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An Efficient Micropropagation System for *Morinda officinalis* How. (Rubiaceae), an Endangered Medicinal Plant

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ABSTRACT

The present work developed an efficient plant regeneration and propagation system *via* direct organogenesis for *Morinda officinalis* How., a valuable endangered medicinal plant. The effects of explant types on shoot induction, plant growth regulators on shoot proliferation and elongation and the subsequent rooting ability of shoots were examined. Among the various types of explant, direct shoot proliferation was successfully achieved from shoot tips and nodal stem segments with around 95% of the explants producing approximately 5 shoots per explant after 8 weeks of culture on optimal medium. On the other hand, leaf and internodal explants did not produce any shoots. The most effective cytokinin on shoot proliferation was 6-benzyladenine. When the concentration of 6-benzyladenine was at 1.0-2.0 mg L^{-1}, a high mean shoot number (about 5 shoots per explant) was achieved. Shoot elongation was obtained satisfactorily by transferring the shoots to Murashige and Skoog basal media containing 2.0-3.0 mg L^{-1} gibberellic acid-3 within 2 weeks. Rooting was 100% on half-strength Murashige and Skoog medium containing 0.2 mg L^{-1} indole-3-butyric acid after 3 weeks of culture. The plantlets were acclimatized in the greenhouse and subsequently transferred to the field with 90% survival rate.

Keywords: Direct regeneration, Explants, Plant growth regulators.

INTRODUCTION

Morinda officinalis How. (Rubiaceae) is one of lianoid shrubs, which grows in Guangdong, Fujian, Guangxi, Hainan and Sichuan provinces in south China. It has been used in traditional Chinese medicine for thousands of years. The root of *M. officinalis* has been traditionally used to treat kidney disorders, impotence, rheumatism, pain, depression and inflammation (Li *et al.*, 2003; Choi *et al.*, 2005; Kim *et al.*, 2005). *M. officinalis* is often an ingredient in herbal sex enhancers (Seo *et al*., 2005). Its strong history and proven effects make it one of the most common herbs in China as well as the Western world.

M. officinalis has been listed in China plant red data book (Ruan, 1991): habitat destruction, over-exploitation for medicinal trade, and low natural regeneration potential resulted in the severe depletion of its natural population. In contrast, recent years have witnessed a rapid growth in the world demand for *M. officinalis* and, as a result, the interest for growing it has been increasing markedly. *M. officinalis* can be propagated by seeds or vine cuttings, however, both propagation methods are inefficient for its commercial production, since the seeds′ moisture content rapidly decreases during the first few days, causing loss of viability, and the planting material has a very low multiplication rate and requires a large area of stock plants. Until now, just a few preliminary Chinese studies have reported on the *in vitro* culture of *M. officinalis* (He and

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Xu, 2002; Chen *et al.*, 2006; Huang *et al.*, 2007) and *in vitro* establishment or regeneration of this species has not been previously reported in English language literature. *In vitro* micropropagation has been proved to be a powerful technique with great potential not only for rapid clonal propagation of plant species but also for the conservation of rare and endangered species (Sarasan *et al.*, 2006; Engelmann, 2011). The aim of this study was to develop an efficient protocol for *in vitro* micro-propagation of *M. officinalis*, which supports cultivation by providing true-to-type plants in large numbers. For this purpose, we aimed to compare shoot induction ability of different explants from *M. officinalis* and to study the effects of plant growth regulators (PGRs) on shoot proliferation, elongation, and rooting.

MATERIALS AND METHODES

Culture Initiation

The young healthy and profusely growing vine of *M. officinalis* was collected from Deqing, Guangdong Province, China, and used as the source of explants. The species was identified by expert consultation. A voucher specimen was deposited in the herbarium of Guangzhou University of Chinese Medicine, under accession number GUCM 010047. Shoot tips and nodal explants with a single axillary bud were washed under running tap water for 30 min and then surface-sterilized with 70% (*v/v*) ethanol for 30 s, followed by 0.1% (*w/v*) mercuric chloride ($HgCl_2$) solution for 10 min and, finally, rinsed four or five times with sterile double-distilled water. The surface sterilized explants were cut into 1-1.5 cm length pieces containing a single node with an axillary bud or a shoot tip with an apical bud. Subsequently, the explants were inoculated vertically on aseptic nutrient media. The new shoots induced from the *in vitro* cultures were further used as explants for adventitious shoot proliferation.

