REGENERATION OF *Leptolaena multiflora* (L.) BY *IN VITRO* SEED GERMINATION AND ZYGOTIC EMBRYO CULTURE

DINAHARILALA Mamisitraka T. G. [1] ; RAVONIARISON. N. [1]

(1) *Plant Physiology Laboratory, unit : Plant Regeneration and micropropagation Laboratory, Faculty of Sciences, University of Antananarivo Madagascar.*

ABSTRACT

Leptolaena multiflora (L.) is endemic specie from Madagascar, renowned for its medicinal properties. It is listed as endangered on the IUCN red list. The seed surrounded by a hard shell is one of the limiting factors of its germination. This study aims to regenerate this specie by in vitro germination techniques and culture of mature zygotic embryo. The evaluation of the effects of types of seed scarification on germination shows that soaking seeds for 48 h improves the rate of seed germination. Besides, the increasing concentration of sucrose from 10 g/l to 40 g/l has a positive effect on the development of the embryo, and promotes the development of the aerial part and the root system of vitroplants. However, the high concentration above 80 g/l reduced the growth and in vitro development of the embryo. Beyond this dose, regeneration and root growth are weak and tend to stop.

Keyword : *in vitro germination, Leptolaena multiflora, mature zygotic embryo, regeneration*

---

1. INTRODUCTION

Leptolaena multiflora (L.) is a forest tree belonging to the Sarcolaenaceae family, a plant family entirely endemic to Madagascar. It has been classified as endangered species (EN) according to IUCN criteria, due to the loss of its natural habitat and the exploitation of its timber [1]. *L. multiflora* is one of the sensitive species threatened called also “species of concern” (SOC) with extinction in the Ambatovy mining site in Madagascar. For its therapeutic virtues, the decoction of its bark is used in the treatment of jaundice and anemia. Its wood is widely exploited for the construction of housing, the manufacture of railway sleepers and the production of charcoal [2].

The ecology study showed that *L. multiflora* has an average natural regeneration rate and its seed germination is difficult in the natural state [3].

The *in vitro* germination techniques and culture of isolated mature embryos are of great interest. They make possible to reduce losses during the germination of the seeds, to quickly provide sterile plants used during the micropropagation of a given specie, to shorten the sexual reproduction cycle of the seedlings, to preserve the embryos of hybrids for the breeding program ([4], [5], [6]).

The originality of this study is based on the fact that it is the first carried out on the *in vitro* embryo culture of the genus *Leptolaena* taking into account the biological particularities of the trees [7], most woody forest species seem recalcitrant to *in vitro* regeneration. Our preliminary study on the *in vitro* micropropagation of this species was unsuccessful. The overall objective of this study is to contribute to the conservation and reproduction of this endemic and endangered species. The specific objective is to develop the *in vitro* regeneration technique of *L. multiflora* from seeds and isolated zygotic embryos. The following hypotheses were put forward *(a)* on one hand, dormancy, seed coat or seed pericarp are major factors inhibiting the germination of *L. multiflora*, and *(b)*

on the other hand, the development of the isolated zygotic embryo cultured *in vitro* requires an optimal sucrose concentration.

2. MATERIAL AND METHODES

2.1 Plant materials

The seeds of *Leptolaena multiflora* (L.) come from ripe fruits of greenish yellow color which are harvested within the mining site of Ambatovy in Madagascar (Photo 1). They are kept at cold temperature (4°C) until their culture. For embryo culture, zygotic embryos, at the cotyledonary stage, 1 mm in length, greenish yellow (Photo 2) were used as explants.

Photo 1: Mature fruits of *L. multiflora*

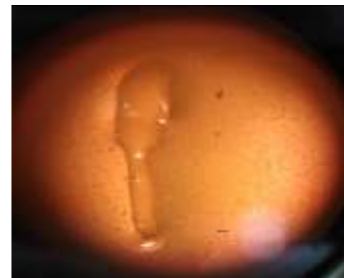

Photo 2: Zygotic embryo of *L. multiflora* at the cotyledonary stage

2.1 In vitro germination of *L. multiflora* seeds

2.1.1 Fruit surface disinfection and seed scarification

Seed disinfection was preceded by fruit decontamination and seed scarification under a laminar flow hood. After washing with soapy water, the fruits are disinfected by immersion for 60 minutes in a Mancozeb antifungal solution at a concentration of 1% (m/v) and for 5 minutes in 70° ethanol. The seeds are extracted from the pulp and the woody endocarp of the fruits. Two types of scarification were done as seed pretreatment. The one type was a wet pre-treatment in which the seeds have been soaked in distilled water for 48 hours. The other type composed a dry pre-treatment which consists of manual scarification of the seeds with a sterile scalpel in order to abrade the seed coat so that the cotyledons are visible. Seed disinfection consists of soaking them in the 20% (v/v) sodium hypochlorite solution for 10 min, 20 min and 30 min. After each disinfection, they are rinsed three times with sterile distilled water.

