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IN VITRO REGENERATION OF THREE FOREST SPECIES (*DISTEPHANUS AFF GARNIERIANA; MEDINILLA MICRANTHA; MELICOPE SP. INDET*) OF AMBATOVY MINE SITE (MADAGASCAR) ACCORDING TO THEIR BIOLOGICAL FORM

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ABSTRACT

The micropropagation biotechnology was tested on the regeneration and the multiplication of three forest species of biological form different. To estimate the behavior of these species towards the technique, three parameters were studied: the axillary buds initiation, the speed of growth, and the multiplication factor. The explants with axillary buds were cultured on suitable culture medium for each species. The obtained results show the success of the *in vitro* micropropagation of these three species. The epiphyte lianescent species "*Medinilla micrantha*» showed an early of bud burst appeared to the 3rd week of culture, a speed of fast growth of 0.5cm in 17 days and a high multiplication factor of 5 in 3 months. As for the herbaceous species "*Distephanus aff garnieriana* ", its axillary buds began to sprout after 6th week of culture; its speed of growth is on average (0.5cm in 47 days) and 3 for its multiplication factors. Concerning the ligneous species *"Melicope sp. Indet* ", the starting up of axillary buds burst was more late observed after the 12th week of culture. It was a slow-growing species achieving 0.5 cm in height in 72 days and the multiplication factor was 2 within 3 months.

KEYWORDS: Biological form, *Distephanus aff garnieriana, Medinilla micrantha, Melicope sp. Indet., in vitro,* micropropagation, regeneration.

I - INTRODUCTION

According to their identification in the field, three native species such *Distephanus aff garnieriana* (Asteraceae) *Medinilla micrantha* (Melastomataceae) and *Melicope sp. indet.* (Rutaceae) originate in Ambatovy evergreen rainforest are among the sensitive, endemic, and endangered species and have priority under conservation.

MEF (Ministry of Water and Forests)/SNGF (National Silo of forest seeds) reported in 2000 that forest genetic resources must be reproduced with appropriate techniques to their sustainability. Thus, biotechnology of *in vitro* micropropagation is one of the spécial conservation program for endemic, and endangered species. This technique constitute a powerful tool to obtain (from a single individual plant), a lot of plants that are genetically identical (clone) with a proliferation rate which could reach 100 to 1000 times higher (Margara, J., 1982; Gambourg et Phillips, 1995; Lydiane et Kleyn, 1996; Ferry et al., 1998; Semal, 1998; http://1).

However, *in vitro* regeneration of most forest trees is particularly hard and often faces several difficulties (high secretion of polyphenols and tannins, hyperhydricity, low rooting capacity, high levels of contamination, ...) due to various internal anatomical factors and physiological and external environmental (Harry, ES et al, 1975; Durand and Nitch, 1977. Rancilla, CM, 1979). The biological form of the species could be among these factors. The objective of this work is to study comparatively the behavior of these species in regard with the *in vitro* micropropagation.

II. MATERIALS AND METHODS

II.1. PLANT MATERIAL

Medinilla micrantha, *Distephanus aff garnieriana* , and *Melicope sp. Indet.* are indigenous, endemic and threatned species in the evergreen rainforest of Ambatovy, in Madagascar. This area is geographically bounded by the coordinates 18° 49' 0.12" south latitude and 48° 18' 00" of East longitude (http://2).

Regarding their biological form: *Medinilla micrantha* is a lianescente species; *Distephanus garnieriana aff.* is an herbaceous, and *Melicope sp. Indet.* is a ligneous species.

II.2. COLLECTION SITE

The explants of Distephanus *garnieriana aff., Medinilla micrantha* and *Melicope sp. Indet*. were harvested in the evergreen rainforest of Ambatovy, in Madagascar. This area is geographically bounded by the coordinates 18° 49' 0.12" south latitude and 48° 18' 00" of East longitude (http://2).

III. 3. METHODS

The biological basis of the method is the development of the previous buds on stem fragment cultured.

