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Micropropagation, Callus Induction and Indirect Organogenesis of *Maytenus octogona* (L'Hér.) DC. (Celastraceae), a Shrub of Peruvian Desert

Magali Sánchez-Chuquicusma[1], Guillermo E. Delgado-Paredes[1]* and Consuelo Rojas-Idrogo[1]

[1]Facultad de Ciencias Biológicas, Universidad Nacional Pedro Ruiz Gallo, Ciudad Universitaria, Juan XXIII No 391, Lambayeque, Perú

*Corresponding Author E-mail: guidelg2001@yahoo.es

ABSTRACT

A micropropagation protocol for Maytenus octogona, a medicinal shrub of Peruvian desert, was established using in vitro germinated 3 to 4-week-old seedlings as a source of explants. Shoot elongation was obtained from apical buds and nodal segments cultured in MS medium supplemented with 0.01 mg/L IAA – 0.25 mg/L BAP and 0.01 mg/L IAA – 0.01 mg/L GA_3, with 37.0 and 36.7 cm, respectively, after 60 days of culture. Isolated individual shoots were cultured on full strength MS medium containing IBA 25 mg/L for 24 h in the dark for induction of roots, followed by transfer to gelled hormone-free full strength MS basal medium, with 100% of shoots rooted. Callus induction was achieved from various explants in MS medium supplemented with 5.0 mg/L 2,4-D + 1.0 mg/L KIN + 25 mg/L cysteine +510 mg/L KH_2PO_4, 2.0 mg/L 2,4-D + 0.2 mg/L KIN, and 5.0 mg/L 2,4-D + 0.2 mg/L KIN. Indirect organogenesis was observed in callus induced from hypocotyls in MS medium supplemented with 1.0 mg/L BAP and 2.0 mg/L 2iP. This study is the first attempt to standardize the shoot elongation and shoot proliferation from apical buds and nodal segments, callus induction from various explants as zygotic embryos, seeds, leaves, hypocotyls and roots, and indirect organogenesis from hypocotyls.

Keywords: *Callus induction, in vitro propagation, Maytenus octogona, shoot elongation, rooting.*

INTRODUCTION

The order Celastrales consists of 12 families and a little more than 2000 species. The Celastraceae (800), Hippocrateaceae (300), Aquifoliaceae (300+), and Icacinaceae (400) are fairly closely related and make up the bulk of the order[11]; however, in the APG classification, the order Celastrales consists of the few families such as the Celastraceae, Lepidobotryaceae and Parnassiaceae[3]. The family Celastraceae encompasses 98 genera comprising approximately 1210 species that are widely distributed throughout Asia, Africa and the Americas[8,32]; these species are trees and shrubs and sometimes climbing or vining. In the Peru the Celastraceae family comprises 8 genera and 27 species, of which the genus *Maytenus* is the largest, with 16 species described[4], and in the world *Maytenus* is a genus of over 200 species, mostly in the tropical and subtropical Africa, Asia and Australia. *Maytenus octogona* (L´Her.) DC. known as "mude" or "realengo" is a shrub or small tree to 1-5 m tall, commonly found in western South America (Ecuador, Peru and Chile) in the coastal zone and arid lowlands.

The Celastraceae family has shown the accumulation of an interesting class of bioactive compounds, the quinone-methide triterpenes (QMTs). These QTMs are secondary metabolites that occur only in species of this family, therefore are considered chemotaxonomic indicators[5,10]. These triterpenes have been intensively studied by several important research groups due their biological activities, such as anti-

inflammatory[19], antioxidant[13], antifungal[17], antitrypanosomal[14], antimicrobial[31], and recently, attention has been focused on the QMT maytenin, because it is highly toxic to a panel of cancer cell lines[2].