2.1.2 Seed sowing and parameters studied

Basal medium was a half-strength basal medium of Murashige and Skoog (1962) (MS/2) supplemented with vitamins, 20 g/l (m/v) of sucrose, 15% green coconut milk and solidified with 8 g/l (w/v) agar were used [8]. One seed per culture flask containing 10 ml of medium was inoculated (Photo 3). Three (3) seed lots with 9 treatments each were established, including the controls. Each treatment was repeated 15 times (Table 1). Five (5) trials were carried out, giving a total of 675 seeds sown. The contamination rate after 2 weeks of culture and the germination rate after 8 weeks of culture were considered as the evaluation parameters for the *in vitro* seed germination study.

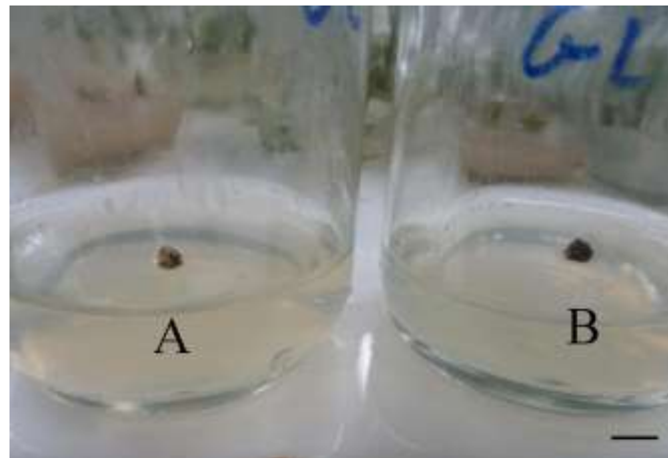

Photo 3: Seeds sown after pretreatment and disinfection, (A) manually scarified seed, (B) seed soaked in distilled water for 48 h, (bar = 1cm)

Table 1: Seed pretreatment and disinfection for *in vitro* germination

Batches	Control			Wet pretreatment			Dry pretreatment		
Seed soaking time in 20% NaOCl (min)	10	20	30	10	20	30	10	20	30
Number of seeds sown	15	15	15	15	15	15	15	15	15

NaOCl: sodium hypochlorite

2.2 Culture of zygotic embryos of *L. multiflora*

2.2.1 Zygotic embryo regeneration media at the cotyledonary stage

The study of the influence of sucrose concentration on the development of zygotic embryo at the cotyledonary stage was carried out. The different sucrose concentrations added in a half-strength basal medium of Murashige and Skoog (1962) (MS/2) (Table 2) were adopted from the work of Jay-Allemand and Cornu (1986) on the *in vitro* culture of embryos isolated from *Cocos nucifera* [9]

Table 2: Different types of culture medium for *L. multiflora* embryo culture

Type of culture medium	E0	E1	E2	E3	E4	E5
Sucrose (g/l)	0	10	20	40	80	160

2.2.2 Zygotic embryo seeding and evaluation parameters

The optimal condition (20% NaOCl solution for 20 min) for seed surface sterilization during *in vitro* germination was used for embryo culture. In order to increase the imbibition of the cotyledons and to facilitate the collection of the embryos, the seeds were rinsed and soaked in sterile distilled water for 48 hours.

The explants consist of zygotic embryos, at the cotyledonary stage, 1 mm in length, greenish yellow (photo 2). They are inoculated into culture flasks containing 10 ml of medium, at the rate of one embryo per culture flask. Six (6) treatments were carried out, each of which was the subject of 10 repetitions, for a total of 60 embryos including those cultured in the control medium. The cultures were maintained in a culture chamber under controlled conditions at a temperature of 25° C±2., under a light intensity of 3000 Lux and a photoperiod of 16 h light/8 h dark. The rate of contamination, the speed of initiation of the embryo corresponding to the time interval between the date of sowing of the embryo and that of the appearance of the radicle, the average length of the

primary root, the average length of the aerial part, and rate of regeneration of the embryos into seedlings were the evaluation parameters considered for the development and the growth of the embryos.