The original explant cultured is a steam fragment with at least one axillary bud. For each species, the node is cut a few millimeters from the stem to avoid damaging the axillary buds. The average size of explants was 1.5cm**.** The explants were decontaminated before their culture in suitable nutrient media. Each species was grown in their own culture medium of bud initiation, growth and multiplication of plantlets.

Basal medium used for these species was a half-strength basal Murashige and Skoog (1962) (MS/2) supplemented with 15% green coconut milk (v/v), 0.5mg/l thiamine-HCl, 0.5mg/l pyridoxine, 0.5mg/l nicotinic acid, 100mg/l myo-inositol.

The culture media for initiation and multiplication was made of basal medium supplemented with or without growth regulators: cytokinin which was 6-Benzylaminopurin (BAP) and auxin constituted by Naphthalene Acetic acid (NAA) or Indol Butyric Acid (IBA) which were suitable and specific for each species. Different concentrations and combinations of growth regulators are summarized in table 1.

Table 1: Concentrations and combinations of growth regulators for initiation, and multiplication of *Distephanus garnieriana aff., Medinilla micrantha* and *Melicope sp. Indet*.

	Distephanus garnieriana aff.		*Medinilla micrantha*		*Melicope sp. Indet.*	
	Initiation	**Multiplication**	**Initiation**	**Multiplication**	**Initiation**	**Multiplication**
BAP (mg/l)	2	1	0	1	0	2
NAA (mg/l)	0.2	0	0	0	0	0.05
IBA (mg/l)	0	0.5	0	0	0	0

NAA: Naphthalene Acetic Acid; BAP: 6-Benzylaminopurin; IBA: Indole-butyric-Acid

One explant per culture vessel was used. The stem fragments were plunged vertically on the culture media. Each culture vessel contains 15ml of solid medium and covered with aluminum foil and parafilm. The cultures are placed in a climatic chamber at temperature of 25°C provided with fluorescent tubes "Phillips-40W" providing an illumination of 2000-2500 lux. The cultures are placed in a climatic chamber at temperature of 25 ° C provided with tubes "Phillips-40W" with an illumination of 2500-3000 lux. The photoperiod was 16 hours of light per day. Eight explants were used for each species and repeated for 3 times.

- **Parameters evaluation**

  In order to evaluate the development of explants of each species, the following criteria were used:

a) ***Bud initiation time***: it is defined as the number of days elapsed between the in vitro cultivation and the beginning of bud burst.

b) ***Vitroplants growth rate***: The explants are transferred to fresh medium every four weeks. The growth rate is defined as the number of days which the plantlets increased the size by 0.5cm.

c) ***Multiplication rate of explants***: the stem is cut into fragments, put back on the fresh medium, will give as many leafy tufts. The multiplication phase consists of the subdivision of leafy shoots and nodes of the rod on the multiplication medium. It is evaluated by the number of new individuals trained every three months.

- **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 treatments is made following NEWMAN- KEULS test (probability threshold of 5%).

III- RESULTS

1. *Bud Initiation*

The following figure shows the results of the statistical analysis of the specific difference in the initiation of axillary buds of the three species.

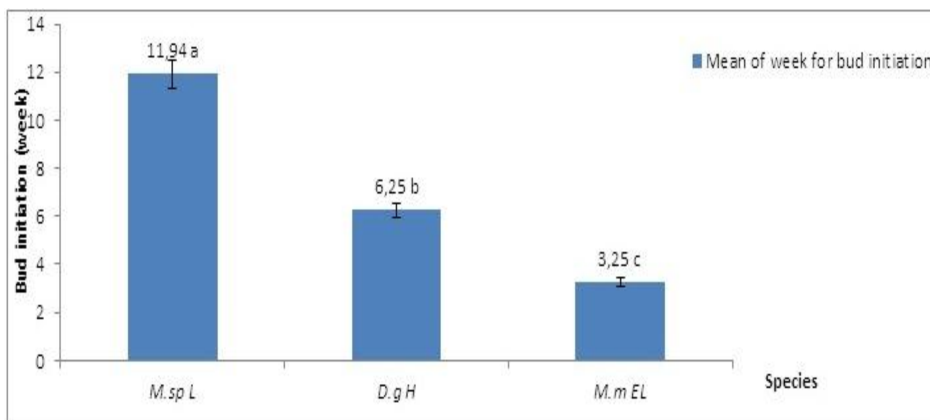

Fig 1: Specific difference in the initiation of axillary buds of the three species

