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COMPARATIVE STUDY OF MICROPROPAGATION OF *EUGENIA SP.NOV.1* AND *SYZYGIUM PARKERI* (MYRTACEAE) UNDER THE INFLUENCE OF BENZYLAMINOPURINE (BAP), NAPHTHALENE ACETIC ACID (NAA) AND POTASSIUM PHOSPHATE DEHYDROGENATE (NaH_2PO_4)

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ABSTRACT

Some researches about the micropropagation of *Eugenia sp.nov.1* and *Syzygium parkeri* (Myrtaceae) threatened of extinction at the mining site of Ambatovy in Madagascar were carried out in order to manage their multiplication and preservation. Surface sterilization tests of the explants, using $CaOCl_2$ at 5% or 7% concentration and soaking time of 10 or 15min, were performed. *In vitro* regeneration of these species were established on MS/2 medium supplemented with 150ml/l of green coconut water, 2g/l of activated charcoal, 0 or 90mg/l of potassium phosphate dehydrogenate (NaH_2PO_4) and 0.5 to 3mg/l of BAP and/or 0 to 0.5mg/l NAA. The aseptic culture establishment using 7% of calcium hypochlorite ($CaOCl_2$) during 10min allowed to obtain the best rate of survival explants (20% of contamination rate and 0% of death rate). 2mg/l of BAP and 90mg/l of NaH_2PO_4 produced 3 shoots/explants for *Eugenia sp.nov.1* and *Syzygium parkeri*. An average length of 2cm of the shoots was obtained in the medium growth regulators free. As for the *in vitro* rooting, different reactions were observed for the two species. 1.5mg/l of BAP permitted to get an average of 0.5 root/explant for *Eugenia sp.nov.1*. For *Syzygium parkeri*, 1.6 roots/explant were obtained with 3mg/l/0.2mg/l of BAP/NAA in combination.

KEYWORDS: *Eugenia sp.nov.1; Syzygium parkeri;* vitropropagation; growth regulators, potassium dehydrogenate phosphate.

INTRODUCTION

Eugenia sp. nov. 1 and *Syzygium parkeri* are forest trees belonging to the family of Myrtaceae. This family includes over 5,500 species. Syzygium and Eugenia are among the genus representative of the Myrtaceae family in Madagascar (Schatz, 2001; http://1). This genus has a huge economic importance because of its fruits, spices, and wood provided and its stake in the pharmaceutical industry. Moreover, almost all fleshy fruits belonging to this family are edible, mentioning guava (*Psidium guajava*) as example (http://1). Several studies have shown the presence of numerous active ingredients in some species belonging to the genus *Syzygium* and *Eugenia* some of which are used in the treatment of diabetes (Bhat, 2008; Tanwar and al., 2011). Other species are used to extract anti-infective agents (Awuah and Ellis, 2001 Jabeen and Javaid, 2009 and Raj, 2011). However, *Syzygium parkeri* and *Eugenia sp Nov.1* are among the endangered species by the massive and alarming destruction of Madagascar's forest. Moreover, among the methods of regeneration, cuttings have proved to be rather difficult in *Eugenia sp. 1 nov*. and in *Syzygium parkeri*. As with many woody species, rooting still remains difficult and needs several optimizations (Francelet A., 1991).

Indeed, the method of vitropropagation was taken to achieve their regeneration and their *ex-situ* conservation. The vitropropagation is a very effective tool for biotechnology and increasingly used in agriculture and horticulture to facilitate the propagation of elite genotypes (Haïcour et al., 2002). Micropropagation is among the techniques of vitropropagation to produce a clone very quickly. It allows a high multiplication rate from an explant in a relatively short time and in a minimum space (Haïcour et al., 2002). On the other hand, with good physical condition (light, temperature, humidity), the vitropropagation requires appropriate chemical condition (culture medium). Guadinovà (1983) confirmed that the deficiency in a macroelement can significantly affect the enzyme system or assimilation system.

In the present work, we investigated *in vitro* regeneration of *Eugenia sp.nov.1* and *Syzygium parkeri* in order to determine whether the reactivity and *in vitro* development of two species belonging to the same family (Myrtaceae) would be similar or different. It was based on the following objectives:

- to establish aseptic culture for *Eugenia sp.nov.1* and *Syzygium parkeri* to achieve viable and healthy explants, allowing their *in vitro* regeneration;
- to optimize and to compare the *in vitro* regeneration of these species by the micropropagation technique, using different hormonal combinations: 6-Benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA) and with additional macronutrients: potassium dehydrogenate phosphate (NaH_2PO_4).

