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BRIEF COMMUNICATION

Seed germination and *in vitro* propagation of *Maytenus canariensis* through regeneration of adventitious shoots from axillary and apical buds

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Abstract

Seed germination and micropropagation protocols of the medicinal species *Maytenus canariensis* (Loes.) G. Kunkel & Sunding were optimized. *In vitro* seed germination occurred (86 to 94.7 %) only after treatment of the seeds with H_2SO_4, followed by surface sterilization and culture on solid nutrient medium without any growth regulators. Micropropagation failed when explants were taken from mature trees, and browning of the nutrient medium frequently occurred despite testing many growth media. Nonetheless, adventitious shoot regeneration was achieved employing axillary or apical buds taken from 2 - 2.5 months old plantlets obtained after *in vitro* germination of seeds, following culture on nutrient media supplemented with benzylaminopurine, kinetin and naphthalenacetic acid (NAA), attaining up to 3.9 shoots per explant, after 4 - 6 months. Root induction was best on a medium containing 4.0 mg dm^{-3} NAA, achieving a 100 % induction. After hardening of rooted plants, survival after transfer to soil was 71.43 %.

Additional key words: growth regulators, micropropagation, seed dormancy.

The genus *Maytenus*, belonging to the *Celastraceae* family, is the largest genus of the family and comprises 255 species, many of which showing medicinal properties. *Maytenus canariensis* is endemic to the Canary Islands, present in all the islands except Lanzarote, and being the only species of this family in the islands. It is a shrub or small tree (2 - 6 m high), located in the thermophilous forest, currently under protection by law enforcement and classified as a vulnerable species. This is a medicinal plant, and a shrub of ornamental interest. Regarding the *in vitro* culture of *Celastraceae* species, in one instance a rapid *in vitro* multiplication and restoration of *Celastrus paniculatus* has been reported, employing nodes, shoot tips, internodes and leaf bases excised from young vines of the flowering woody climber (Nair and Seeni 2001); in another report, micropropagation of the same species was achieved using internode explants (Rao and Purohit 2006). Furthermore, suspension cultures of *M. hookeri* have been established from callus cultures induced from leaves and young stems and their chemical constituents were determined including a novel triterpenoid, as well as 8 known compounds, together with studies on the accumulation of polyhydroxyfatty acids in salicylic acid elicited cultures (Lu *et al.* 2002, Li *et al.* 2004). Similarly, the *in vitro* propagation of *M. ilicifolia* as a potential source for anti-tumour and antioxidant quinomethyde triterpenes production has also been reported (Buffa-Filho *et al.* 2004).

Here, we report on the optimization of seed germination and *in vitro* multiplication of the vulnerable medicinal plant *M. canariensis* as a means of propagation by establishing successful protocols employing different cytokinin and auxin regimes, and thus begin the biotechnological exploitation of this species.

Seeds of *Maytenus canariensis* (Loes.) G. Kunkel & Sunding were collected from trees growing near La Laguna University at different times (December 2004 and June 2005). Fruits containing an average of 3 seeds per fruit were allowed to dry at room temperature for 3 - 4 weeks, and seeds were removed from them

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Abbreviations: BAP - benzylaminopurine; B_5 - Gamborg´s medium; Kin - kinetin; MS - Murashige and Skoog´s medium, NAA - naphthaleneacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid.

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and kept at room temperature until required.

After removal of the aril present in the seed using a pair of forceps, seeds were surface-sterilized by a brief immersion (30 s) in ethanol (70 %), followed by different surface-sterilization treatments: *a*) 7 % aqueous solution of sodium hypochlorite (v/v) from a commercial bleach, for 15 min and then four washes with sterile distilled water; *b*) aqueous solution of H_2O_2 (20 %) (v/v) for 5 or 10 min, and washed four times with sterile distilled water; *c*) 1 % aqueous solution of $HgCl_2$ (m/v) for 15 or 25 min together with a treatment of H_2O_2 (20 %) (v/v) for 5 min, and finally washed four times with sterile distilled water. Prior to surface-sterilization, the following treatment was performed to stimulate seed germination. Seeds were immersed in H_2SO_4 (95 - 98 % purity, 18.76 M) for 15 min, washed with distilled water and then surface-sterilized by the treatments *a*) or *c*) described above.