2.2.3 Statistical processing of results

Data analysis is performed by percentage calculations and/or analyzes of variance using R Version 4.1.2 software. The separation of the homogeneous groups observed between several means is made according to the Newman-Keuls test at the probability threshold of 5%.

3. RESULTS

3.1 Effect of soaking time in a sodium hypochlorite (NaOCl) solution on the rate of contamination.

Table 3 reports the effects of the duration of seed soaking in a 20% sodium hypochlorite solution observed after 2 weeks of culture. According to the results obtained, the rate of contamination is significantly lower with the soaking times of 20 min and 30 min, than that of 10 min. The mean rate of contamination is 1.3% for the soaking time of 20 min, and 30 min, however it is 9.3% for the duration of 10 min.

Table 3: Results for surface disinfection of after 2 weeks of culture

Time (min)	10	20	30
Contamination rate (%)	9,3 ± 0,2 a	1,3 ± 0,1 b	1,3 ± 0,1 b

The values followed by the same letter show no significant difference according to the Newman and Keuls test at the 5% level.

3.2 Influence of pretreatments and soaking time in chlorine solution on the germination capacity of *L. multiflora* seeds

Figure 1 summarizes the variation in the germination rate of *L. multiflora* seeds depending on the type of pretreatment and the soaking time of the seeds in a 20% sodium hypochloride solution. The wet and dry pretreatments promoted seed germination with highly significant values compared that of the control. The germination rates of seeds soaked in distilled water for 48 h or wet pretreatment were higher (20 to 22.6%) than those obtained by the dry pretreatment (8 to 9.3%).

The soaking time of seeds in 20% NaOCl has no influence on the germination rate of wet and dry pretreated seeds. For the same pretreatment, the germination rates of the seeds placed in culture remained more or less constant regardless of the soaking time of the seeds in 20% NaOCl. These germination rates are respectively around 21% and 8% for wet and dry pretreated seeds.

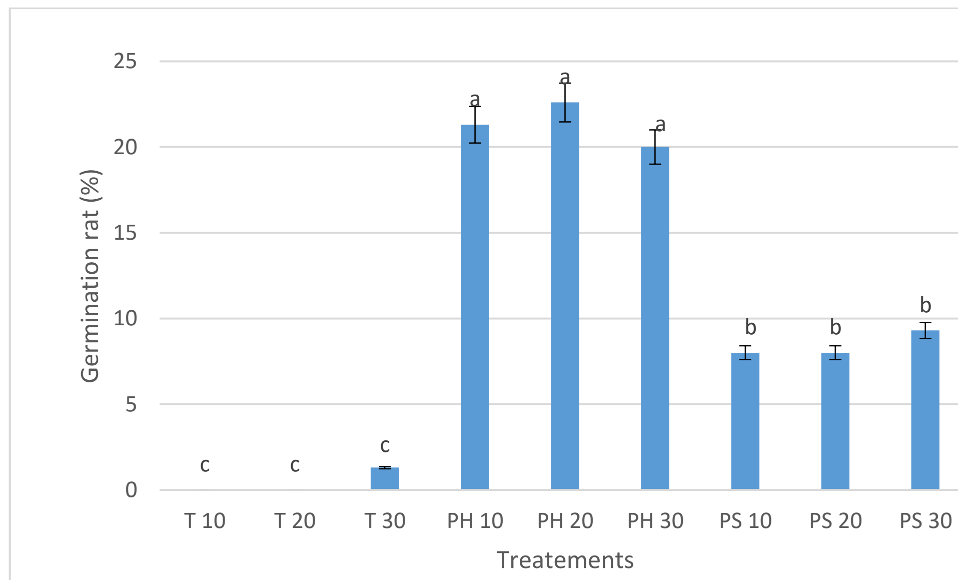

Figure 1: Effects of seed pretreatments and soaking time in 20% sodium hypochlorite of *L. multiflora* seeds on the germination rate after eight weeks of cultivation

T: Control; PH: Wet pre-treatment; PS: Dry pre-treatment; 10, 20, 30: soaking time (in minutes) of seeds in 20% sodium hypochlorite.