MATERIALS AND METHODS

1. Collection Site

The wildlings *Eugenia sp.nov.1* and *Syzygium parkeri* 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).

2. Plant Material

Segments of younger steam, 2cm of length with one node and a single axillary bud were used as the initial explants.

3. Methods

3.1 Explant surface disinfection

Surface disinfection of *Eugenia sp. nov.1* and *Syzygium parkeri* explants was similar and performed under laminar flow hood. After washing with soap and water, these explants were soaked in a mancozeb solution 3% (w/v) for 30min; in 70 ° ethanol for 5min and then in a calcium hypochlorite solution of various concentrations of 5% and 7% (w/v) for 10min and 15min for each concentration. For the best work of disinfectants, a few drops of Tween 20 have been added. Each treatment was repeated 20 times. Effects of different treatments were quantified on the basis of infection and mortality rates.

3.2 *In vitro* multiplication and rooting

Eugenia sp.nov.1 and *Syzygium parkeri* microcuttings were placed on culture media in order to promote their growth, multiplication and rooting.

Basal medium 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 multiplication and rooting were made of basal medium supplemented with 90mg/l of potassium dehydrogenate phosphate (NaH_2PO_4) and different concentrations and combinations of a cytokinin which was 6-Benzylaminopurin (BAP) and an auxin constituted by Naphthalene Acetic acid (NAA).

The cultures were maintained in a culture chamber at 25±2 ◦C under 16h/8h photoperiod with a light intensity of 3000lux. The cultures were maintained through regular subculturing at 4 week intervals on fresh medium with the same composition.

Effects of different treatments were quantified on the basis of the mean number of shoot per explant (microcutting), mean length of new shoots, the mean number of roots per *vitroplant*

Table 1: Different types of culture media for micropropagation of *Eugenia sp.nov.1* and *Syzygium parkeri.*

Type de milieu de culture	L0	C0	C1	C2	C3	C4	C5	C6	C7	C8	C9
NaH_2PO_4 (mg/l)	0	90									
BAP (mg/l)	0	0	1	2	3	1	2.5	3	1.5	2	2.5
NAA (mg/l)	0	0	0	0	0	0.2	0.2	0.2	0.5	0.5	0.5

NAA: Naphthalene Acetic Acid; BAP: 6-Benzylaminopurin ; L0: control medium ;
C0: medium with NaH_2PO_4, and growth regulator free; C: media with NaH_2PO_4 and various concentrations and combinations of growth regulators

Table 2: Different types of culture media for rooting of *Eugenia sp.nov.1* and *Syzygium parkeri*

Type de milieu de culture	L0	C0	C1	C2	C3	C4	C5	C6	C7	C8	C9
NaH_2PO_4 (mg/l)	0	90									
BAP (mg/l)	0	0	0.5	1.5	3	1.5	2.5	3	0.5	1.5	2
NAA (mg/l)	0	0	0	0	0	0.2	0.2	0.2	0.5	0.5	0.5

NAA : Naphthalene Acetic Acid ; BAP : 6-Benzylaminopurin ; L0 : control medium
C0 : medium with NaH_2PO_4, and growth regulator free; C : media with NaH_2PO_4 and various concentrations and combinations of growth regulators.

3.3 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%). For the percentage calculation, data had to undergo an angular transformation to obtain averages that were subsequently treated by software "STAT-ITCF" Version 4.

RESULTS

1. Influence of the soaking time and concentrations of $caocl_2$ on the surface disinfection of *Eugenia sp.nov.1* and *Syzygium parkeri* explants.

The effects of different treatments for surface disinfection of *Eugenia sp.nov.1* and *Syzygium parkeri* explants are summarized in Table 3.

Regarding the contamination rate, table 3 shows that the reactions of *Eugenia sp.nov.1* and *Syzygium parkeri* were similar. The contamination rate of the explants treated with calcium hypochlorite ($CaOCl_2$) was the inverse of the soaking time.

In 7% of $CaOCl_2$ for 15min (T4), the lowest levels of contamination were obtained. These rates were respectively 10.6% for *Eugenia sp.nov.1* and 10.3% for *Syzygium parkeri*. Contrariwise, contamination rates were increased to 20.6% for *Eugenia sp.nov.1* and 20% for *Syzygium parkeri* when the soaking time was reduced to 10min (T3).

In 5% of $CaOCl_2$ for 15min (T2), explants contamination rates were increased to 30.3% for *Eugenia sp.nov.1* and to 30.6% for *Syzygium parkeri*. For soaking time of 10min (T1), the levels of contamination increased more and more up to 40% for *Eugenia sp.nov.1* and up to 40.3% for *Syzygium parkeri*.

