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Full Length Research Paper

Effects of growth regulators 6-Benzylaminopurine and 2-Naphtalene Acetic Acid on the *in vitro* shoot multiplication from nodal segment of *Medinilla mandrakensis* (Melastomataceae)

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Medinilla mandraken **is a threatened epiphyte forest endemic of Madagascar. Micro propagation regeneration is an appropriate way for propagation and conservation of this species. The study aims to find the best combination of growth regulators for the rapid proliferation *in vitro* of shoots by nodal segment. Micro cuttings was from aseptic germination of seeds grown on Murashige and Skoog in half strength medium (MS/2), supplemented with 15% of green coconut water. The MS/2 added 0, 0.5, 1, or 2mg/L of 6-Benzylaminopurine (BAP), alone or in combination with 0.1 or 0.5 mg/L of 2-Naphtalene Acetic Acid (NAA), were tested for its multiplication. The effects of growth regulators were evaluated in function of means number of regenerated shoots, their size, callogenesis, and rooting rate. The best shoot growth was obtained in the medium growth regulators free (13.3 mm in 45 days). Culture Medium added of 2 mg/L of BAP promotes maximum proliferation of shoots (27.5 shoots per explants in 45 days). The combined use of BAP and NAA regardless of their concentrations reduced the production of shoots and improved the cals formation. Rooting was noted in the medium growth regulator free.**

Key words: Growth regulators, *Medinilla mandrakensis,* regeneration, micropropagation, microcutting.

INTRODUCTION

Madagascar is recognized not only by the richness of its biodiversity but also by the high endemicity of its fauna and flora. Madagascar Island sheltered the 5% of plant and animal species on the planet (Ravoahangy, 2011). The decrease of forest area could lead to the extinction of many endemic species of the island. According to Myers et al. (2000), Madagascar has already lost 90% of its primary forests. Because of the rapid demographic growth, the anthropogenic pressures are the main causes of the destruction of the natural resources of Madagascar.

A mine project of large-scale in the deposits of Ambatovy and Analamay is located in the East-Central part of Madagascar. The project concerned the exploitation of nickel and cobalt under forest cover (Dickinson, 2010). Among the programs of biodiversity management of this project, the threatened plant species or species of concern (SOC) are subject to a special *ex situ* preservation through biotechnology *in vitro* culture. In the Ambatovy Mining Area, *Medinilla mandrakensis* belongs to the family Melastomataceae and ranked among the threatened species or "SOC". It is an epiphytic plant forest growing on trees around 1200 to 1400 m and endemic to Madagascar. Moreover, like all other epiphytes, *Medinilla mandrakensis* is not immune to threats of habitat loss caused by the deforestation in Madagascar.

A susceptible alternative to implement the conservation and the multiplication of the species *Medinilla mandrakensis* proves indispensable. Biotechnology of the micro propagation has been undertaken because it offers opportunities more advantageous than conventional propagation methods. In fact, it allows, on the basis of a

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Table 1. Different types of culture media in BAP (6-benzylaminopurine) and NAA (2-Naphthalene Acid Acetic).

CULTURE MEDIA		M0 (control)	M1	M2	M3	M4	M5	M6	M7
Growth regulator (mg/L)	BAP	0	0.5	1	2	0.5	1	2	0.5
	NAA	0	0	0	0	0.1	0.1	0.1	0.5

BAP : 6-benzylaminopurine
NAA : 2-Naphthalene Acid Acetic

single individual (plant), to obtain a lot of plants that are genetically identical (clone) to the mother plant, with a rate of proliferation which could reach 100 to 1000 times higher (http://1, Ferry et al., 1998 ; Semal, 1998).

To our knowledge, no study has been performed on the *in vitro* culture of gender *Medinilla*. This study aims to establish a protocol for the rapid *in vitro* shoot proliferation of the species, to contribute to its preservation and multiplication.