In Vitro Culture Media and Conditions

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) was used for shoot induction and elongation, and half strength MS basal medium was used for *in vitro* rooting. All media were supplemented with 3.0% (*w/v*) sucrose and 0.7% (*w/v*) agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. The cultures were incubated for a 16/8 h (light/dark) photoperiod at 25±2 °C under a fluorescent light.

Shoot Induction

For the comparison of shoot induction ability of different explants, the shoot tips, nodal and internodal stem segments (1 cm), and leaf segments were excised from the previously micropropagated shoots bearing 3-4 internodes and were inoculated on MS basal media supplemented with 1.0 mg L^{-1} 6-benzyladenine (BA). To investigate the effects of different PGRs on shoot proliferation, nodal stem segments were inoculated on MS basal media supplemented with different concentrations (0.0, 0.5, 1.0, 2.0, and 4.0 mg L^{-1}) of cytokinins BA, kinetin (Kin) and thidiazuron (TDZ). All of the cultures were subcultured onto the fresh medium with the same composition at two weeks intervals. The percentage of shoot proliferation, number of shoots per explant, and mean shoot length were recorded every week for eight weeks.

Shoot Elongation

The isolated shoots with an average height of 1 cm were transferred to MS medium supplemented with gibberellic acid-3 (GA_3) at different concentrations (0.0, 1.0, 2.0, 3.0, and 4.0 mg L^{-1}). The average height of shoots elongated of microshoots was recorded after two weeks of culture.

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Rooting and Acclimatization

The elongated shoots were excised aseptically and implanted on half-strength MS medium supplied with auxins α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) at different concentrations (0.0, 0.2, 0.5, and 1.0 mg L^{-1}). The rooting percentage, mean root number and mean root length were recorded after three weeks of culture.

Regenerated plants with well-developed roots were taken out from the culture medium, washed gently under running tap water to remove agar gel adhered to the roots, and then transferred to plastic pots containing sterile sand and garden soil for hardening. The plantlets were kept in a transparent polythene membrane at 80% relative humidity, 25±2 °C under a 16/8 h (light/dark) photoperiod. After acclimatization, the hardened plantlets were then transferred to black polybags in the nursery under normal day length conditions. The survival rates of plants were recorded after this period.

Data Collection and Analysis

The complete randomized design was used for all experiments. There were three replicates in each treatment, and each experiment was repeated three times. Data were analyzed using the SPSS software (version 17). Significant differences were assessed by using Duncan's Multiple Range Test at the 5% probability level. All the data were expressed as mean ± standard error (SE).

RESULTS

Effect of Different Explants on Shoot Proliferation

Little buds sprouted from some of the shoot tip and nodal stem explants without intervening callus formation within one week of culture, while leaf and internodal stem explants did not respond even after eight weeks of incubation (data not shown). More than 95% of shoot tip and nodal stem explants produced new axillary shoots after four weeks of culture. With increasing culture time, more shoots per explant appeared to be produced (approximately 5 shoots after 8 weeks of culture) (Table 1, Figure 1*a-b*). There were no significant differences in proliferation frequency and number of shoots per explant between shoot tip and nodal stem explants, however, the length of shoots induced from nodal explants was greater than those formed on shoot tip explants.