From results, increasing the concentration of $CaOCl_2$ up to 7% and the soak time for 15min decreased the mean contamination rate up to 10% in both species.

Les résultats obtenus dans le tableau 4 indiquent que quelle que soit la concentration en $CaOCl_2$ (5% ou 7%), un taux de mortalité nul a été constaté lorsque la durée de trempage a été de 10mn (T1 et T3).

The results in table 4 indicate that whatever the concentration of $CaOCl_2$ (5% or 7%), zero mortality rate was found when the soaking time was 10min (T1 and T3).

Furthermore, these results showed that the species *Eugenia sp.nov.1* was more sensitive than *Syzygium parkeri* in regard with the action of $CaOCl_2$. In *Syzygium parkeri*, only the

treatemet with 7% of $CaOCl_2$ during 15min induced 20.6% of explants mortality rate. Neverthless, in *Eugenia sp.nov.1* for each $CaOCl_2$ concentration (5% and 7%), 15min soaking time has caused to the explants mortality of 10% and 40.3% relatively. From statistical analysis, explants mortality rate increased with the concentration of $CaOCl_2$ and the soaking time.

Thus, for both species, the treatment with 7% $CaOCl_2$ for 10min (T3) was the most effective because it has achieved the highest survival rate of explants, so a fairly low contamination rate (20.6 %) for *Eugenia sp.nov.1*, (20%) for *Syzygium parkeri* and a zero rate of mortality for both species.

Table 3: Results for surface disinfection of microcuttings *Eugenia sp.nov.1* and *Syzygium parkeri*

Treatment	Contamination rate (%)		Mortality rate (%)	
	Eugenia sp.nov.1	***Syzygium parkeri***	***Eugenia sp.nov.1***	***Syzygium parkeri***
T1	40±0.3a	40.3±0.2a	0±0d	0±0d
T2	30.3±0.2a	30.6±0.3a	10±0.2c	0±0d
T3	20.6±0.4ab	20±0.3b	0±0d	0±0d
T4	10.6±0.2b	10.3±0.2b	40.3±0.3a	20.6±0.4b

$CaOCl_2$: Calcium Hypochlorite

T1 : $CaOCl_2$ 5%, 10min ; T2 : $CaOCl_2$ 5%, 15min ; T3 : $CaOCl_2$ 7%, 10min ; T4 : $CaOCl_2$ 7%, 15min.

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 at 5%

2. Multiplication

2.1 Influence of growth regulators and NaH_2PO_4 on the number of shoots produced

From Table 4, in the medium supplemented with NaH_2PO_4, BAP and NAA, 100% of explants of *Eugenia sp.nov.1* and *Syzygium parkeri* produced shoots but the number of newly formed shoots was varied following the concentrations of BAP and NAA. In the control medium L0 and C0, a single shoot per explant was produced in both species (figures 1, 2, 4 and 5).

In culture medium supplemented exclusively with BAP and NaH_2PO_4 (C1 to C3), the number of shoots produced was dependent on the concentration of BAP. This medium promoted bud development in both species.

With 1 to 2mg/l BAP (C1 to C2) and NaH_2PO_4, number of shoots was further increased gradually as the concentration of BAP (Figure 3 and 6). However, at 3mg/l of BAP (C3), the number of shoots has decreased. There was only an average of 1.2 shoots per explant for *Eugenia sp.nov.1* and 1.5 shoots per explant for *Syzygium parkeri*.

In the media supplemented with 0.2mg/l of NAA and 1 to 3mg/l of BAP (C4-C6) and NaH_2PO_4, a reduction in the number of shoots was observed in both species. This rate was further reduced when the concentration of NAA was increased to 0.5mg / l (C7-C9).

The optimum concentration of BAP for bud development was 2mg/l (C2). For this concentration, on average, 3 buds per explant were produced for *Eugenia sp.nov.1* and for *Syzygium parkeri*.

According to the statistical analysis, the reactivity of *Eugenia sp.nov.1* and *Syzygium parkeri* regarding these growth regulators showed no significant difference. Besides, callogenesis phenomena located on the base of the explants were observed. A maximum rate of callus (100%) was observed on the medium supplemented with 0.2mg/l NAA and 2.5mg/l BAP (C5) and NaH_2PO_4.

Table 4: Average number of shoots per explant depending on concentration and combination of growth regulators and NaH_2PO_4, in *Eugenia sp.nov.1* and *Syzygium parkeri* (after 4 months).