MATERIALS AND METHODS

Plant material

The explants used were the micro cuttings (nodal segment) from *in vitro* seed germination of *Medinilla mandrakensi* (Melastomataceae).The seeds were from ripe fruits which were harvested in the dense humid forest of Ambatovy in Madagascar. This area is geographically bounded by the coordinates 18 ° 49 '0.12'' south latitude and 48 ° 18' 00'' of longitude East. Micro cuttings with a node, 5mm of length, without leaf and 45 days old, were from the middle portion of the seedlings.

Media and culture conditions

Germination

After washing with tap water, fruits were sterilized by immersion in a solution of NaOCl 20% (w/v) for 20, 25 and 30 minutes, then by soaking in ethanol 70° for 1 minute and in a solution of fungicide 6% (w/v) (mancozeb) for 60 minutes.

The culture medium used for *in vitro* germination medium was Murashige and Skoog (1962) in half strength (MS/2), growth regulators free but supplemented with green coconut water to 15% (v/v).

Multiplication

The basic medium used was Murashige and Skoog (1962) in half strength (MS/2) supplemented with vitamins and amino acid (0.1 mg/L thiamine, 0.5 mg/L pyridoxine, 2 mg/L glycine, 0.5mg/L nicotinic acid and 100 mg/L myo-inositol) and 3% (w/v) of sucrose. The compositions of culture media, with phytohormones BAP and NAA, are summarized in (Table 1.) Notice that each treatment is repeated 8 times.

These media are solidified with 0.8% (w/v) agar. The pH of the culture medium is adjusted to 5.5 ± 0.1. The culture media were sterilized by autoclaving at 121°C for 20min. Seedlings of 45 days old from in vitro germination were cut into small cuttings 5mm in length and have a node. Eight (8) explants per treatment were grown. They are planted vertically, basal pole in the culture medium. The cultures were incubated in a culture room at a temperature of 25°C under a light intensity of 3000lux and a photoperiod of 16h (light) / 8h (darkness). The multiplication rate, the average size of shoots produced, the rate of callus formation, the rate of explants producing shoots vitrified were considered to evaluate the effects of growth regulators on the proliferation of shoots.

Expressions of results

For data analysis, analysis of variance (ANOVA) and comparison of means were performed using the software "STAT-ITCF" Version 4. The separation of homogeneous groups observed between several medium is made following the test-NEWMAN- KEULS (probability threshold of 5%). To calculate percentage, means were obtained after angular transformation, and then treated with software STAT-ITCF.

RESULTS

Germination

No culture was contaminated whatever the duration of soaking fruits (20, 25 and 30 minutes) in the solution of sodium hypochlorite (NaOCl), 20% (w/v). Besides, whatever the duration of soaking fruit in the solution of

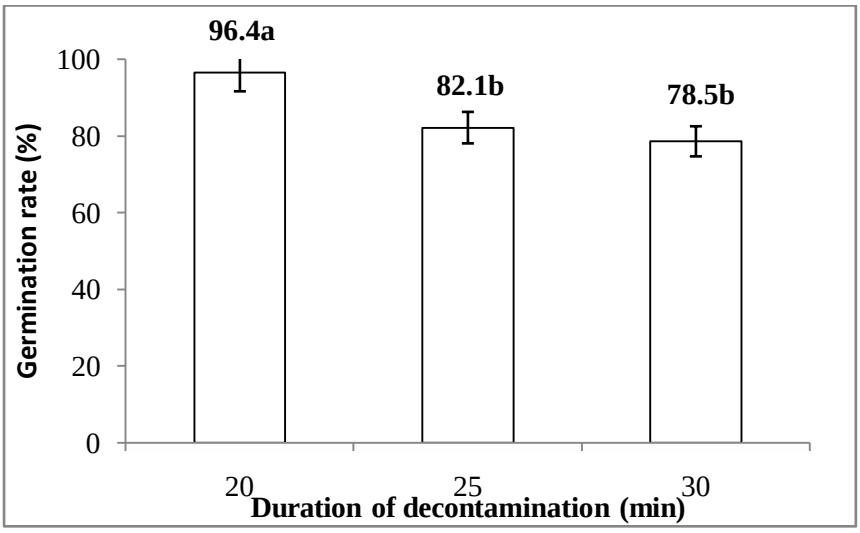

Figure 1. Effects of soaking time in NaOCl solution (20%) of *Medinilla mandrakensis* fruit on the germination rate after 45 days of culture.