After these treatments, 10 - 12 seeds were placed on solid MS (Murashige and Skoog 1962) medium supplemented with 3 % sucrose, 0.7 % agar, and pH 6.0 was adjusted before autoclaving at 121 ºC for 20 min. Petri dishes, which were sealed with parafilm, were placed in a culture chamber (temperature of 25 ± 2 °C, 16-h photoperiod and irradiance of 35 μmol m^{-2} s^{-1} supplied by cool-white fluorescent tubes).

Germinated seeds were then transferred onto the same solid culture medium but in glass vessels (100 cm^3) with *Magenta B* cups (*Sigma-Aldrich*, Madrid, Spain), and the jars were then placed under the same culture conditions to allow further plant growth. Axillary or apical buds taken from 2.0 - 2.5 months old plants were employed for *in vitro* multiplication. Similarly, leaf fragments (1 × 1 cm) and small cuttings containing buds (3 - 4 cm long) taken from young branches of growing trees were also employed as explants. These were initially washed with water and detergent, and then surface sterilized following treatment *a*.

For induction and multiplication of shoots, axillary and apical buds taken from *in vitro* germinated seeds, leaf fragments or small cuttings were cultured on different growth media composed of either MS or B_5 (Gamborg *et al*. 1968) basal salts, with 3 % sucrose, and supplemented with different plant growth regulators (Table 1). Similarly, for root induction of regenerated shoots the media composed of the same basal salts and 3 % sucrose but supplemented with different auxins were tested (Table 2; 9 - 12 shoots were employed for each treatment).

For acclimatization to soil, 25 micropropagated rooted *M. canariensis* shoots (*ca*. 3 - 5 cm in length, 50 to 60-d-old) were individually potted in plastic cups containing commercial peat after thoroughly removing the agar trapped in the roots. The substrate was watered to saturation and then the pot and plant covered with a translucent plastic bag, to which water was previously sprayed, to provide a moist environment and thus to prevent desiccation of plants. Potted plants were then placed in a phytotron at 26 °C, 16-h photoperiod with an irradiance of 50 μmol m^{-2} s^{-1} and were watered regularly. The plastic bags were gradually opened by cutting one of the corners after 10 d and then another after 16 d, and finally the bags were removed after 25 d.

The means and standard error of the means were calculated to assess the variation within replicates. Tukey's tests and analysis of variance (*ANOVA*) were conducted to evaluate differences between means of the different treatments. Moreover, a χ^2-test was employed to assess differences with the root induction and shoot induction response data. All analyses were performed at a significance level of 0.05.

Seed germination was attempted in order to obtain young and surface sterile plant material for the subsequent establishment of *in vitro* propagation protocols, necessary for the intended exploitation of this medicinal plant through biotechnological means. To the best of our knowledge, there are not reports on *in vitro* seed germination trials of *M. canariensis*; besides, the plant is known to produce abundant seeds although germination rate and plantlet survival are very limited, resulting in a poor multiplication in its natural habitats.

Regarding *in vitro* germination of seeds, there was a total lack of germination when seeds were just surface sterilized regardless of the surface-sterilization protocol followed, and cultured on solid medium even after inoculating 200 - 300 seeds per treatment and after 70 - 80 d in culture, results in agreement with the known low germination rate of this species. Furthermore, mechanical scarification was also practiced by scraping the seeds with fine sand paper, followed by surface sterilization and *in vitro* culture, but germination failed (data not shown). On the contrary, in *M. senegalensis* germination was achieved following scraping of the pericarp with sand paper (Matu *et al.* 2006). Nevertheless, germination was drastically boosted after seeds were treated with H_2SO_4, and then surface-sterilized by either method *a* or *c*, resulting in a germination percentage of 86 and 94.7 after a period of 35 - 55 d in culture, respectively. Similar results were reported for the germination of *Parkia biglobosa* which was also increased after H_2SO_4 treatment but for shorter times 1, 3 and 5 min (Aliero 2004). These results suggest that H_2SO_4 did not only scarify the testa of seeds, which was also achieved after scraping the seeds with sand paper, but somewhat influenced the physiological state of the embryo leading to seed germination. The growing plantlets were then employed as a source of axillary and apical buds which were subsequently used for *in vitro* propagation.

In order to induce multiple shoots of *M. canariensis*, axillary and apical buds taken from *in vitro* germinated 2 - 2.5 months old growing plantlets, or leaf fragments and small cuttings containing buds taken from young branches of growing trees were used as a source of plant material. These were cultured on different media (basal salts MS or B_5) containing different amounts of BAP or Kin, ranging from 1 - 4 mg dm^{-3}, and 0.250 mg dm^{-3} of NAA (Table 1).