In *in vitro* tissue culture, suspension cell cultures of *Maytenus hookeri*, collected from Xishuangbanna (Yunnan, China), were established from the calli induced from the leaves and young stems on MS media with the supplement of 2.0 mg/L 2,4-D and 0.1 mg/L KIN, and the ethyl acetate extract of the cultures did not yield maytansine instead of affording nine compounds including one novel triterpenoid, named 2,3-diacetoxyl maytenusone, and eight known ones including squalene, salaspermic acid, maytenonic acid, and others[20]. In another study, cell culture of *M. ilicifolia* was established with callus induced from leaf explants in order to produce and to quantify the antitumoral and antioxidant QMTs, 22β-hydroxymaytenin and maytenin, and the QMTs showed maximum accumulation in the logarithmic phase growth of cell culture[6]. Likewise, explants of *M. aquifolium* were induced to form callus and, subsequently, suspension cultures, and the isolation of natural products from callus led to the identification of the cytotoxic triterpene quinonemethides, maytenin and 22β-hydroxymaytenin by reverse phase high performance liquid chromatography[9]. Recently, cell suspensions of *Maytenus ilicifolia*, derived from the long-term cell line retained their capacity to synthesize and accumulate QMTs, and presented levels of maytenin, 22β-hydroxymaytenin, celastrol and pristimerin that were, respectively, 1.96-, 2.48-, 8.85- and 3.29-times higher than those in the roots of 5-year old plants[7].

In the other hand, in some *Maytenus* species was observed *in vitro* clonal propagation. In a pioneer study, an *in vitro* method for cloning and mass multiplication of *Maytenus emarginata*, a highly drought resistant tree of the Indian Desert, was developed; in this study, shoot segments harvested from a "plus" tree of 30-year-olds to produce multiple shoots on MS medium containing 0.1 mg/L IAA and 2.5 mg/L BAP[30]. Axillary buds of *M. aquifolium* were cultured in MS medium, and shoot proliferation induced with 3.0 mg/L BAP was greater than in MS medium with KIN at the same concentration; addition of 0.2 mg/L IAA to the medium contributed to an increase in plantlet height, and rootless plantlets were rooted successfully *in vitro* or *ex vitro*[26]. In *M. ilicifolia*, micropropagated shoots were obtained from axillary buds cultured in MS medium supplemented with 3.0 mg/L BA, and the addition of 0.2 mg/L IAA to the medium increased shoot elongation[27]. In this same species, in the micropropagation of nodal and apical segments, the solution with 0.05% ascorbic acid showed the best regeneration rates and the slowest oxidation rates, and 2.0 mg/L BAP was more effective than KIN with regard to percentage of explants regenerative[15]. Mature plants of *M. canariensis*, an endemic species to the Canary Islands, Spain, were established for apical meristems cultures using branches of two-month-olds collected during the first seven weeks of spring, and the most satisfactory results were obtained using a modified WPM.2 macronutrients medium and MS micronutrients medium supplemented with 0.5 mg/L GA_3, 0.5 mg/L BAP and 0.2 mg/L IAA[23]. In this species, *in vitro* seed germination occurred only after treatment of the seeds with H_2SO_4, and adventitious shoot regeneration was achieved employing axillary or apical buds taken from 2 – 2.5 months old seedlings, following culture on nutrient media supplemented with BAP, KIN and NAA[18]. Likewise, a micropropagation protocol for *M. senegalensis* was established using *in vitro* germinated 6-week-old seedlings as a source of explants; in this study was observed that low levels of BA (0.5 or 1.0 mg/L) were optimal for shoot induction and growth, and generally promoted the average number of shoots produced per explant, while KIN promoted the mean length of shoots[22].

At the present are not known studies in tissue culture of *Maytenus octogona*, therefore the present investigation focused on factors affecting the efficient micropropagation, callus induction for the establishment of cell suspensions, and indirect organogenesis, of this important species of the Peruvian desert.

MATERIALS AND METHODS

Plant material

Seeds of *Maytenus octogona* (L´Hér.) DC. (Celastraceae) were collected from the Dunas La Virgen, Lambayeque, Peru. Botanical identification was performed by Doctor Guillermo E. Delgado Paredes from Universidad Nacional Pedro Ruiz Gallo (UNPRG) based on taxonomic description realized by Macbride[21]. The botanic specimen vouchers were deposited at same herbarium of the institution (HPR).