M.spL: Melicope sp. Indet. (ligneous); D.gH: Distephanus aff. garnieriana (herbaceous); M.mEL: Medinilla micrantha (epiphyte lianescent)

The start of pre-existing buds or buds sprout is manifested by swelling the growing point. According to the analysis of variance, there is an early bud sprout for epiphytic lianescente species compared to herbaceous species and woody species. A comparison of means using the Newman Keuls test at 5% probability level shows a highly significant difference with three heterogeneous groups. Indeed, *Medinilla micrantha* (epiphytic lianescent) and *Distephanus aff garnieriana* (herbaceous) show an early bud that appeared respectively in 3rd and 6th week of culture whereas in the woody *Melicope sp. Indet*, it was later and only appeared after the 12th week.

2. *Plantlets in vitro growth*

The following figure shows the plantlets growth rate according the species biological forms:

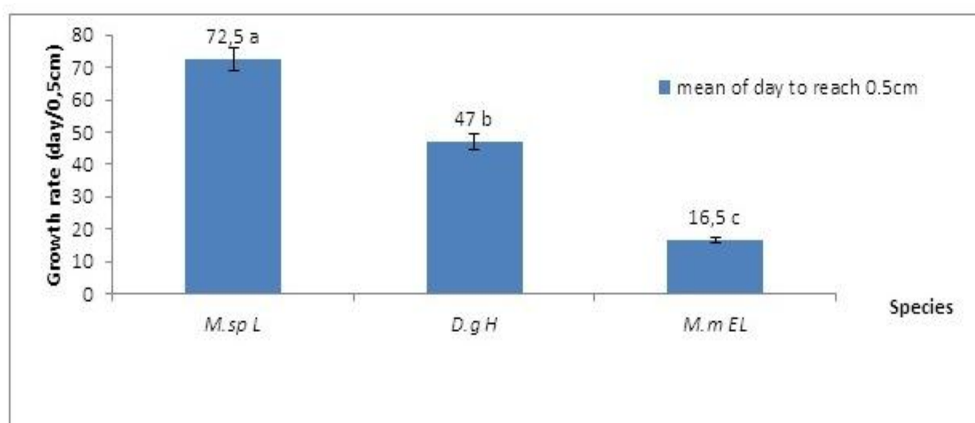

Figure 2: Specific difference on the *in vitro* plantlets growth rate

M.spL : Melicope sp. Indet. (ligneous) ; D.gH : Distephanus aff. garnieriana (herbaceous); M.mEL : Medinilla micrantha (epiphyte lianescent)

The growth rate is estimated by the number of days which the seedlings size reach 0.5cm. In terms of comparative tests carried out to follow the *in vitro* culture growth, the ANOVA revealed a highly significant difference between the 3 species that are composed of three heterogeneous groups (Figure 2).

The developed plants in vitro can have a slow, medium or rapid growth, depending on the species. Thus, the height of the *Medinilla micrantha* explant can reach 0.5cm in 17 days that of the species *Distephanus aff garnieriana* reaches 0.5 cm in 47 days whereas for *Melicope sp. Indet*, the explant reaches 0.5cm in 72 days. Indeed, according to their biological forms, the epiphytic lianescent species (*Medinilla micrantha*) has a fast growth compared to herbaceous species (*Distephanus aff garnieriana*) whose growth is intermediate and the woody species *Melicope sp. Indet* is very slow growing.

3- *In vitro multiplication rate*

Every 3 months, in vitro multiplication of the three species rate was estimated. The following figure shows the specific difference according their biological forms.