Medium	Average number of shoots/explant	
	Eugenia sp.nov.1	***Syzygium parkeri***
L0	1±0d	1±0d
C0	1±0d	1±0d
C1	2.2±0.4abcd	2.5±0.8abc
C2	3±0.7a	3.2±1a
C3	1.2±0.4cd	1.5±1.3cd
C4	1.1±0.2cd	1.4±0.5cd
C5	2.6±1.3abc	3±1.1a
C6	2.1±1.6abcd	2.5±0.7abc
C7	2±1abcd	2.4±0.6abc
C8	1.4±0.4cd	1.5±0.5cd
C9	1.2±0.4cd	1.4±0.5cd

L0 : control medium ; CO : with NaH_2PO_4 (90mg/l) ;

C1 : BAP (1mg/l) + NaH_2PO_4 (90mg/l) ; C2 : BAP (2mg/l) + NaH_2PO_4 (90mg/l) ; C3 : BAP (3mg/l) + NaH_2PO_4 (90mg/l) ; C4 : BAP (1mg/l) + NAA (0.2mg/l) + NaH_2PO_4 (90mg/l); C5 : BAP (2.5mg/l) + NAA (0.2mg/l) + NaH_2PO_4 (90mg/l) ; C6 : BAP (3mg/l) + NAA

(0.2mg/l) + NaH_2PO_4 (90mg/l); C7 : BAP (1.5mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/l) ; C8 : BAP (2mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/l) ; C9 : BAP (2.5mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/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 at 5%

Figure 1 : shoot of *Eugenia sp.nov.1* on control medium L0 after 4 months.

Figure 2 : shoot of *Eugenia sp.nov.1* on medium C0 after 4 months.

Figure 3 : shoot of *Eugenia sp.nov.1* on medium C2 after 4 months.

Figure 4 : shoot of *Syzygium parkeri* on control medium L0 after 4 months.

Figure 5 : shoot of *Syzygium parkeri* on medium C0 after 4 months.

Figure 6 : shoot of *Syzygium parkeri* on medium C2 after 4 months.

2.2. Influence of growth regulators and NaH_2PO_4 on elongation of shoots

According to figure 7, no significant difference was observed in the average size of shoots of *Eugenia sp.nov.1* and *Syzygium parkeri*. In the control medium (L0), the average shoot length was 1.5cm for *Eugenia sp. nov.1* and 1.6cm for *Syzygium parkeri* (figures 8 and 10). Furthermore, the addition of potassium phosphate dehydrogenate (NaH_2PO_4) in the culture medium promoted shoot development in both species. The maximum length of shoots with an average size of 2.1cm to 2.2cm for both species was achieved in the medium growth regulators free, supplemented with NaH_2PO_4 (figures 9 and 11).

It has been shown that in culture medium added only of BAP at concentration of 1 to 3mg/l (C1, C2 and C3), and with NaH_2PO_4, the size of shoots was decreased up to an average of 1cm for both species. Besides, after 4 months of culture, the shortest shoots were observed in the medium supplemented simultaneously with NaH_2PO_4, 1mg/l BAP and 0.2mg/l NAA (C4). The average shoot length was only 0.8cm for both species.

An increase in the concentration of NAA 0.5mg/l (C7, C8 and C9) further improved shoot length. It was 1.5cm in *Eugenia sp.nov.1* and 1.6cm in *Syzygium parkeri*.