Establishment of aseptic cultures

Seeds surface were sterilized in a solution of 50% commercial bleach (2.0 – 2.5% active chlorine) for 15 min plus one drop of detergent Tween 20, followed by 1 min immersion in 70% ethanol. The material was rinsed three times with sterile, distilled water in a clear air cabinet, one to three surface-sterilized seeds were placed into test tubes (18x150 mm) containing the nutrients of the MS formulation[24] supplemented with 3% sucrose, 100 mg/L myo-inositol, 1.0 mg/L thiamine.HCl and 8 g/L agar-agar.

Plantlet mass propagation

Prior to the study of *M. octogona* morphogenesis, *in vitro* mass propagation was carried out through axillary bud propagation. First and second axillary buds emerging were excised from germinated plantlets, vertically placed onto the shoot multiplication MS medium (shoots and roots induction) with sucrose, vitamins and several plant growth regulator interactions, and sub-cultured after 60 days of growth. In order to investigate *M. octogona* clonal propagation potential, explants were cultured in a different combinations (0.01, 0.05 and 0.1 mg/L) of the auxin indole-3-acetic acid (IAA) and 0.25, 1.0 and 2.5 of the cytokinin 6-benzylaminopurine (BAP) and 0.01 and 0.02 mg/L IAA with 0.01 and 0.02 mg/L GA_3 (gibberellic acid); likewise, some treatments were supplemented with Plant Prod (PP) fertilizer (N 100%, P_2O_2 52%, K_2O 10%). In the rooting process the explants were cultured in 0.01, 0.1 and 0.5 mg/L IBA (indole-3-butyric acid); in additional experiment, explants were treated with 25 mg/L IBA for 24, 48 and 72 hours prior to cultivating them in culture medium without hormones. In each treatment 15 repetitions for each experimental unit were tested, and the experiments were repeated twice. This strategy permitted both obtaining large quantities and achieving a synchronized growth of the plantlet culture for the study of morphogenetic responses in this species.

Culture conditions

The pH of the medium was adjusted to 5.8 and solidified with 8 g/L agar-agar prior to autoclaving at 121 ^oC for 20 min. The cultures were incubated at 26±2 ^oC under a 16-h photoperiod (30 µmol m^{-2} s^{-1} PFD) using cool white fluorescent tube lights and 70% relative humidity.The materials grown in the dark (seeds germination and callus induction) were cultured in the same growth-room at 26 ^oC.

Morphogenetic potential study

The seeds, zygotic embryos, hypocotyls (~ 1.5 cm), leaves (~ 1.0 cm^2) and roots (~ 1.0 cm) were excised from 60 days old *in vitro* grown plantlets. All explants were horizontally placed onto the culture medium. Leaf sections were placed with the abaxial side in contact with the culture medium. Zygotic embryos were cultured in a basal medium containing different combinations (0.5, 1.0, 2.5 and 5.0 mg/L) of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and the cytokinin kinetin (KIN) 0.5 and 1.0 mg/L, with some treatments supplemented with 25 mg/L cystein and 510 mg/L KH_2PO_4. Seeds were cultured in a basal medium containing different combinations (0.2 and 2.0 mg/L) of the auxins 2,4-D, 1-naphthalene acetic acid (NAA) and IAA and 0.2 mg/L of the cytokinins BAP, KIN and $N^6(\Delta^2$-isopentenyl) adenine (2iP), and leaves, hypocotyls and roots were cultured in a basal medium containing different combinations (2.0 and 5.0 mg/L) of 2,4-D and 0.2 and 0.5 mg/L of KIN. In each treatment 15 repetitions for each experimental unit were tested, and the experiments were repeated twice.