Table 2. Influences of BAP and NAA on the number and size of shoots from microcuttings of *Medinilla mandrakensis* (Melastomataceae) produced after 45 days of culture.

Medium type	Number of shoots/explants	Size of shoot (mm)
M0	**2 ± 0,0 f**	**13.3 ± 0.6 a**
M1	**15.3 ± 0.9 c**	**4.9 ± 0.6 f**
M2	**21 ± 0.7 b**	**4.1 ± 0.5 g**
M3	**27.5 ± 0.7 a**	**4 ± 0.4 g**
M4	**13.2 ± 0.4 d**	**9.1 ± 0.3 c**
M5	**15.5 ± 0.5 c**	**7.7 ± 0.6 d**
M6	**16 ± 1.4c**	**6.8 ± 0.5 e**
M7	**9.2 ± 0.7 e**	**10.6 ± 0.5 b**

Legends: M0 (control): BAP/NAA (0/0); M1: BAP (0.5 mg/l); M2: BAP (1mg/l); M3: BAP (2mg/l) M4: BAP (0.5 mg/l) + NAA (0.1mg/l); M5: BAP (1mg/l) + NAA (0.1mg/l); M6: BAP (2mg/l) + NAA (0.1mg/l), M7: BAP (0.5mg/l) + NAA (0.5mg/l). For each column, the values represent the mean ± standard deviation. Values followed by the same letter are not significantly different according to the Newman-Keuls test.

sodium hypochlorite 20% (w/v), the seeds began to sprout from the fourth week. Figure 1 summarizes the changes in the rate of seed germination of *Medinilla mandrakensis* depending on the duration of soaking fruits in NaOCl. The highest germination rate (96.4%) was obtained from seeds from fruits soaked for 20 minutes in the NaOCl solution 20% (w/v).

However, for soaking periods of 25 minutes and 30 minutes, germination rates decrease respectively 82.1% and 78.5%.

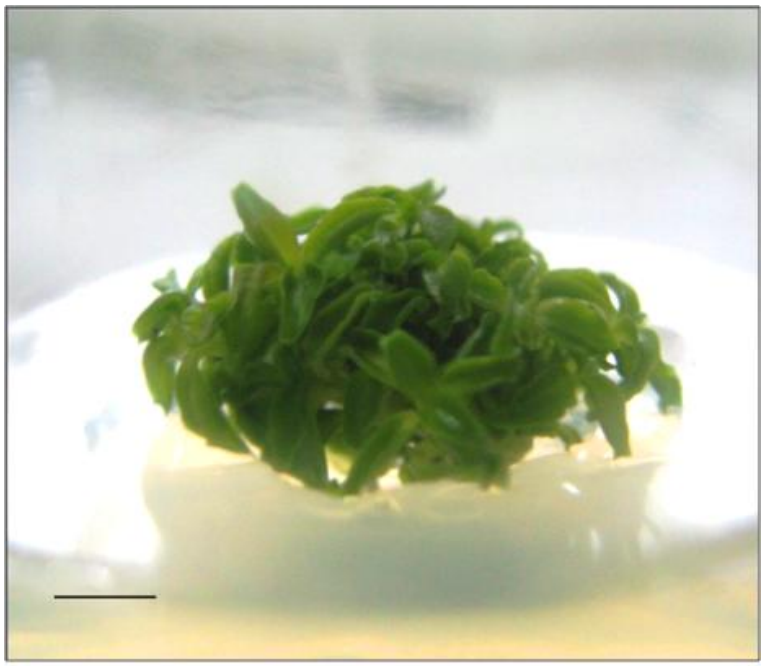

Figure 2. Shoots from a microcutting of *Medinilla mandrakensis* (Melastomataceae) produced on MS/2 medium supplemented with 1mg/L BAP (M2) after 45 days [bar = 5mm].