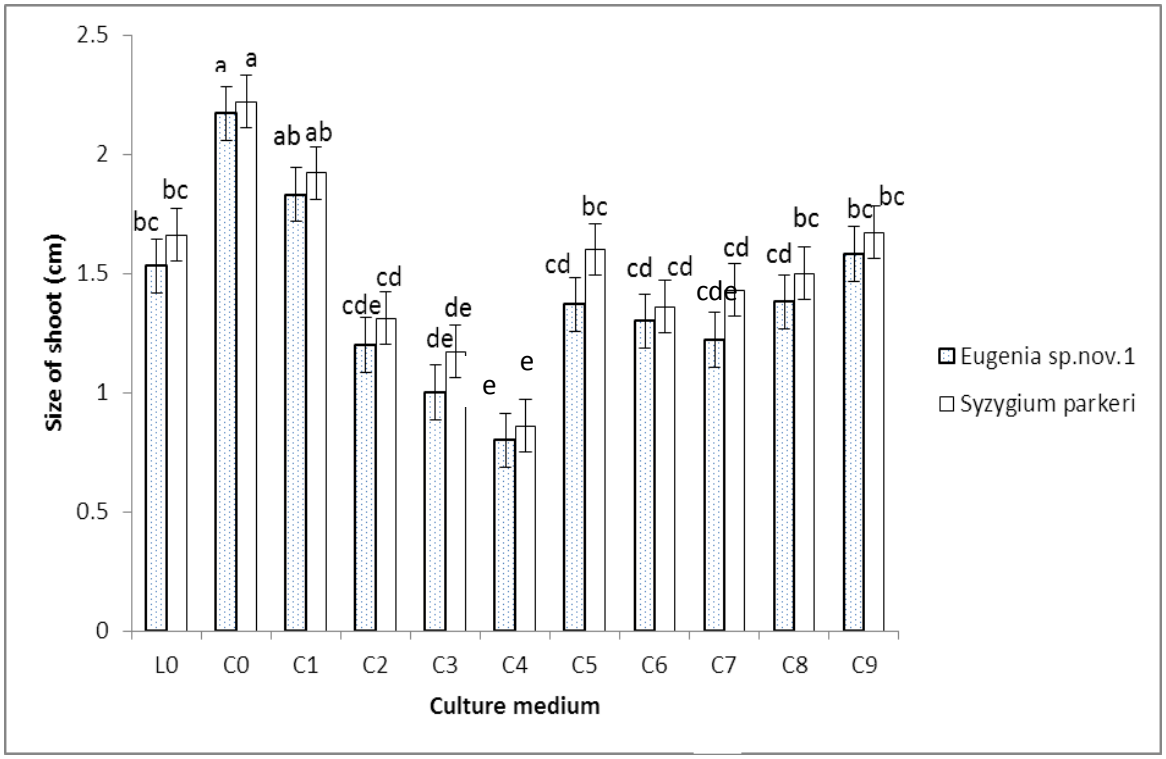

Figure 7: Influence of the combination of BAP / ANA and NaH_2PO_4 on the mean length shoot in *Eugenia sp. nov.1* and in *Syzygium parkeri* (after 4 months).

L0 : control medium ; CO : with NaH_2PO_4 (90mg/l) ; C1 : BAP (1mg/l) + NaH_2PO_4 (90mg/l) ; C2 : BAP (2mg/l) + NaH_2PO_4 (90mg/l) ; C3 : BAP (3mg/l) + NaH_2PO_4 (90mg/l) ; C4 : BAP (1mg/l) + NAA (0.2mg/l) + NaH_2PO_4 (90mg/l); C5 : BAP (2.5mg/l) + NAA (0.2mg/l) + NaH_2PO_4 (90mg/l) ; C6 : BAP (3mg/l) + NAA (0.2mg/l) + NaH_2PO_4 (90mg/l); C7 : BAP (1.5mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/l) ; C8 : BAP (2mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/l) ; C9 : BAP (2.5mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/l). The Values followed by the same letter are not significantly different according to the Newman-Keuls test at 5%.

Figure 8: shoot of *Eugenia sp.nov.1* on medium (L0) after 4 months.

Figure 9 : shoot of *Eugenia sp.nov.1* on medium C0 after 4 months

Figure 10 : shoot of *Syzygium parkeri* on the medium L0 after 4 months

Figure 11 : shoot of *Syzygium parkeri* on the medium C0 after 4 months

3. ROOTING

3.1 Influence of growth regulators and NaH_2PO_4 on the number of roots

The results obtained on the rooting of seedlings according to the concentration of growth regulators and the presence of potassium dehydrogenate phosphate (NaH_2PO_4) are summarized in table 5. Statistical analysis revealed the different responses between the rooting rates of both species.

In control medium (L0), the mean number of root produced per shoot was 0.3 for *Eugenia sp. nov.1* and 1 for *Syzygium parkeri*.

In the medium growth regulators free supplemented with NaH_2PO_4 (C0), the mean number of roots per seedling produced in *Eugenia sp.nov.1* and *Syzygium parkeri* were respectively 0.3 and 0.9.

Whatever the concentration of growth regulators (BAP, ANA), the average numbers of roots produced by Eugenia sp.nov.1 were always lower (0 to 0.5 root/seedling) than *Syzygium parkeri* (0.2 to 1.6 roots/seedling).

For *Eugenia sp.nov.1*, the maximum number of roots (average 0.5) was obtained on medium supplemented simultaneously with 0.2mg/l of NAA, 2.5 to 3mg/l of BAP and 90mg/l of NaH_2PO_4. For *Syzygium parkeri*, it was on average of 1.6 roots/explant with combination of 90mg/l NaH_2PO_4, 0.2mg/l NAA and 2.5mg/l BAP.