The histograms surmounted by the same letter do not show any significant difference according to the Newman and Keuls test at the 5% threshold.

3.3 Embryo culture contamination rate

L. multiflora embryo culture contamination rate is 0%, no culture was contaminated. This result shows the effectiveness of the disinfection method performed. In fact, fruit disinfection with 1% Mancozeb for one hour and soaked in 70° alcohol for 5 min, followed by immersion of its seed isolated in a 20% sodium hypochlorite solution for 20 min were the optimal conditions for surface sterilization of seeds.

3.4 Influence of the sucrose concentration on the root development of the embryo

The root growth kinetics (Figure 2) shows that the root part of the embryo has evolved according to the concentration of sucrose in the culture medium.

Figure 2 shows that in the culture medium without sucrose (control), the embryo began to develop its radicle after 3 weeks of culture. In culture media containing 10, 20, 40, 80 g/l of sucrose, embryos showed a beginning of evolution by elongation of radicles at the first week of culture. On the other side, with high concentration of sucrose (160 g/l), the embryos only developed their root system from the fourth week of cultivation.

Regarding the growth in length of the radicle, it varied according to the concentration of sucrose in the culture medium. After eight weeks of culture, the length of the embryos radicles was the greatest, reaching 7.8 mm in the culture medium with 80 g/l of sucrose. For the embryos on the media containing 20 and 40 g/l of sucrose, their radicles have respective average lengths of 3.2 mm and 4.5 mm in the medium without sucrose (control) and that supplemented with 160 g/ L of sucrose, the lengths of radicles were the shortest, 0.2 mm and 0.1 mm respectively.

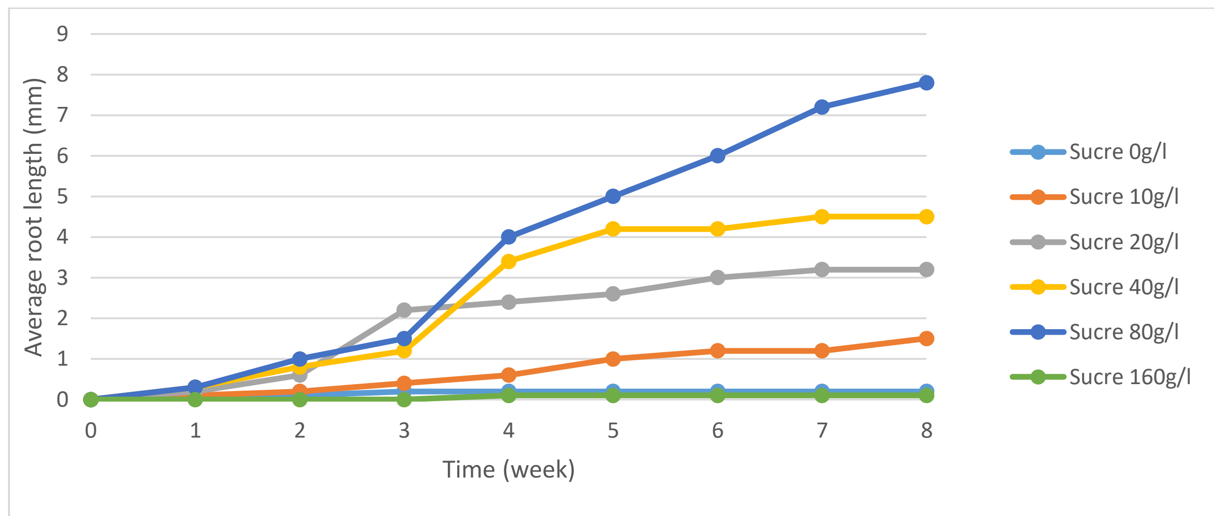

Figure 2: Speed of initiation and evolution of root length according to sucrose concentration

3.5 Influences of sucrose concentration on seedling growth

During eight weeks of cultivation, the growth kinetics of seedlings from zygotic embryos (Figure 3) varied according to the sucrose rate in the culture medium. Maximum growth in seedling length (4.5 mm) was obtained at the optimum sucrose concentration of 20 g/l. Sucrose concentrations of 10, 40, 80 g/l have no significant influence on the length growth of seedlings according to the Neman and Kewls test at 0.5%. The growth kinetics were less efficient there and the average lengths of the seedlings are 2.8 mm, 3 mm, 2.4 mm, respectively.