Table 1. *Maytenus canariensis* shoot induction after culture on MS or B_5 medium supplemented with 0.250 mg dm^{-3} NAA and different concentrations of BAP and Kin [mg dm^{-3}]. Each value is the mean of 3 replicates, 9 - 12 explants ± SE. Response indicates the percentage of buds responding to the different shoot induction and multiplication treatments after 6 months. IM1 to IM-12 refers to the different shoot induction and multiplication media, concentrations of growth regulators added presented as mg dm^{-3}. Values followed by different letters in a column differ significantly at $P < 0.05$ (χ^2-test at d.f. 1).

Medium		Number of shoots induced after 2 months	3 months	4 months	5 months	6 months	Response [%]
IM-1	MS + BAP (1.0)	1.10 ± 0.35ab	1.10 ± 0.35ab	1.10 ± 0.35a	1.10 ± 0.35ab	1.10 ± 0.35ab	60.00bc
IM-2	MS + BAP (2.0)	1.64 ± 0.31bc	1.64 ± 0.31b	2.00 ± 0.36ab	2.27 ± 0.45abc	2.27 ± 0.45abc	81.87ab
IM-3	MS + BAP (4.0)	2.60 ± 0.60c	3.30 ± 0.67c	3.60 ± 0.87b	3.90 ± 1.07c	3.90 ± 1.07c	90.00a
IM-4	MS + Kin (1.0)	0.55 ± 0.17ab	0.55 ± 0.17ab	1.00 ± 0.24a	1.00 ± 0.24ab	1.00 ± 0.24ab	55.50c
IM-5	MS + Kin (2.0)	0.10 ± 0.10a	0.10 ± 0.10a	0.50 ± 0.16a	0.50 ± 0.16ab	0.50 ± 0.16a	50.00cd
IM-6	MS + Kin (4.0)	0.22 ± 0.22ab	0.22 ± 0.22ab	0.22 ± 0.22a	0.22 ± 0.22a	0.22 ± 0.22a	11.10ef
IM-7	B_5 + BAP (1.0)	1.00 ± 0.24ab	1.11 ± 0.26ab	1.33 ± 0.24a	2.88 ± 0.84bc	3.11 ± 0.87bc	88.88a
IM-8	B_5 + BAP (2.0)	0.18 ± 0.18ab	0.18 ± 0.18ab	0.09 ± 0.09a	0.09 ± 0.09a	0.09 ± 0.09a	9.10ef
IM-9	B_5 + BAP (4.0)	1.45 ± 0.39abc	1.63 ± 0.36b	1.91 ± 0.58ab	1.91 ± 0.58abc	1.91 ± 0.58abc	81.81ab
IM-10	B_5 + Kin (1.0)	0.00 ± 0.00a	0.00 ± 0.00a	0.33 ± 0.16a	0.33 ± 0.16a	0.33 ± 0.16a	33.33de
IM-11	B_5 + Kin (2.0)	0.00 ± 0.00a	0.00 ± 0.00a	0.20 ± 0.20a	0.20 ± 0.20a	0.20 ± 0.20a	20.00de
IM-12	B_5 + Kin (4.0)	0.22 ± 0.15ab	0.22 ± 0.15ab	0.67 ± 0.24a	0.67 ± 0.24ab	0.66 ± 0.24ab	55.55c

Table 2. *Maytenus canariensis* root induction after culture on different media. Each value is the mean of 4 - 6 explants ± SE. RI-1 - MS + 1.0 mg dm^{-3} NAA, RI-2 - MS + 4.0 mg dm^{-3} NAA, RI-3 - B_5 + 4.0 mg dm^{-3} NAA, RI-4 - MS + 4.0 mg dm^{-3} 2,4-D, RI-5 - B_5 + 4.0 mg dm^{-3} 2,4-D, IM1 to IM-12 refers to the different shoot induction and multiplication media assayed (Table 1). Values followed by different letters in a column differ significantly at $P < 0.05$ (χ^2-test at d.f. 1).