Values followed by the same letter are not significantly different according to the Newman-Keuls test.

Multiplication

After 8 days of culture, 100% of micro cuttings developed in regardless of the type and concentration of growth regulators studied.

Influences of phytohormones on the number and size of shoots produced. The effects of different concentrations of BAP and NAA on the number and size of shoots produced per explant of *Medinilla mandrakensi*s (Melastomataceae) are summarized in Table 2.

Influences of BAP

Analysis of variance revealed highly significant effects ($P < 0.01$) of the BAP on the number of shoots obtained. The addition of BAP has improved the number of shoots produced per explant. Each explant produced an average of more than 15 shoots per micro cuttings in the medium with BAP (Figure 2), against only two shoots on the BAP free medium or control medium (M0). Besides, the number of shoots increases with the concentration of BAP. Thus, the BAP promotes the growth of shoots. The maximum rate of increase of 27.5 shoots per explant was achieved on medium supplemented with 2mg /L of BAP (M3).

Conversely, the addition of BAP in the culture medium further reduced the size of the newly formed shoots. In the control medium (M0), the average shoot length was 13.3mm. Where as in the presence of 2 mg/L BAP (M3), they were on average 4 mm, so there was a reduction of 70%.

Influences of NAA

From table 2, treatment with combined BAP and NAA improved the production of at least 9 shoots per explant compared to the control medium that provides only two shoots per explant.

Compared to the effect of BAP alone, the addition of NAA in the culture medium resulted in a reduction of number of newly formed shoots. The main reduction (9 shoots per explant) was obtained with the medium containing 0.5 mg/L of NAA and 0.5mg/L of BAP (M7).

As for the size of the shoots, they have a length greater than 5mm in all media supplemented with NAA and BAP (M4, M5, M6, and M7). NAA promotes shoot elongation contrary to media containing only BAP on which the maximum mean shoot length is 4.9mm. During this stage of multiplication, roots and callus were observed.

Influences of NAA and BAP on rooting

Rooting was found only in growth regulators free medium (M0) with a high rate of 100% (Figure 3). However, the roots

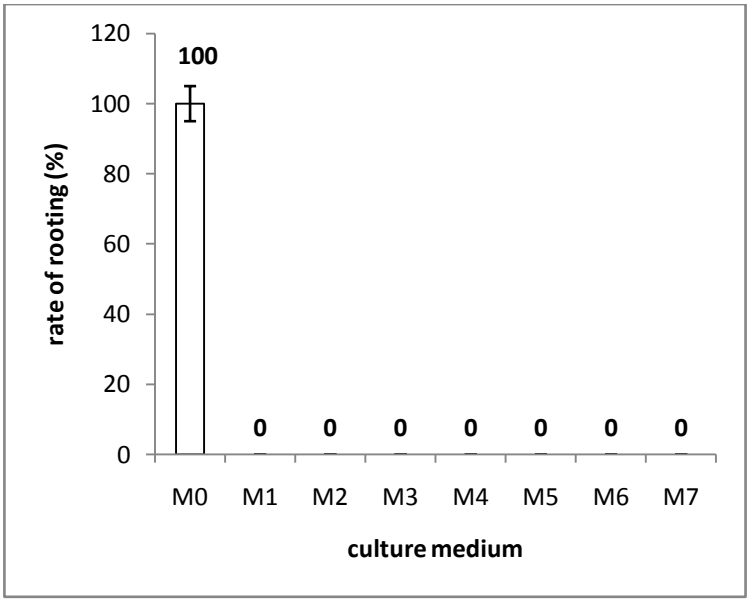

Figure 3. Influence of NAA and BAP on the rooting rate shoots of *Medinilla mandrakensis (Melastomataceae).*
Legends: M0 (control): BAP/NAA (0/0); M1: BAP (0.5 mg/L) ; M2: BAP (1mg/L) ; M3: BAP (2mg/L) M4: BAP (0.5 mg/L) + NAA (0.1mg/L) ; M5: BAP (1mg/L) + NAA (0.1mg/L) ; M6: BAP (2mg/L) + NAA (0.1mg/L), M7:BAP(0.5mg/L) + NAA (0.5mg/L).