Statistical analysis

For shoot elongation, shoot proliferation, leaves, nodes and roots formation data were analyzed by analysis of variance and the means were compared with Duncan's multiple range test. All the statistical analysis were carried out the Statgraphics Plus 5.0 software (StatPoint; Warrenton, Virginia, USA).Data of callus induction were expressed in percentages.

RESULTS AND DISCUSSION

***In vitro* seed germination**

In agar-gelled MS nutrient medium without any growth regulators, on seed germination radicles emerges in 5 – 7 days and complete expansion of cotyledons between 25 to 30 days. After 15 days, the germination of seeds was 90% in seeds cultivated 10 days after they were collected.

In cultivated seeds, 20 and 30 days after collected the germination rate was 83.3 and 75.7%, respectively (data not showed in table). After 6 months seeds were collected, they showed completely lost their germination capacity. The contamination rate was relatively low with 1 to 5% for bacteria and fungi, respectively, and no shoot hyperhydricity was observed in the *in vitro* cultures.

In *Maytenus canariensis in vitro* seed germination occurred between 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[18].These results were similar to those presented in our study, although it was not necessary to use any method of seeds scarification. In other study, a micropropagation protocol for *M. senegalensis* was established using *in vitro* germinated 6-week-old seedlings as source of explants[22].

Effect of IAA-BAP and IAA-GA_3 on shoot elongation and nodes formation

The effect of different concentration of IAA in combination with BAP or GA_3 was examined on shoot elongation, nodes formation and others morphological traits of first and second nodal segment of seedlings (Table 1). The maximum shoot length (37.0 and 36.7 mm), and the maximum number of nodes formation per explant (6.1 and 6.0) was observed on medium supplemented with 0.01 mg/L IAA – 0.25 mg/L BAP and 0.01 mg/L IAA – 0.01 mg/L GA_3, respectively, outperforming other treatments tested. Raising the concentration of BAP (0.25 to 2.5 mg/L) increased number of shoot, but inhibited shoot elongation, and rooting was observed only in the IAA-GA_3 treatments.

Examination of the effect of nutrient strengths (MS and half-strength MS) supplemented with IAA-BAP and the fertilizer PP, showed that the shoot elongation (22.4 mm) and nodes formation (4.4) was achieved on full-strength MS medium supplemented with 0.02 mg/L IAA, 0.02 mg/L BAP and 2.5 mg/L PP (Table 2).

In a pioneering research in *M. emarginata*, shoot segments were cultured to produce multiple shoots (10-15 shoots/explant) on MS medium containing 0.1 mg/L IAA and 2.5 mg/L BAP, and *in vitro* produced shoots were cut into segments and cultured on shoots proliferation medium but with only 1.0 mg/L of BAP to further multiply the shoots[30]. In *M. aquifolium*, shoot proliferation of axillary buds induced in MS medium supplemented with 3.0 mg/L BAP was greater than in MS medium with KIN at the same concentration; addition of 0.2 mg/L IAA to the medium contributed to an increase in plantlet height[26]. In *M canariensis*, whole shoots of juvenile branches of mature plants were used for *in vitro* regeneration of multiple shoots and best results (3.7 to 4.4) were achieved with WPM.2 in combination with 1.0 mg/L BAP and 0.5 mg/L IAA or 0.5 mg/L NAA[23]. In this same species, adventitious shoot regeneration was achieved employing axillary or apical buds taken from 1 – 2.5 months old plantlets obtained after *in vitro* germination of seeds, following culture on nutrient media supplemented with BAP, KIN and NAA, attaining up to 3.9 shoots per explants, after 4-6 months[18]. Likewise, in *M. senegalensis* the presence of cytokinin BA (0.5 or 1.0 mg/L) was necessary for shoot induction and growth and generally promoted the average number of shoots produced per explant, while KIN promoted the mean length of shoots; however, inclusion of auxin (IAA and IBA) in the shoot induction and growth medium did not have significant effects on the mean length of shoots[22]. These results partially agree with the results shown in this study, especially in relation to the use of low concentrations of BAP but disagree respect to the use of auxins, especially IAA; however, in the study of Pereira et al.[27], micropropagated shoots of *M. ilicifolia* were obtained from axillary buds cultured in MS medium supplemented with BA, and the addition of IAA to the medium increased shoot elongation, although the number of shoots formed was also influenced by degree of juvenility of the explant, and by bud explant position on the stem.