The most significant decrease in growth rate was recorded with the control medium and with that supplemented with 160 g/l of sucrose. On these culture media, the average lengths of the seedlings were respectively 1.6 mm and 1.4 mm after eight weeks of culture.

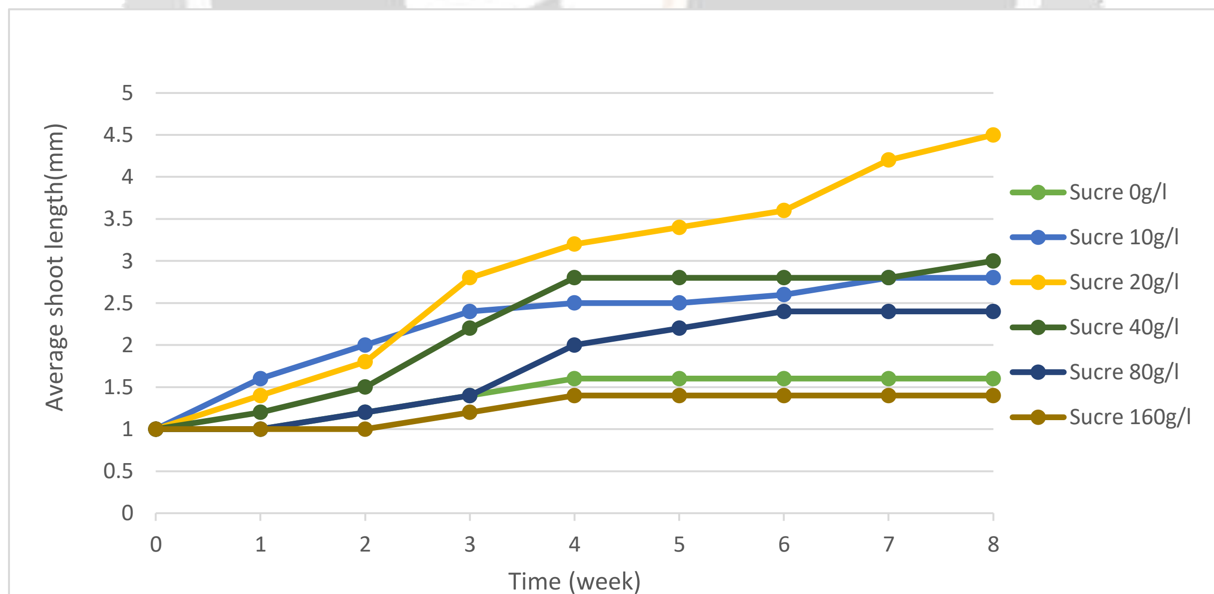

Figure 3: Growth kinetics and evolution of the average length of the seedlings according to the sucrose concentration.

3.6 Influences of sucrose concentration on the rate of embryos regeneration into plantlets

The sucrose amount in the culture medium strongly influences the embryo regeneration rate into a seedling. According to Table 4, after eight weeks of culture on the different media, the regeneration rates of the embryos into complete plantlets, with root, stem and leaves, varied according to the sucrose concentrations. The addition

of sucrose in the medium promotes the regeneration of the embryo into a seedling in *L. multiflora.* with 10 to 40 g/l of sucrose, the rate of embryo regeneration was increasingly high, up to 100%, as the sucrose concentration increased. However, for the high doses of 80 g/l and 160 g/l, the regeneration rates decrease to 50 and 10% respectively.