Table 5: average number of roots per plantlet depending on the growth regulators (BAP/ANA) concentration in *Eugenia sp.nov.1* and in *Syzygium parkeri* (after 4 months).

Culture medium	Average number of roots/plantlet	
	Eugenia sp.nov.1	***Syzygium parkeri***
L0	0.3±0.5bc	1±0.7ab
C0	0.3±0.5bc	0.9±1.1ab
C1	0±0c	0.1±0.4bc
C2	0.5±0.5bc	0.6±0.8bc
C3	0±0d	0±0c
C4	0±0d	0.2±0.4bc
C5	0.5±0.5bc	1.6±0.6a
C6	0.5±0.5bc	1.1±0.8ab
C7	0.5±0.8bc	1±0.7abc
C8	0.4±4bc	0.6±0.8bc
C9	0.4±0.6bc	0.5±0.5bc

L0 : control medium ; CO : with NaH_2PO_4 (90mg/l) ; C1 : BAP (1mg/l) + NaH_2PO_4 (90mg/l) ; C2 : BAP (2mg/l) + NaH_2PO_4 (90mg/l) ; C3 : BAP (3mg/l) + NaH_2PO_4 (90mg/l) ; C4 : BAP (1mg/l) + NAA (0.2mg/l) + NaH_2PO_4 (90mg/l); C5 : BAP (2.5mg/l) + NAA (0.2mg/l) + NaH_2PO_4 (90mg/l) ; C6 : BAP (3mg/l) + NAA (0.2mg/l) + NaH_2PO_4 (90mg/l); C7 : BAP (1.5mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/l) ; C8 : BAP (2mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/l) ; C9 : BAP (2.5mg/l) + NAA (0.5mg/l) + NaH_2PO_4 (90mg/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 at 5%

DISCUSSION

Regarding the effects of disinfection on survival rate of explants, the aseptic is one of the parameters which determine the success of the *in vitro* regeneration of plants. The sodium hypochlorite and calcium hypochlorite are often used because of their high antimicrobial activities. However, the sodium hypochlorite is less recommended for long term use because it can penetrate into cells and thus to become toxic (Boccon-Gibod, 1989 ; Boxus P. and al., 2003).

From the results, contamination rate decreased gradually as the soak time of the explants and/or the $CaOCl_2$ concentration was increased for *Eugenia sp. nov.1* and for *Syzygium parkeri*. Similar results have obtained by Rasoanandrasana (2010) in *Prunus Africana*. In this case, the $CaOCl_2$ requires use during a prolonged time for best results. Rakotoarisoa (2013) obtained a total disinfection in *Psorospermum sp.nov.B* seeds using 5% $CaOCl_2$ for 15 minutes.

Moreover, the high percentages of mortality rate (40.3% for *Eugenia sp.nov.1* and 20% for *Syzygium parkeri*) were recorded with a high dose of $CaOCl_2$ (7%) and prolonged soaking (15min). These results are in agreement with those obtained by Rasoanandrasana (2010) on the disinfection of nodal segment explant of *Prunus africana*.

Therefore, the type of explant used (stem, leaf, seed, ...) and their characteristics (herbaceous, woody, pubescent, ...) could be the factors making the explants disinfection harder. In presence of the bristles, the air bubble traps prevents the disinfectant to be in direct contact with the surface of the explants, thus, the pubescent explants were more difficult to decontaminate (Skirvin and al., 1999). As for the significant differences in mortality rates

between *Eugenia sp.nov.1* and *Syzygium parkeri*, the presence of thick bristles on their stems could be a factor making explants disinfecting harder.

Besides, the size of the explants could be a parameter that can affect the results of disinfection. Researches by Kasual and al. (2005) have demonstrated from disinfection experiments of axillary buds of apple that if the explant was large, the survival rate was reduced. In this case, the best results (lower mortality and maximal survival rates) were obtained with small explants between 0.2cm and 0.6cm.

Regarding the influences of growth regulators on the number of newly shoots produced, the medium growth regulators free has been effective in inducing the bud axillary development. However, medium with BAP in increase of the concentration (1 to 2mg/l) improved the buds break in *Eugenia sp. nov.1* and in *Syzygium parkeri* except the highest dose of BAP (3mg / l).