Cytokinins, in general, are known to suppress apical dominance and thus stimulate shoot multiplication[12]; likewise, the beneficial effect of BA over the other cytokinins for shoot multiplication or organogenesis is well documented[34]. The requirement of auxins (NAA or IAA) in low concentrations for shoot multiplication and elongation could be due to its reported role in elimination of phenolic substances by competing for the active sites of auxin oxidase enzyme involved in oxidation of phenols, as has been hypothesized in the adventitious shoot regeneration from *in vitro* cultured leaves of apricot[28] and the micropropagation of *Eucalyptus tereticornis*[1].

Rooting

Examination of the effect of various concentrations of IBA (0.01, 0.1 and 0.5) of rooting efficiency of microshoots, showed that the maximum number of roots per shoots (3.1) was achieved on MS medium supplemented with 0.1 mg/L IBA, although this result was statistically similar to that observed with the treatment 0.01 mg/L IBA; the rooting of maximum number of shoots (100.0 %) was achieved in all treatments (Table 3). In other experiments, shoots produced the maximum number of roots (6.5) when treated with 25.0 mg/L of IBA in full strength MS medium for 24 h in the dark, followed by transfer to gelled, hormone-free full strength MS basal medium; however, the maximum shoot elongation (38.8) was achieved when treated with 25.0 mg/L IBA in full strength MS medium for 48 h in the dark (Table 3). Similar results were reported for *M. emarginata* by Rathore *et al*.[30], following a quite similar protocol. In another study in *M. senegalensis*, rooting was achieved in 2 stages. In the first stage, 8-week-old shoots (2-3 cm long) were pulse-treated in the dark using ½ strength MS liquid medium containing auxins and then transferred to a solid hormone-free medium at a 12/12 h photoperiod; the shoots pulse-treated with 25 mg/L IBA for 120 h produced the highest number of roots per shoot[22]. In others *Maytenus* species as *M. canariensis*, the percentage of rooting was markedly different ($p<0.05$) in WPM.2 and half-strenght WPM.2 (less than 46% plantlets obtained with an average rooting between 1.7 and 2.3), or in quarter-strenght WPM.2 supplemented with 0.3 mg/L IAA and 0.3 mg/L NAA (71% of shoots with roots); this treatment was better because lower mineral content, and is thought to be more suitable for *in vitro* rooting of this species[23]. In addition, also in *M. canariensis* root induction was best on a medium containing 4.0 mg/L NAA, achieving 100% induction[18]. Other species of *Maytenus* where rooting *in vitro* has been successfully reported are *M. aquifolium*[26] and *M. ilicifolia*[6,27].

Callus induction and growth

For establishment of callus cultures, explants including zygotic embryos, seeds, leaves, hypocotyls and roots were inoculated in MS media with different hormone combinations and concentrations. From the five types of explants tested, zygotic embryos (Table 4), seeds (Table 5) and leaves (Table 6) were the most responsive in terms of friable callus induction followed by hypocotyl and root explants (Table 6). In the case of zygotic embryos, seeds, and leaves, the maximum callus induction percentage (100%) was observed on medium supplemented with 5.0 mg/L 2,4-D + 1.0 mg/L KIN +25 mg/L cysteine + KH_2PO_4, 2.0 mg/L 2,4-D + KIN 0.2 mg/L, and 5.0 mg/L 2,4-D + 0.2 mg/L KIN, respectively. Roots induction from callus (10 to 60%) was observed in all explants, except the root explants; the higher concentrations of IAA (2.0 mg/L) showed the maximum callus induction of about 60%, in embryos zygotic explants, followed by NAA/BAP, NAA/KIN and NAA/2iP (40%) in seeds, and 2,4-D/KIN (40%) in leaves.