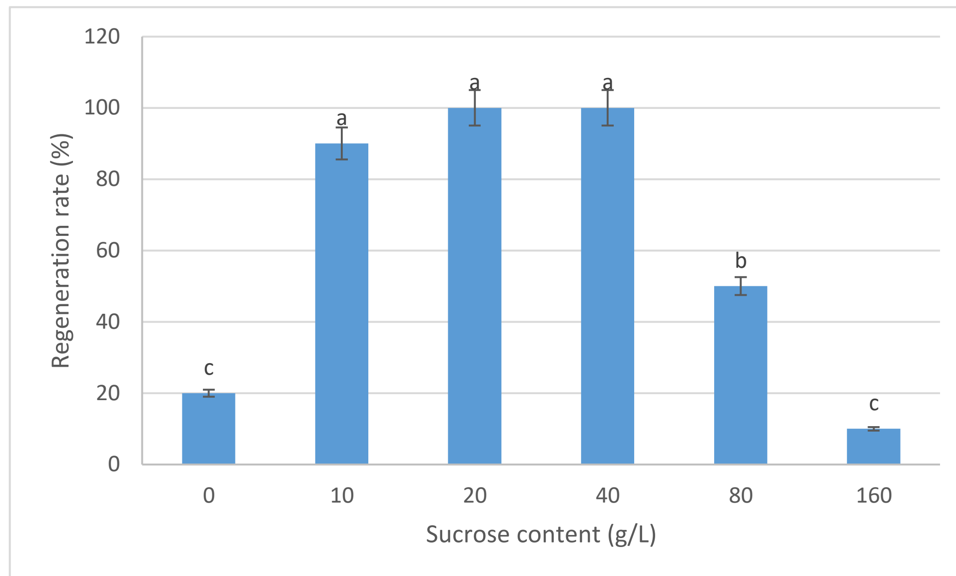

Figure 4: Effect of sucrose concentration on the rate of regeneration of embryos into seedlings after eight weeks of culture

The histograms surmounted by the same letter do not show any significant difference according to the Newman and Keuls test at the 5% threshold.

4. DISCUSSION

Soaking seeds in sterile distilled water for 48 hours before sowing (wet pretreatment) achieved the highest germination rate (22.6%). This could be due to the pre-treatment carried out, which softened the seed coats, making them more permeable to water and gases. For this purpose, dormancy will be breaking and the process of germination will be triggered. This result agrees with that of Rakoarisoa, (2013) who reported that the wet pretreatment method allowed the softening of the seed coat [10]. In addition, seed volume increased after 48 hours of water imbibition, which is reflected in water ingress into the seeds. This result is in agreement with that obtained by Aoudjit (2006) on the germination of *Pistacia atlantica* [11]; he reported that imbibition significantly increased the rate of seed germination.

Increasing the sucrose concentration (up to 40 g/l) accelerated embryo initiation, while the highest concentration of 160 g/l slowed the growth of the root system. This could be due to the inhibitory effect of the high dose of sucrose in the culture medium. Similar results were obtained by Bah et al (1989) on the *in vitro* culture of zygotic coconut palm embryos *Cocos nucifera* (L) [12]. According to Thomas (1980), sucrose in the *in vitro* culture conditions affects growth proportion and the location of mitoses [13]. This author specified that with a sucrose concentration of 100 g/l, cell divisions of mature *Pinus sylvestris* embryos slowed down. According to Ruven (1952), high doses of sucrose could inhibit cell elongation and differentiation. The composition of mineral salts in culture media, the supply of vitamins and especially sucrose in the culture medium are very important to obtain complete development of embryos cultured *in vitro* [14]. [15], [16], [17].

5. CONCLUSION

Leptolaena multiflora (L.) is endemic specie threatened with extinction of the Ambatovy mining site in Madagascar. The *in vitro* method of germination and culture of mature zygotic embryo of this species has highlighted interesting results. Seed surface sterilization conditions, *in vitro* seed germination and zygotic embryo culture from seed were optimized. Thus, the optimal condition for surface sterilization with a low

contamination rate of 1.3% has been developed. This involves soaking the seeds in a NaOCl solution at a concentration of 20% (m/v) for 20 minutes. At the end of the two pretreatment methods performed (wet and dry), the dry pretreated seeds showed a low germination capacity between 8 and 9.3%. In contrast, wet pretreatment by soaking seeds in sterile distilled water for 48 hours improved seed germination capacity with average germination rates of 20–22.6%

Regarding embryo culture, the results obtained have demonstrated that in vitro regeneration of L. multiflora is possible. The addition of sucrose at a concentration of 20 g/l in the culture media further promotes the regeneration of the embryos with a regeneration rate of 100%. However, with the high quantity of sucrose (160g/l), the regeneration of the embryo into a seedling was inhibited. Compared to the germination method, it should be emphasized that the embryo culture method is more advantageous because of the palpable results such as a higher regeneration rate of up to 100%, a faster regeneration rate, and early development with elongation of the radicles since the first week of culture in medium containing 20 g/l of sucrose

BIBLIOGRAPHY

[1] Schatz, G. E., 2001. Generic Tree Flora of Madagascar. Royal Botanic Garden et Missouri Botanical Garden.