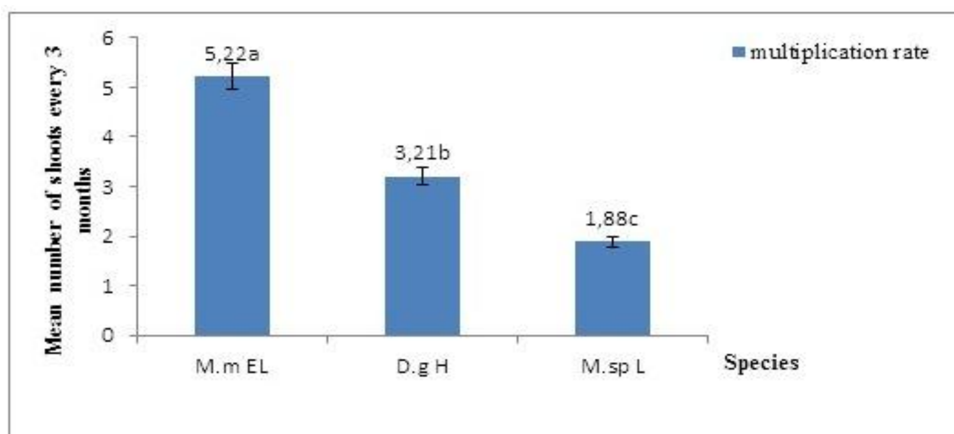

Figure 3: Specific difference in vitroplantlets multiplication rate every 3 months according the species biological form

M.spL : Melicope sp. Indet. (ligneous) ; D.gH : Distephanus aff. garnieriana (herbaceous); M.mEL : Medinilla micrantha (epiphyte lianescent)

Once the well elongated explants in their growth medium have been cut and transplanted to their specific multiplication medium, axillary and/or adventive shoots are formed on them. From Figure 3, it was found that after three months, five leafy shoots were formed on *Medinilla micrantha*. Shoots planted separately will regenerate up to five new seedlings. Concerning the species *Distephanus aff garnieriana,* three individuals have been produced

and who will regenerate the three other seedlings. For *Melicope sp. Indet*. species, there were two seedlings that were the origin of two new plantlets. From these results, it was shown that the transplantation of seedlings has increased every three months the number of plants by a factor of five in *Medinilla micrantha ;* a factor of five in *Distephanus aff garnieriana ;* and only one in *Melicope sp. Indet*. Thus, these results show the superiority of the multiplication rate in epiphytic lianescent compared to that of herbaceous and woody species.

DISCUSSION AND CONCLUSION

In vitro propagation of forest trees is recognized difficult because of the lack of published scientific papers and experimental protocols which are variable and specific to each species or even for each variety. In this work, we have purposely chosen for the three species of different biological forms, like the majority of forest species, propagation in vitro multiplication of axillary buds (Favre, J.M., Junker, B., 1987.).

It appears from this experiment that the process allowed the *in vitro* regeneration of *Distephanus garnieriana aff., Medinilla micrantha* and *Melicope sp. Indet*. species. However, clear differences were observed regarding the parameters studied. In fact, the epiphytic lianescente species showed the most favorable behavior like a time short time for bud development, a fast growth and hight rate of multiplication. However, the woody species (*Melicope sp. Indet*.) has required more time to bud development besides its rate of growth and multiplication is the lowest. So This result seems to be caused by the the fact that the lignification of stem tissue (more or less advanced) could be a hindrance to the growth and development of plant cultivation, which is consistent with the results obtained by Montarone, M.; Savignac, D. Maricot, C. en 1997, concerning the behavior of cuttings. In other words, the changing behavior of each species could be due to the lignification level on the epidermis of stem of the mother plant.

Distephanus aff garnieriana showed intermediate characteristics compared to the other two species. It seems that at an advanced stage of lignification of the stem, there is progressive decrease in the ability to organogenesis and the speed of growth during the *in vitro* culture. The ability to bud development and organogenesis is better when tissues are still young (case of *Medinilla micrantha* and *Distephanus garnieriana aff*), This is according to the result obtained by Franclet, NY, in 1979 and Bonga. JM in 1982, about the rejuvenating principle of the adult tree.

So we can reasonably think that this highly significant difference in the parameters studied, comes from the different biological form of the species. The development of *in vitro* micropropagation of these species suggests a conceptual model for the *ex situ* conservation of other priority species susceptible or threatened endemic species and their propagation.

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