Callus induction percentage was mainly dependent on the cytokinin (KIN) and auxin (2,4-D) ratio rather than their independent concentrations. When the concentration of KIN/2,4-D was increased significantly from 0.2:2.0 to 1.0:5.0 mg/L the induction percentage was always 100%. In the absence of growth regulators (KIN, BAP, 2iP,2,4-D, NAA or IAA), alone or in various combinations, there was no callus induction. Similar results were observed in the callus culture and *in vitro* biosynthesis of cardiac glycosides from *Calotropis gigantea*[33], and in the plant regeneration in *Holarrhena antidysenterica* from shoot segment- derived callus[29]. Likewise, roots induction and growth of *M. octogona* mostly depended upon the nature of explants as well as the concentration and combination of plant hormones, specifically cytokinin and auxin.

In *Maytenus ilicifolia* callus induction was observed in leaf explants from 8 years old micropropagated plants in MS medium supplemented with 1.0 mg/L KIN and 2,4-D, respectively; in this study cell culture was established in order to produce and to quantify the antitumoral and antioxidant quinonemethide, and 22β-hydroxymaytenin and maytenin[6]; likewise, in this same species, *M. ilicifolia*, callus cultures were initiated from leaves of plants that had been micropropagated *in vitro* over a period of 5 years, and cell suspensions employed in the assays were obtained from callus material that had been subcultured every 45 days over a 10-year period on MS medium supplemented with 3% sucrose, 1.0 mg/L 2,4-D and 0.5 mg/L KIN[7], and in *M. hookeri* the callus was induced from leaves and young stems on MS medium supplemented with 2.0 mg/L 2,4-D and 0.1 mg/L KIN, and the suspension cell cultures were established in SH media with the same formulations[20].

Similar to the reported in the present work, in both species of *Maytenus* was necessary to supplement the culture medium with KIN and 2,4-D.

Callus induction and posteriorly establishment of cell suspension cultures capable of producing medicinal compounds at a rate similar or higher than intact plants have accelerated over the last few years, mostly due to optimization of the cultural conditions, selection of high-producing strains and employment of precursor feeding, transformation methods and immobilization techniques[25,35].

Indirect organogenesis

In our study, the percentage for shoot regeneration from calli is shown in table 7. The highest regeneration percentage was achieved in MS medium supplemented with 1.0 mg/L BAP (50% with 1 – 3 shoots per explant) and 2.0 mg/L 2iP (40% with 1 – 3 shoots per explant). This is the first report on indirect regeneration in a species of the *Maytenus* genus.

Indirect organogenesis is the formation of plant organs from a determined tissue in order to form complete plants, and the process occurs from previously callus in the initial explants[36]; this process is a useful tool for the induction of somaclonal variation because the tissues may be cultured with little differentiation or allows the culture of isolated cells. The genetic variability induced by tissue cultures could be used as a source of variability to obtain new genotypes[16], and consequently a higher production of secondary metabolites.

Table 1: Effect of several hormone interactions on morphogenic responses induction (buds) in apical bud explants of *M. octogona*, after 60 days of culture

Treatments (mg/L)			Shoot elongation (mm)	Number of shoots	Number of leaves per shoot	Number of nodes per shoot	Number of roots per shoot
IAA	**BAP**	**GA_3**					
0.01	0.25		37.0 ± 1.1 a	1.8 ± 1.1 b	5.7 ± 2.3 a	6.1 ± 2.4 a	0 b
0.05	1.0		19.1 ± 1.1 b	1.9 ± 1.3 b	3.2 ± 2.0 b	3.5 ± 1.8 b	0 b
0.1	2.5		18.5 ± 1.2 b	2.8 ± 1.9 a	2.7 ± 1.9 b	3.4 ± 1.8 b	0 b
0.01		0.01	36.7 ± 1.8 a	0 c	5.3 ± 2.9 a	6.0 ± 2.8 a	1.5 ± 1.4 a
0.02		0.02	25.8 ± 1.4 b	0 c	3.0 ± 0.9 b	3.6 ± 0.7 b	1.4 ± 1.6 a