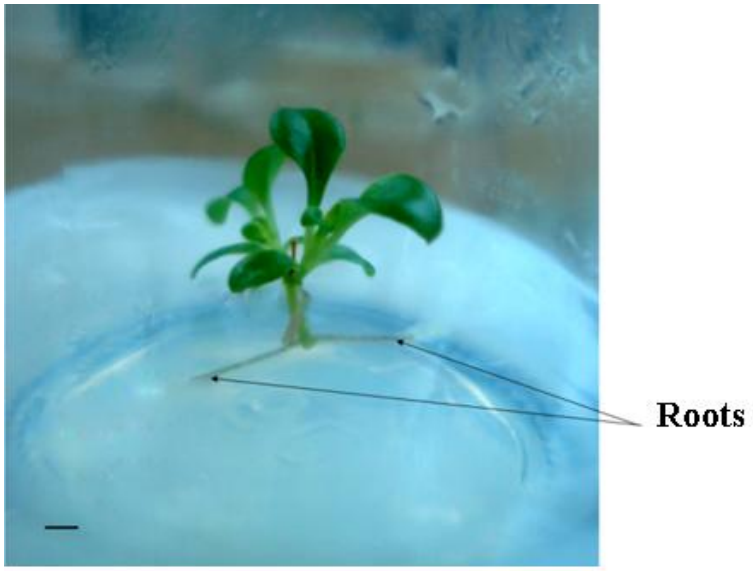

Figure 4. Seedling of *Medinilla mandrakensis* (Melastomataceae) with two small roots on the control medium (MS/2) growth regulators free (M0) after 45 days [bar = 5mm].

were fewer (2 per explant), frail and small (average length was 13.3 mm per explant) (Figure 4).

Influences of NAA and BAP on the rate of callus

Figure 5 shows the rate of callus recorded during the multiplication stage. Calluses were observed only on media supplemented with NAA whatever its concentration (M4, M5, M6, and M7). The contribution of this growth regulator strongly induced callus. Therefore, 100% of explants cultured in the medium supplemented with NAA produce callus at the base of explant (Figure 6).