Effect of Different Cytokinins on Shoot Proliferation

The morphogenetic responses of nodal stem explants to BA, Kin and TDZ are summarized in Table 2. An average of 1-2 shoots per explants was obtained from 86.11% of explants when cultured on MS medium without PGRs. BA was more efficient than other cytokinins with respect to initiation and subsequent proliferation of shoots. More than 93% of cultured explants could form multiple shoots at all the BA concentrations tested. With increasing concentration of BA, more shoots were produced, but shoot growth was reduced. In general, when the concentration of BA was at 1.0-2.0 mg L^{-1}, a high number and longer lengths of shoots (approximately 5 shoots per explant and about 1 cm in length) were achieved after 8 weeks of culture. Kin instead of BA did not cause a significant increase in shoot proliferation response, but led to corresponding decrease in the average shoot number (fewer than 3 shoots per explant). TDZ has been shown to have negative effects on shoot proliferation from nodal stem explants. The proliferation rate and final shoot scale

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Table 1. Shoot proliferation from shoot tip or nodal stem explants of *M. officinalis* cultured on MS medium containing 1.0 mg L^{-1} BA. [a]

Explant	Shoot proliferation frequency (%)			Average no. of shoots/explant			Average length of shoots (cm)		
	After 4 wk	After 6 wk	After 8 wk	After 4 wk	After 6 wk	After 8 wk	After 4 wk	After 6 wk	After 8 wk
Shoot tip	98.33±1.67a	100.00±0.00a	100.00±0.00a	2.34±0.10a	3.20±0.20a	4.74±0.23a	0.52±0.02b	0.68±0.02b	0.84±0.05b
Nodal stem segment	96.67±1.67a	96.67±1.67a	96.67±1.67a	2.43±0.06a	3.17±0.35a	4.80±0.26a	0.59±0.03a	0.83±0.03a	1.08±0.01a

[a] Values represent the mean ± SE. Means within each column followed by the same letter are not significantly different at P<0.05 according to Duncan's Multiple Range Test.

Table 2. Effect of different cytokinins on direct shoot proliferation from nodal stem explants of *M. officinalis.*[a]

Cytokinins (mg L^{-1})			Shoot proliferation frequency (%)			Average no. of shoots/explant			Average length of shoots (cm)		
BA	Kin	TDZ	After 4 wk	After 6 wk	After 8 wk	After 4 wk	After 6 wk	After 8 wk	After 4 wk	After 6 wk	After 8 wk
0.0	0.0	0.0	86.11±0.96b	86.11±0.96b	86.11±0.96b	1.80±0.03ef	1.84±0.05de	1.84±0.05f	0.58±0.01b	0.87±0.02a	1.28±0.08a
0.5	-	-	93.89±0.96a	93.89±0.96a	93.89±0.96a	2.36±0.07b	2.82±0.09b	3.26±0.05c	0.62±0.02a	0.79±0.03b	1.09±0.04b
1.0	-	-	93.96±2.91a	93.96±2.91a	93.96±2.91a	2.73±0.18a	3.57±0.09a	4.88±0.32b	0.58±0.02b	0.76±0.04bc	1.04±0.08bc
2.0	-	-	96.11±3.47a	96.11±3.47a	96.11±3.47a	2.35±0.05b	3.68±0.21a	5.09±0.17b	0.40±0.02e	0.70±0.04d	0.92±0.04cde
4.0	-	-	96.44±3.36a	96.44±3.36a	96.44±3.36a	2.58±0.20a	3.79±0.21a	5.71±0.26a	0.36±0.02f	0.49±0.04f	0.81±0.02ef
-	0.5	-	96.11±3.47a	96.11±3.47a	96.11±3.47a	1.98±0.04de	2.11±0.19d	2.24±0.13e	0.56±0.03b	0.74±0.04bcd	1.03±0.07bc
-	1.0	-	98.33±2.89a	98.33±2.89a	98.33±2.89a	1.79±0.06ef	2.03±0.23d	2.22±0.12e	0.57±0.02b	0.74±0.03bcd	0.98 ±0.09bcd
-	2.0	-	84.45±3.85b	84.45±3.85b	84.45±3.85b	2.10±0.17cd	2.44±0.22c	2.53±0.13d	0.56±0.02b	0.72±0.04cd	1.08±0.10b
-	4.0	-	73.33±6.67c	73.33±6.67c	73.33±6.67c	2.21±0.18bc	2.56±0.20bc	2.62±0.13d	0.45±0.04d	0.57±0.02e	0.74±0.06fg
-	-	0.5	56.59±5.00d	56.59±5.00d	56.59±5.00d	1.51±0.14g	1.51±0.14f	1.51±0.14g	0.52±0.03c	0.60±0.04e	0.87±0.11de
-	-	1.0	46.20±1.59e	46.20±1.59e	46.20±1.59e	1.50±0.07g	1.50±0.07f	1.50±0.07g	0.32±0.03g	0.51±0.03e	0.62±0.03g
-	-	2.0	43.75±1.25e	43.75±1.25e	43.75±1.25e	1.68±0.17fg	1.68±0.17ef	1.68±0.17fg	0.23±0.03h	0.40±0.04g	0.42±0.05h
-	-	4.0	42.22±2.55e	42.22±2.55e	42.22±2.55e	1.87±0.02ef	1.87±0.02de	1.87±0.02f	0.23±0.02h	0.37±0.03g	0.40±0.05h