[2] Hong-Wa, C., 2003. Description, distribution, écologie, utilisation et risques d'extinction des espèces du genre *Leptolaena Thouars sensu stricto* (Sarcolaenaceae, famille endémique malgache). Mém. DEA en Ecologie Végétale, Département de Biologie et Ecologie Végétales, Univ. Antananarivo. 60p.

[3] Dinaharilala M. T. G. ; Ramanampamonjy R. N., 2017. Caractérisation écologique des habitats et régénération naturelle de *Leptolaena multiflora* (Sarcolaenaceae), espèce menacée au sein du site d'exploitation minière d'Ambatovy en vue de sa conservation. Actes du Forum de la Recherche 14-15-16 juin 2017 à Antananarivo.

[4] Yildirim, H., Tilkat, E., Onay, A., Ozen, H.C. 2007. International Journal of Science and Technology, 2, 2, 99-104.

[5] Lachkar, A., Bayoudh C., Majdoub A. et Jraidi, B. 2011. Sauvetage *in vitro* d'embryons des variétés précoces d'abricotier (*Prunus armeniaca* L.) en Tunisie. ATBT

[6] Brigitte, M. et Yannick B. 1987. Utilisation de la culture in vitro d'embryons immatures Agronomie, 7 (5). pp 313-319.

[7] Champagnat. P., 1983. Quelques particularités biologiques des arbres, Bulletin de la Société Botanique de France. Actualités Botaniques, 130 (2). pp 11-20.

[8] Dinaharilala, M., T., G., 2012. Substitution des phytohormones synthétiques par des additifs organiques sur la micropropagation de *Aeranthes antennophora* et *Bulbophyllum peyrotii* deux espèces d'orchidées endémiques, menacées du site minier d'Ambatovy. Mém. D.E.A. en Physiologie Végétale, Dép. Ecologie et Biologie Végétales, Univ. Antananarivo, 47p.

[9] Jay-Allemand, C. et Cornu, D., 1986. Culture in vitro d'embryons isolés de noyer commun (Juglansregia L.). Ann. Sci. 43 (2). pp 1889 – 198.

[10] Rakoarisoa, J. M. 2013. Régénération *in vitro* et *in vivo* de *Dracaena sp. nov. 3* (DRACAENACEAE) et de *Psorospermum sp. nov. b* (CLUSIACEAE), deux especes forestieres menacees du sited'exploitation miniere d'ambatovy. Mém. D.E.A. en Physiologie Végétale, Dép. Ecologie et Biologie Végétales, Univ. Antananarivo, 41p.

[11] Aoudjit, M. H. 2006. Etude de la germination des grains du *Pistacia atlantica Desf.* (Pistachier de l'Atlas) et essai de multiplication de *Pistacia vera* L. (pistachier vrai) en pépinière selon deux types de greffes écusson et « chip budding ». Mém. D.M en Sciences Agronomique. Option Foresterie et Conservation de la Biodiversité. Institut National Agronomique El Harrach, 93p.

[12] Bah, A. B., Durand-Gasselin., Engelmann. 1989. Culture *in vitro* d'embryons zygotiques de cocotier *(Cocos nucifera L.).* Oléagineux, 44 (11).

[13] Thomas, M. J., 1980. Applications physiologiques de la culture d'embryon isolé. *In* : Connaissance de la biologie des arbres par les cultures *in vitro*. 2ème réunion de la section Francaise de l'IAPT sur l'arbre *in vitro*. 14-15 nov. 1980 à Paris.

[14] Ruven, A. H. G. C., 1952. *In vitro* studies on the embryo of *Capsella bursa pastoris*. Actu.Bot. Neerl. 1. pp. 157-200.

[15] Bouharmont, J. 1961. Embryo cultur of rice on sterile medium. Euphytica. 10. pp. 283-293.

[16] Lepage-Degiry, M. T. 1967. Développement *in vitro* d'embryons encore immatures au moment de la dissémination des semences chez quelques plantes ligneuses. Thèse de 3ème Cycle. Paris. 122p.

[17] Thomas, M. J. 1970. Premières recherches sur les besoins nutritifs des embryons isolés du *Pinus Sylvestris* (L). Embryons différenciés. C.R. Acad. Sci. 270. pp. 2648-2651.