Similar results have been obtained in *Withania somnifera* (Sen and al., 1991), in *Holarrhena antidysenterica* (Raha and Roy, 2001), in *Syzygium francissi* (Shatnawi and al., 2004) and in *Syzygium cuminii* (Randriamampionona, 2010). This ability of BAP to improve the buds break is due to the properties of cytokinins such as the stimulation of mitotic activity insuring the improvement of cell division, the removal of apical dominance and the awakening of dormant axillary buds (Anjali et al., 2000 ; Youmbi et al., 2001, http://3).

However, the researches of Randriamampionona (2010) were revealed that high multiplication rate; an average of 7.5 shoots per explant was obtained in *Syzygium cuminii* with the low dose of BAP (1mg/l). The maximum multiplication rate of *Eugenia sp nov.1*and *Syzygium parkeri* has been obtained in 2mg/l of BAP concentration. In this case, no remarkable difference has been observed even the work of Lane and Mc Dougald (1982) has shown the variation of multiplication rate of explants according to the species. This reaction could be the result of the need of exogenous supply of BAP, in endogenous concentration of BAP and the efficient absorption of BAP according to the species in culture.

The addition of NAA in the culture medium leads to a significant decrease in multiplication rate in *Eugenia sp. nov.1* and in *Syzygium parkeri*. It may be linked to the inhibitory effect of NAA at a dose of 0.5mg/l on the action of BAP in the bud development of *Eugenia sp.nov.1* and *Syzygium parkeri*. This result corroborates that obtained by Ephrem, (2012) on *in vitro* propagation of *Medinilla mandrakensis*.

As for the influences of NaH_2PO_4 on the newly shoots production, taking into account the combination of BAP and NAA, the results showed that the addition of NaH_2PO_4 in the culture medium promoted especially shoot formation in *Eugenia sp. Nov. 1* and *Syzygium parkeri*. For adventive bud multiplication, the NaH_2PO_4 was often used to improve the multiplication rate (Monette, 1986; http: // 4). However, Taylor and Knauss (1978) could not obtain an improvement in the propagation rate for *Dieffenbachia maculata*, even by doubling the concentration of NaH_2PO_4 in the culture medium. Researches on the tissue culture carried out by Heller (1953) have also shown that the optimal concentration for each of the macronutrients (N, Ca, K, P, ...) varies with the species. Furthermore, Miller and Murashige (1976) were able to show from searches on some species of foliage plants, *vitroplant* reactivity in regard with the concentration of NaH_2PO_4 varied depending on the species.

In 1986, Monette added 170mg / l of NaH_2PO_4 in MS (Murashige and Skoog, 1962) so that to promote the proliferation shoots of *Actinidia chinensis*.

For in vitropropagation of a variety of peach-tree (GF-557), the addition of 90mg/l NaH_2PO_4 in media MS circles and LQ (Lepoivre Quoirin and 1977) used alternately, allowed to prevent problems linked to phenomena of yellowing of shoots, callus at the base of the explants and hyperhydricity of *vitroplant* (Ghorbel et al., 1994).

Concerning of influence of growth regulators on shoots development, in *Eugenia sp.nov.1* and in *Syzygium parkeri*, the maximal length of shoots was obtained in the medium growth regulators free (C0). One reason for the explants to benefit from this ability could be explained by the capacity of items such as macronutrients, microelements, organic supplements, the carbon source and vitamins ... present in the culture medium to promote *in vitro* plant development (George et al, 2008; http. // 3).

However, the presence and increase of the concentration of BAP in the medium further reduces the size of the shoots. Except the growth regulators free medium, the maximum height of the shoots in both species was obtained on medium containing only a low concentration of BAP (1mg/l). Similar results were observed in *Balanites aegyptica* (Ndoye, 2003), with *Quercus robur* (Chalupa, 1984) and in *Syzygium cuminii* (Randriamampionona, 2010).

Moreover, in combination with NAA, BAP has an essential role in cell division stimulating the cytokinesis or cell partitioning during mitosis and in the expansion of cells and the formation of new buds (Boxus, 1995; Rahman and al, 1993; http:. // 3). In this case, the NAA and BAP would possess inhibitory effects on shoot elongation for *Eugenia sp. nov. 1* and *Syzygium parkeri* when employed simultaneously at the dose 0.2mg/l of NAA with 1 to 3mg/l BAP. The results obtained in these two species corroborate those obtained by Randriamampionona (2010). This author found that shoot elongation was reduced when the concentration of NAA and BAP used separately or in combination was high. Murashige (1973) also indicated that the optimum concentration of auxin or cytokinin depends on one or the other when used in combination.