[a] Values represent the mean ± SE. Means within each column followed by the same letter are not significantly different at P<0.05 according to Duncan's Multiple Range Test.

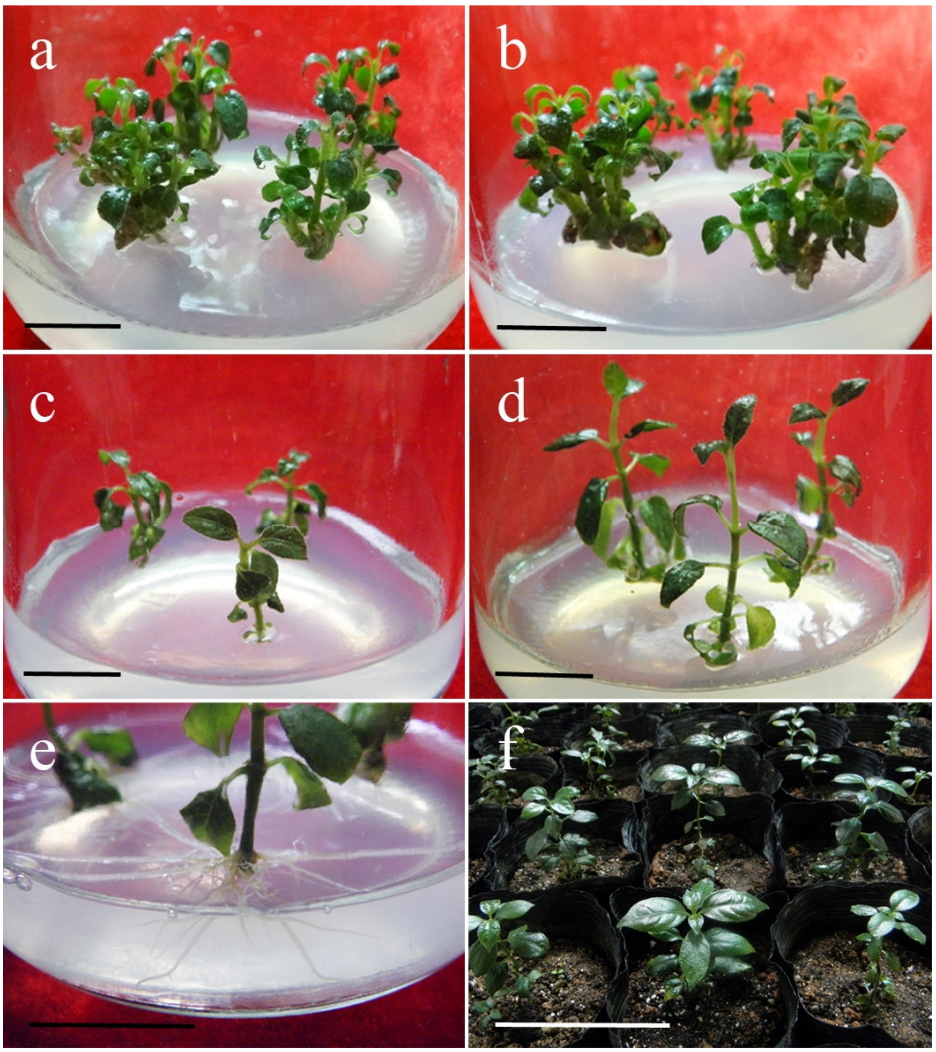

Figure 1. Shoot induction and plantlet regeneration in *M. officinalis* (a) Regenerated shoots from shoot tip after 8 weeks of culture on MS medium containing 1.0 mg L^{-1} BA. *Scale bar* = 1 cm. (b) Regenerated shoots from nodal stem segment after 8 weeks of culture on MS medium containing 1.0 mg L^{-1} BA. *Scale bar* = 1 cm. (c) Elongation of shoots after 2 weeks of subculture on MS medium. *Scale bar* = 1 cm. (d) Elongation of shoots after 2 weeks of subculture on MS medium containing 3.0 mg L^{-1} GA_3. *Scale bar* = 1 cm. (e) Rooting of the elongated shoots after 5 weeks of culture on half-strength MS medium containing 0.2 mg L^{-1} IBA. *Scale bar* = 1 cm. (f) Acclimatized plants of *M. officinalis* after 6 weeks. *Scale bar* = 10 cm.

and their size were significantly less than those of the control.

Effect of GA_3 on Induced Shoot Elongation

Although the shoot induction from nodal stem segments was achieved in all media, the proliferated shoots grew slowly and most of them were small, reaching around 1 cm in length after 8 weeks of culture (Table 2). Figure 2 shows the height of shoots elongated after 2 weeks of culture. In the absence of