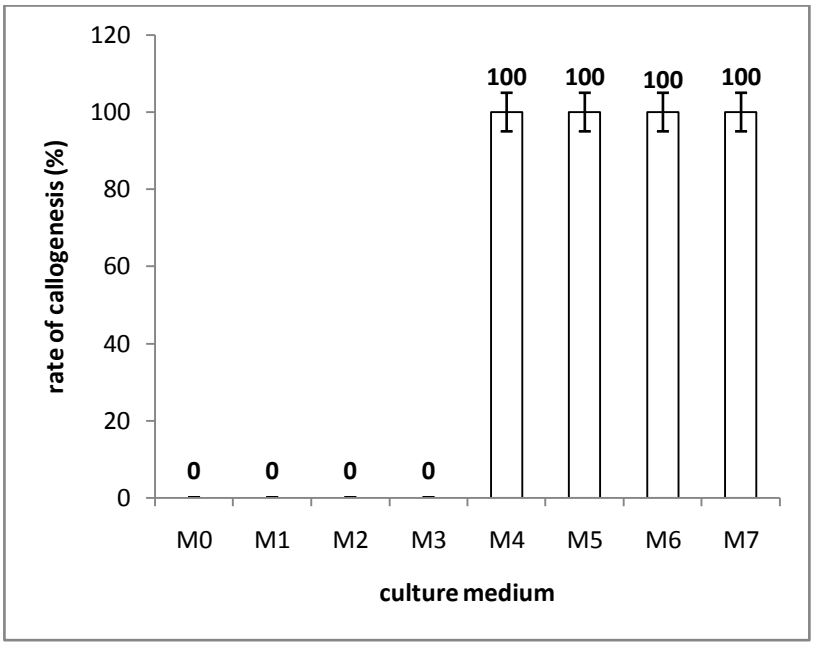

Figure 5. Influences of NAA and BAP concentrations on callogenesis rate
Legends: M0 (control): BAP/NAA (0/0), M1: BAP (0.5 mg/L) ; M2: BAP (1mg/L) ; M3: BAP (2mg/L) M4: BAP (0.5 mgLl) + NAA (0.1mgLl) ; M5: BAP (1mg/L) + NAA (0.1mg/L) ; M6: BAP (2mgL) + NAA (0.1mg/L), M7:BAP(0.5mg/L) + NAA (0.5mg/L)

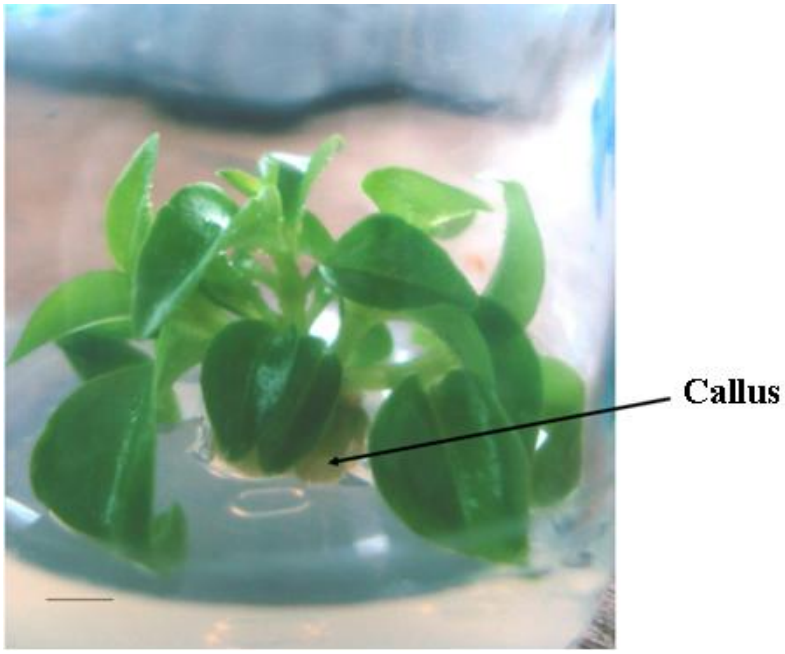

Figure 6. Callus at the base of shoots of *Medinilla mandrakensis* (Melastomataceae) on culture medium (MS/2) supplemented with 0.5mg/L NAA and 0.5mg/L BAP (M7) [bar = 5mm].

DISCUSSION

In vitro multiplication of *Medinilla mandrakensis* (Melastomataceae) is possible. Different types of culture media, even the control medium, allowed obtaining shoots on micro cuttings. These results agree with those obtained by Randriamampionona (2010), using similar concentrations of growth regulators on *in vitro* culture of

Syzygium cuminii. However, Pandeya et al. (2010) have had new shoots of *Clitoria ternatea* after addition of growth regulators. High rate with high concentration of BAP in the culture medium was detected. Similar results were reported by Naz et al. (2007) in *Cicer arietinum* and in *Dianthus caryophyllus* (Mahdiyeh et al., 2011). This ability of BAP to stimulate new formation of shoots is due to the properties of cytokinins such as:

- The removal of apical dominance and the awakening of dormant axillary buds (Sayanika and Sharma, 2009; Meftahizade et al., 2010; Mudasir and Ashok, 2010)
- The stimulation of cell division.