Values with different letters in the same column are significantly different ($P \leq 0.05$; *t* test)

Table 2: Effect of several hormone interactions in the improvementon morphogenic responses induction (buds) in apical bud explants of *M. octogona*, after 60 days of culture

Treatments (mg/L)				Shoot elongation (mm)	Number of nodes per shoot	Number of leaves per shoot
Mineral salts	**IAA**	**BAP**	**PP**			
MS	0.02	0.02		11.4 ± 1.7 b	3.0 ± 3.7 b	2.7 ± 3.3 b
MS	0.02	0.02	2.5	22.4 ±1.0 a	4.4 ± 2.2 a	4.0 ± 2.4 a
MS/2	0.02	0.02		9.8 ± 0.8 bc	2.1 ± 1.7 bc	1.9 ± 1.9 bc
MS/2	0.02	0.02	2.5	12.8 ± 1.1 b	2.1 ± 2.0 bc	1.6 ± 1.9 c

MS, MS full-strength; MS/2, MS half-strength; PP, Plant Product fertilizer

Table 3: Effect of IBA on morphogenic responses induction (roots) in apical bud explants of *M. octogona*, after 60 days of culture

Treatments IBA (mg/L)	Exposure time (hours)	Shoot elongation (mm)	Number of roots per shoots
0.01	0	-	2.7 ± 3.1 d
0.1	0	-	3.1 ± 3.0 d
0.5	0	-	2.1 ± 2.6 de
25.0*	24	30.4 ± 1.3 b	6.5 ± 6.3 a
25.0*	48	38.8 ± 1.9 a	4.4 ± 4.2 c
25.0*	72	22.3 ± 0.9 c	5.0 ± 4.6 b

Values with different letters in the same column are significantly different ($P \leq 0.05$; *t* test)

*Plantlets received pre-treatment with IBA in different exposure times before grown in culture medium without growth regulators.

Table 4: Effect of auxin and cytokinin on callus induction and roots from zygotic embryos of *M. octogona*, after 60 days of culture

Treatments (mg/L)				Callus induction (%)				Roots formation (%)	
2,4-D	**KIN**	**Cist.**	**KH_2PO_4**	**-**	**+**	**++**	**+++**	**-**	**+**
0.5	0.5	-	-	100	-	-	-	-	-
1.0	0.5	-	-	90	10	-	-	-	-
2.5	0.5	-	-	90	10	-	-	-	-
5.0	0.5	-	-	80	10	10	-	-	-
5.0	0.5	-	-	90	10	-	-	90	10
5.0	0.5	-	510.0	80	10	10	-	80	20
5.0	0.5	25.0	510.0	10*	50*	30*	10*	70	30
5.0	0.5	25.0	-		70	30		80	20
5.0	1.0	-	510.0	-	40*	30*	30*	90	10
5.0	1.0	25.0	510.0	-	20*	30*	30*	90	10

*Friable callus

Table 5: Effect of several auxins and cytokinins on callus induction and roots from seeds of *M. octogona*, after 60 days of culture[1,2]

Treatments (mg/L)						Callus induction (%)				Roots formation (%)			
2,4-D	**NAA**	**IAA**	**BAP**	**KIN**	**2iP**	**-**	**+**	**++**	**+++**	**-**	**x**	**xx**	**xxx**
0.2	-	-				70	30	-	-	100	-	-	-
2.0	-	-				40	50	10	-	90	-	10	-
-	0.2	-				100	-	-	-	80	10	-	10
-	2.0	-				90	10	-	-	90	10	-	-
-	-	0.2				100	-	-	-	60	-	20	20
-	-	2.0				90	10	-	-	-	20	20	60
2.0	-	-	0.2	-	-	-	40	40	20	80	20	-	-
2.0	-	-	-	0.2	-	-	30*	30*	40*	80	10	-	10
2.0	-	-	-	-	0.2	-	50	40	10	60	10	20	10
-	2.0	-	0.2	-	-		30	50	20	30	20	10	40
-	2.0	-	-	0.2	-		50	40	10	40	20	-	40
-	2.0	-	-	-	0.2		70	30	-	30	10	20	40
-	-	2.0	0.2	-	-		40	40	20	50	10	10	30
-	-	2.0	-	0.2	-		80	10	10	90	10	-	-
-	-	2.0	-	-	0.2		80	20	-	20	20	20	40