GA_3, height of elongated shoots only reached 0.36 cm (Figure 1*c*). Supplying 2.0-3.0 mg L^{-1} GA_3, height of elongated shoots could reach around 1.5 cm, and the elongated shoots appeared to be normal and healthy (Figure 1*d*), however,

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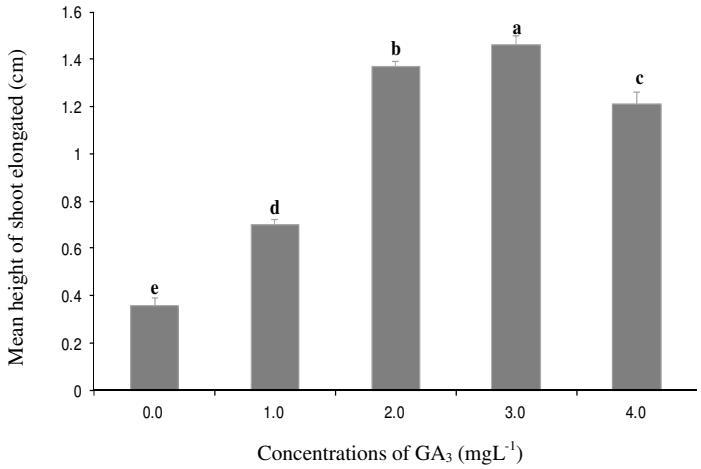

Figure 2. Effect of different concentrations of GA_3 on shoot elongation from induced shoots of *M. officinalis* cultured on MS medium. Means with *different letters* are significantly different at P<0.05 according to Duncan's Multiple Range Test. *Vertical bars* represent the standard error. The data were recorded after 2 weeks of culture.

GA_3 concentration up to 4.0 mg L^{-1} resulted in reduction in shoot elongation. Hence, GA_3 induced a marked elongation in the length of shoots.

Rooting and Acclimatization

In the medium without auxins, the rooting percentage was 70.18% (Table 3), while, among the three auxins used, IBA caused more positive response on rooting as compared to NAA and IAA. Especially in the case of IBA at 0.2 mg L^{-1}, 100% of shoots produced roots without the formation of a distinct intermediate callus (Figure 1*e*). NAA and IAA have been shown to have negative effects on root induction of shoots. In the presence of NAA or IAA, the rooting percentage and root length were significantly less than those of the control.