Concerning the influence of NaH_2PO_4 on shoots elongation, in order to improve the shoot elongation, the addition of NaH_2PO_4 in the culture media was effective. This helped to show that the addition of macronutrient can positively affect the explants *in vitro* growth. In 1976, Miller and Murashige discovered through vitropropagation of some tropical plants that the number and length of shoots increased progressively with the concentration of NaH_2PO_4 supplemented in the culture medium up to a certain threshold from 85 to 340mg/l depending on species.

The increase of the sodium ion concentration and phosphate ion in the culture medium with or without growth regulators promotes the proliferation and growth of cells by stimulating shoot elongation. According to Heller (1949), as some macronutrients, sodium ions have important role in stimulating the *in vitro* multiplication (Heller, 1949). As phosphate ions, they constitute an essential component for the progress of the respiration during the *in vitro* proliferation (http: // 3).

Rooting of *Eugenia sp.nov.1* and *Syzygium parkeri* was possible without exogenous growth regulators. However, NAA and BAP in the culture media promoted especially their rooting. Different reactions, according to the combinations of plant growth regulators have been observed between the rooting rate of *Eugenia sp.nov.1* and *Syzygium parkeri*. For *Eugenia sp.nov.*, the combination of growth regulators used to induced roots has not led to satisfactory results.

Taking into account the influence of different concentrations of growth regulators, cytokinin shows little influence on the induction of rooting plantlets compared to other growth

regulators (Simona et al., 2012). On the contrary, Magyar-Tabori and al (2001) have proved, due to the micropropagation of apple-tree that combined with activated charcoal, NAA, improves *vitroplants* rooting rate.

For *Syzygium parkeri*, the results showed that rooting was possible, even in the medium growth regulators free. Thus, rooting could be due to high levels of endogenous auxin, inducing eventually high rooting capacity (Auderset and al., 1994).

CONCLUSION

This work was undertaken in order to contribute to the conservation of Madagascar's flora. Two species, *Eugenia sp.nov.1* and *Syzygium parkeri*, endangered in the mining site Ambatovy were multiplied *in vitro* to ensure their multiplication and conservation. Belonging to the family of Myrtaceae, the study on vitropropagation of these two species was focused on their reactivity towards the explants surface sterilization, multiplication by axillary bud and rooting induction.

From this study, it follows that the surface sterilization conditions of *Eugenia sp.nov.1* and *Syzygium parkeri* have been optimized. The use of calcium hypochlorite ($CaOCl_2$) at a concentration of 7% during 10min allowed a total survival of explants (100%) with a low contamination rate (20%) for *Eugenia sp.nov.1* and *Syzygium parkeri*.

Concerning the multiplication, the results have shown that the vitropropagation of *Eugenia sp.nov.1* and *Syzygium parkeri* from microcuttings was possible. During multiplication, the reactions of the two species were always similar:

- The optimal concentration for axillary buds proliferation was 2mg/l of BAP for the both species (3 shoots/explant);
- However, BAP inhibited shoot elongation. The addition of growth regulator NAA in the culture media with BAP counteracted this inhibition and promoted further shoot development depending on the concentration of BAP and NAA;
- The medium growth regulators free were much more favorable to the shoot elongation. The addition of NaH_2PO_4 in the culture medium is recommended to optimize the shoot elongation. The maximum average size (2cm for *Eugenia sp.nov.1* and *Syzygium parkeri*) was obtained on the medium supplemented with NaH_2PO_4.

- A significant difference was observed, at rooting of *Eugenia sp.nov.1* and *Syzygium parkeri*. It shows that the optimum concentration for induction of rooting depends on species. *Syzygium parkeri* has a high rooting capacity than *Eugenia sp.nov.1:*
- For *Eugenia sp.nov.1*, despite the low rooting rates, the combination of BAP / NAA at concentrations of 0.5 / 0.5mg/l achieved the higher rooting rate (0.5 root/seedling)
- For *Syzygium parkeri*, the appropriate hormonal combination for the best rooting was composed of 0.2mg/l NAA associated with 2.5mg/l BAP (1.6 roots/seedling)
- In the medium growth regulators free, the addition of NaH_2PO_4 did not improve the rooting of *Eugenia sp.nov.1* and *Syzygium parkeri*.
- The simultaneous presence of BAP/NAA and NaH_2PO_4 in the culture medium improved the rooting seedlings of *Syzygium parkeri*.
- At each stage of micropropagation, similarities and dissimilarities towards the responses to different treatments were observed in *Eugenia sp.nov.1* and in *Syzygium parkeri*. *In vitro* regeneration by micropropagation of *Eugenia sp.nov.1* and *Syzygium parkeri* was successful.

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