[1]-, without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explant; +++, callus covers the whole explant.
*Friable callus
[2]-, without roots formation; x, 1 – 3 roots formation; xx, 4 – 6 roots formation; xxx, > 6 roots formation.

Table 6: Effect of several auxins and cytokinins on callus induction and roots from leaves, hypocotyls and roots of *M. octogona*, after 60 days of culture[1,2]

Treatments (mg/L)		Leaves				Hypocotyls				Roots			
2,4-D	**KIN**	**Callus**			**Roots**	**Callus**			**Roots**	**Callus**			**Roots**
		+	**++**	**+++**	**xx/ xxx**	**+**	**++**	**+++**	**xx/ xxx**	**+**	**++**	**+++**	**xx/ xxx**
2.0	0.2	50*	40*	10*	40	80*	-	20*	20	100	-	-	-
5.0	0.2	30*	50*	20*	10	80*	10	10*	-	90	-	10	-
5.0	0.5	50*	30*	20*	-	-	-	-	-	-	-	-	-

[1]-, without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explant; +++, callus covers the whole explant.
*Friable callus
[2]-, without roots formation; x, 1 – 3 roots formation; xx, 4 – 6 roots formation; xxx, > 6 roots formation.

Table 7: Effect of cytokinins on indirect organogenesis from hypocotyls of *M. octogona*, after 180 days of culture

Treatments (mg/L)			Shoots per explant (%)	
BAP	**KIN**	**2iP**	**0**	**1 – 3**
0.5			50.0	50.0
1.0			60.0	40.0
2.0			90.0	10.0
	0.5		0.0	0.0
	1.0		0.0	0.0
	2.0		0.0	0.0
		0.5	0.0	0.0
		1.0	0.0	0.0
		2.0	60.0	40.0

MS + vitamins + 510 mg/L KH_2PO_4 + 50 mg/L cysteine+ 3% sucrose

Fig.1:

Maytenus octogona. a) Wild plant with fruits. b) Plantlets with roots formation after 180 days of culture. c) Root induction and d) Plants established to greenhouse conditions after 12 months.

CONCLUSIONS

From this study, the highest percentage of seed germination (90%) was obtained in the treatment with MS medium supplemented with 3% sucrose. The best response of shoot elongation (37.0 and 36.7 mm), and maximum number of nodes formation per explant (6.1 and 6.0) was observed on medium supplemented with 0.01 mg/L IAA – 0.25 mg/L BAP and 0.01 mg/L IAA – 0.01 mg/L GA_3, respectively. Shoots produced the maximum number of roots (6.5) when treated with 25.0 mg/L of IBA in full strength MS medium for 24 h in the dark, followed by transfer to gelled, hormone-free full strength MS basal medium. Profuse friable callus (100%) were induced in zygotic embryos, seeds and leaves on medium supplemented with 5.0 mg/L 2,4-D + 1.0 mg/L KIN + 25 mg/L cysteine + KH_2PO_4, 2.0 mg/L 2,4-D + KIN 0.2 mg/L, and 5.0 mg/L 2,4-D + 0.2 mg/L KIN, respectively, and indirect organogenesis was achieved in MS medium supplemented with 1.0 mg/L BAP. The *in vitro* production of QMTs on a large-scale would open new possibilities for the development of novel pharmaceuticals with several biological activities.

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