Plantlets with six to eight fully expanded leaves and well-developed roots were transferred to pots containing sterile sand

Table 3. Effect of different auxins at various concentrations on *in vitro* root induction of *M. officinalis.* [a]

Auxins (mg L^{-1})			Rooting Percentage (%)	Average no. of roots/plantlet	Average root length (cm)
NAA	IAA	IBA			
0.0	0.0	0.0	70.18±3.84b	4.92±0.22ab	0.41±0.02a
0.2	-	-	27.78±4.01e	3.93±0.81ab	0.17±0.04fg
0.5	-	-	45.83±5.25cd	4.83±0.30ab	0.35±0.01abc
1.0	-	-	7.87±3.96f	3.33±2.03b	0.13±0.07g
-	0.2	-	51.51±2.69c	5.05±0.15ab	0.31±0.01bcd
-	0.5	-	52.31±5.34c	4.58±0.30ab	0.22±0.01ef
-	1.0	-	36.01±3.37de	3.09±0.21b	0.26±0.01de
-	-	0.2	100.00±0.00a	5.80±0.32a	0.32±0.01bcd
-	-	0.5	74.26±2.28b	5.97±0.37a	0.37±0.01ab
-	-	1.0	37.82±2.43de	4.00±0.57ab	0.27±0.03cde

[a] Values represent the mean ± SE. Means within each column followed by the same letter are not significantly different at P<0.05 according to Duncan's Multiple Range Test. Data were recorded after three weeks of culture.

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and garden soil for hardening. The hardened plantlets were then transferred to black polybags in the nursery (Figure 1*f*). A survival rate of 90% was achieved from the rooted plantlets after 6 weeks of acclimatization. The regenerated plants did not show any visible variation in morphological or growth characteristics when compared with the donor plants.

DISCUSSION

The successful micro-propagation in plant tissue culture is based on an appropriate choice of explant. Explants containing axillary and apical buds have quiescent or active meristems depending upon the physiological stage of the plant. These buds have the potential to develop into complete plantlets, however, only limited numbers of axillary meristems have the capacity to develop into branches *in vivo*. Using tissue culture, the rate of shoot multiplication can be greatly enhanced by performing axillary bud culture in a nutrient medium containing suitable PGRs (Faisal *et al.*, 2007). Due to continuous availability of cytokinin, buds already present in the initial explant (shoot tip or nodal stem segment) develop into axillary buds, which may grow directly into shoots (Ramadevi *et al.*, 2012). In this study, among the tested explants from *M. officinalis*, only shoot tips and nodal stem segments could produce shoots with maximum 5 shoots per explant. Shoots proliferated from nodal stem segments were longer than those from shoot tips. This might be due to shoot tips exerting strong apical dominance which inhibit shoot development (Lakshmanan *et al.,* 1997). Similar result has also been reported in *Mentha viridis* L. (Raja and Arockiasamy, 2008).

As a promoting cytokinin, BA is most commonly used in plant micropropagation work (Bairu *et al.*, 2007; Sharafi *et al.*, 2014). Some reasons for superiority of BA may be attributed to its being more readily metabolized than other synthetic PGRs in plant tissues or to the ability of BA to induce production of natural hormones such as zeatin within the tissue (Zaerr and Mapes, 1982). Among the cytokinins tested in this study, BA was the most effective. On MS medium without PGRs, only 1-2 shoots per explant were obtained from 86.11% of explants. The addition of BA greatly promoted multiple shoot formation. Shoot number was increased to about 5 shoots per explant. Kin was less effective than BA, only 2-3 shoots per explant were obtained. TDZ was proved to show negative effect on shoot proliferation. Similar results have been reported in other plant species such as *Sarcostemma brevistigma* Wight and Arnott (Thomas and Shankar, 2009) and *Withania somnifera* (L.) Dunal (Nayak *et al.*, 2013).