Induction medium	Rooting medium	Rooting [%]
IM-1	RI-2	81.82ab
	RI-4	0.00
IM-2	RI-2	6.25e
	RI-4	0.00
IM-3	RI-1	0.00
	RI-2	50.00c
IM-4	RI-4	0.00
	RI-2	40.00c
IM-5	RI-4	0.00
	RI-2	0.00
IM-6	RI-4	0.00
	RI-2	50.00c
IM-7	RI-4	0.00
	RI-3	71.43b
IM-8	RI-5	0.00
	RI-3	100.00a
IM-9	RI-5	0.00
	RI-3	22.22d
IM-10	RI-5	0.00
IM-11	RI-5	0.00
IM-12	RI-5	0.00
	RI-3	0.00

Regardless of the shoot induction media employed, when leaf fragments and small cuttings containing buds taken from trees were used, shoot induction failed, which might indicate that this plant material did not react to the stimulus applied by the different growth regulator regimes and nutrients; besides, the surface-sterilization treatment employed also likely affected the integrity of these explants. Moreover, when culturing these explants, browning exudates covered most of the solid medium, which affected the capacity of explants to respond to the different treatments. This contrasts with results on *Celastrus paniculatus* micropropagation which was effective when employing BAP (1 mg dm^{-3}) with maximum number of regenerated bud (3.6) and a frequency of 94 % after 6 weeks when employing explants excised from young vines of flowering plants (Nair and Seeni 2001). Also, micropropagation of the same species, employing only internode segments, was achieved with the same amount of BAP but obtaining a much larger number of induced shoots (14 - 15) with a regeneration frequency of 100 % after 9 weeks (Rao and Purohit 2007).

Nonetheless, shoot induction and multiplication of *M. canariensis* took place only when axillary or apical buds taken from *in vitr*o germinated seeds were employed, following culture on the different media assayed (Table 1). It can be seen that the use of BAP was more effective than Kin, together with the presence of NAA (0.250 mg dm^{-3}), in contrast to *C. paniculatus* where the presence of the auxins IAA or NAA did not improve response rather promoted callusing (Rao and Purohit 2007). The best results were obtained with medium IM-3, supplemented with 4.0 mg dm^{-3} BAP, giving a maximum number of buds of 3.9 after 4 - 5 months with 90 % of the buds responding to this treatment. This was followed by medium IM-7, showing an induction of 3.1 buds after 6 months with 88.8 % of the explants responding to the treatment. There was no statistical difference between the number of buds induced

on both media ($P < 0.05$), similar values being reported in *C. paniculatus* (Nair and Seeni 2001). IM-7 medium contained B_5 nutrients and only 1.0 mg dm^{-3} BAP, and increasing the amount of this growth regulator did not increase the number of buds induced (Table 1). On the other hand, the time needed to reach these values with *M. canariensis* was 4 - 6 months unlike *C. paniculatus* which required shorter time around 6 - 9 weeks (Rao and Purohit 2006, Nair and Seeni 2001).

Induced plantlets were allowed to grow to 3 - 4 cm and then were excised and transferred to root inducing medium (Table 2). Regardless of the shoot induction and multiplication media employed, root induction took place only on RI-2 and RI-3 media, which were supplemented with 4 mg dm^{-3} NAA but having different basal salts. Lowering the concentration of NAA prevented root induction; analogously, when NAA was replaced by 2,4-D, root induction failed, suggesting the appropriateness of NAA for inducing roots in *M. canariensis* plantlets (Table 2). The best root induction medium was RI-3, reaching 100 % induction for shoots derived from medium IM-8. This was followed by 81.82 % root induction on medium RI-2 for shoots derived from medium IM-1. Concerning shoots derived from the best shoot induction and multiplication media (IM-3, IM-7), root induction was 50.0 and 71.4 %, respectively (Table 2).

Finally, acclimatization of *M. canariensis* rooted plants to soil was successful with a high survival rate (71.43 %), similar to that achieved with *M. senegalensis* which varied between 73 to 86 %, depending on the substrate mixture employed (Matu *et al.* 2006), and like that reported for *C. paniculatus*, 70 - 80 % when plants hardened under high humidity conditions (Rao and Purohit 2006). However, these survival rates were lower than that obtained with *Celatrus paniculatus* which reached 95 % (Nair and Seeni 2001).

The data presented demonstrate the establishment of efficient germination and micropropagation protocols for this medicinal species, which allows the regeneration of new plants from axillary or apical buds. Furthermore, if these multiplication rates were maintained, approximately 7 viable plants would be obtained from a single apical or axillary bud per year, a method which could be applied for its conservation. This protocol would also provide sufficient juvenile plant material to attempt to exploit this important medicinal species by biotechnological means for the production of valuable secondary metabolites.

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