA at different concentrations produced more than 75 % of rooted shoots and were

significantly higher than all other treatments ($p < 0.05$) (Table 4). IBA treatment alone or combined with other auxins (IAA, NAA) had less than 50 % of the rosette shoots rooted (data not shown). There was a synergistic effect between the auxins and in certain woody plant species, the IBA was less effective for root initiation compared to NAA or IAA but was effective in stimulation root development and elongation (Mederos-Molina, unpublished data). Moreover, the optimal rooting media (Table 4) had the highest vigorous shoots and these results are consistent with others (Mederos-Molina 1995, 2001; Mederos et al. 1997 a, b). *In vitro Maytenus canariensis* plantlets are susceptible to desiccation once transferred to soil (Mederos-Molina, unpublished data). More experiments therefore need to be done for the hardening phase.

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