Stems of micro-propagated shoots often fail to elongate. This is especially the case when the explants are taken from mature trees (Bonga and von Aderkas, 1992). On the other hand, continuous culture in the medium which contained cytokinins used for adventitious budding also caused stunted growth of the induced shoots (Hajong *et al.*, 2013). In this study, both shoot tips and nodal stem segments were taken from mature vines of *M. officinalis*. The induced shoots from the explants were dwarfed with short internodes. In the presence of cytokinins, the shoots were even shorter. With increasing concentration of cytokinins, the shoot growth was further inhibited. Similar observations were recorded by other investigators (Tefera and Wannakrairoj, 2006; Kumar *et al.*, 2013). Stem elongation is often a prerequisite for rooting of the shoots. GA_3 has been used in shoot elongation in many species, such as *Cenchrus ciliaris* L. (Kumar and Bhat, 2012), *Clitoria ternatea* L. (Singh and Tiwari, 2012), and *Artemisia sieberi* Besser (Sharafi *et al.*, 2014). It might be

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due to GA_3 promoting stem elongation by increasing cell division and elongation (Gaspar *et al.*, 1996). In this experiment, addition of GA_3 (2.0-3.0 mg L^{-1}) to the medium also induced a marked shoot elongation (around 1.5 cm) after 2 weeks of culture.

Auxins play an important role in root development. Among the three auxins used, IBA showed more positive response on rooting of *in vitro* shoots of *M. officinalis* as compared to NAA and IAA. Especially in the case of IBA at 0.2 mg L^{-1}, 100% of shoots produced roots. IBA resulted in similar rooting success in many species, such as *Achillea millefolium* L. (Shatnawi, 2013), *Jatropha curcas* L. (Khemkladngoen *et al.*, 2011), and *Pterocarpus santalinus* L. (Balaraju *et al.*, 2011).

In summary, we have established an *in vitro* propagation system for the valuable, but endangered, medicinal species *M. officinalis*. This developed protocol would be useful for large-scale multiplication and production of true-to-type plants without harming wild populations. This protocol will be also useful for *in vitro* conservation or cryopreservation of this valuable genetic resource. In addition, the system developed in this investigation for this important plant could be a useful tool for the genetic modification through mutagenesis or genetic transformation.

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سامانه ای کارآمد برای ریزتکثیری (ریزافزایی) یک گیاه دارویی در خطر انقراض *Morinda officinalis* How. (Rubiaceae)

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چکیده

این پژوهش برای ایجاد سامانه ای کارآمد برای باززایی و تکثیر از راه اندام زایی مستقیم یک گیاه دارویی ارزشمند و در خطر انقراض به نام *Morinda officinalis* How. (Rubiaceae), انجام شد. تاثیر نوع ریز نمونه ها روی انگیزش ساقه دهی و اثر تنظیم کننده های رشد گیاه روی پُرآوری و توسعه و دراز شدن ساقه ها و درنتیجه روی توان ریشه زایی بررسی شد. در میان انواع ریز نمونه ها، پُرآوری مستقیم ساقه از نوک ساقه ها و بخش های گرهی ساقه به گونه ای موفق آمیز انجام شد و نزدیک به ۹۵٪ ریز نمونه ها ۸ هفته بعد از کشت روی بستر مناسب هرکدام تقریبا ۵ ساقه ایجاد کردند. بر خلاف این ها، از ریز نمونه های تهیه شده از برگ ها و قسمت های بین گرهی هیچگونه ساقه ای رشد نکرد. موثرترین سایتوکاینین روی پُرآوری ساقه، بنزیل آدنین-۶ بود.هنگامی که غلظت این ماده ۲-۱ میلی گرم در لیتر بود میانگین بالایی از تعداد ساقه (حدود ۵ ساقه در هر ریز نمونه) به دست آمد.با انتقال ساقه ها به بستر پایهMurashige وSkoog که حاوی ۳-۲ میلی گرم در لیتر جیبرالیک اسید-۳ بود، طی دوهفته ساقه ها رشد طولی رضایت بخشی نشان دادند. ریشه زایی روی محیطی که نیم-قدرت Murashige وSkoog بود و ۰/۲ میلی گرم در لیتر ایندول-۳-بوتیریک اسید داشت، سه هفته بعد از کشت در حد ۱۰۰٪ بود. گیاهچه ها بعد از سازگاری در گلخانه به مزرعه منتقل شدند و در آنجا نرخ بقای آنها ۹۰٪ بود.

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