-With a small amount of auxin (endogenous auxins), cytokinins stimulate cell cytokinesis and partitioning leading the cells to regain their meristematic character (dedifferentiation) (Gautheret, 1959; Gamborg and Phillips, 1995; Crété, 2009). Then they stimulate redifferentiation in recent buds or shoots.

Contrariwise, the combination of NAA / BAP leads to a decrease in the number of shoots per explant as the concentration of NAA increases. Similar results were observed in *Quercus suber* (El Kbiach et al., 2002); in *Mecardonia tenella* (Alderete et al., 2006); in *Gynura procumbens* (Keng et al., 2009) and in *Clitoria ternatea* (Pandeya et al., 2010).

The inhibitory effect of NAA may be linked to the antagonist roles of these two hormones on the direction of organogenesis. Therefore, the presence of this plant hormone in the culture medium could weaken the ability of BAP to induce cell differentiation in buds (shoots).

The longest shoots were obtained on medium free growth regulators. These results corroborate those obtained by Randriamampionona (2010) and Mahdiyed et al. (2011), respectively in *Syzygium cuminii* and *Dianthus caryophyllus*. The ability of plant species to grow and to lie in phytohormones free medium could be explained among other things by the nitrogen ($NH4^+$) and potassium (K^+) richness of the base medium of Murashige and Skoog. Nitrogen occurs in the vegetative development, and potassium in cell division (Margara, 1989). Moreover, the appearance of roots that absorb nutrients could also work on the shoot elongation. The callus was induced only on media supplemented with the NAA /BAP combination. It could be due to exogenous auxin (NAA) in the culture medium. So, it follows in the ratio'' auxin/cytokinin "to a value suitable for callus formation (equal to or nearby 1). Similar results were obtained in *Qercus suber* (El Kbiach et al., 2002). These authors found that the addition of auxin in the culture medium increased the rate of callus to 93%. Regarding the type of auxin, they reported that the NAA clearly favors the formation of callus on explants.

Regarding the phenomenon of hyperhydricity, it was found only in the culture medium supplemented exclusively with BAP only at a higher dose (2mg/l). Mukherjee et al. (2010) reported the same effect in *Vitis champinii Planch* grown on MS medium added 2mg/l BAP. Moreover, Mahdiyed et al. (2011) indicated that the BAP is inducing hyperhydricity that kinetin, especially at high doses. This hyperhydricity phenomenon is due to ethylene accumulation and other gases (Zobayed et al., 1999) such as CO_2 in the culture tube (Chien-Chou et al., 2005). But according to Ziv (1991), this physiological disorder is related to physical and chemical conditions under which the explants grow as a high humidity, a carbohydrate excess, a low brightness and a high concentration of growth regulators.

CONCLUSION

It follows from this work that the forest species endangered of Ambatovy mine site in Madagascar, *Medinilla mandrakensis* (Melastomataceae) can be propagated in vitro from microcuttings or nodal segment. The results showed that the exclusive use of BAP clearly favors shoot proliferation and inhibits the formation of roots and callus. The most effective concentration of BAP for shoot proliferation was 1 mg/L. Beyond this dose (2 mg/L), this phytohormone induced hyperhidricity newly regenerated shoots.

Counter to the use of BAP, the NAA has led to reduced numbers of newly formed shoots. In addition, the contribution of this phytohormone also promoted callus formation at the base of all explants regardless of the dose used.

Multiplication of *Medinilla mandrakensis* through nodal segment *in vitro* culture is very important for the conservation of this threatened species because it may produce large number genetically uniform plants or clones. This promising alternative could be also a conceptual model to undertake for micro-propagation of the other gender or family forest plants.

In our future studies, we intend to study the regeneration through other organ and/or tissue culture (leaf, calls culture) of *Medinilla mandrakensis*. It will be another way for multiplication of this species. Extension of this study to other genders or species seems important.

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