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IN VITRO ASYMBIOTIC SEEDS GERMINATION OF 51 ENDEMIC, THREATENED ORCHID SPECIES OF MADAGASCAR

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ABSTRACT

The "Orchidaceae" is well represented in Madagascar with about 1500 species. Its extinction is related to the deforestation and to the massive and illegal collect. One technique of the conservation of endemic and threatened orchids of Madagascar is the *in vitro* asymbiotic seeds germination for their rapid reproduction. Besides, this method has the advantage of not damaging the mother plant. 51 species of orchids from Madagascar were the subject of this study. The seeds are from ripe capsules or unripe and dehiscent capsules or not. After surface sterilization of capsules, the seeds were sterilized in 0.5% of mancozeb for 50 min, then 10% of sodium hypochlorite for 15 min. The germination culture medium was composed of MS/2, supplemented with vitamins, 3% of sucrose and then solidified with 1% of agar. The results show that this culture medium is favorable for germination of 48 of the 51 species tested. The speed and capacity of germination varies with the species studied; respectively between 4 to 18 weeks and 15 to 92%. The best rate of germination (100%) was obtained with seeds from indehiscent ripe capsules. Non-germinated seeds were either from dehiscent capsules or green immature capsules. Thus, seeds from ripe indehiscent capsules are recommended for *in vitro* germination of Malagasy Orchids.

KEYWORDS: Asymbiotic, germination, *in vitro*, Madagascar, Orchids, seeds, symbiosis.

INTRODUCTION

Plant micropropagation technique has been widely used for commercial production of ornamental plants, food plants (Bajaj, YPS, 1991), medicinal and aromatic plants (Bajaj, YPS, et al. 1988) as well as forest and fruit trees, especially those the propagation is difficult. (BAJAJ, Y., 1991). This technique was used to propagate about 1000 plant species (MURASHIGE, T, 1989).

The intensive multiplication technique of endangered endemic Orchids of Madagascar was adopted in order to obtain healthy plants in all seasons, with very high speed of multiplication.

Furthermore, mass production for trading made the Orchids so popular. *In vitro* culture methods could also be used to save some endangered species due to the degradation of their habitat and abusive collection (BOURRIQUET, R. 1985).

Positive results have already been obtained on propagation from the apex of the bulb of *Bulbophyllum rauhii*, of axillary buds of *Angrecum sororium* and protocorms sections of *Angraecum eburneum superbum* (RAHELIVOLOLONA, R., 1999). Indeed, further work is very interesting to be undertaken in order to preserve endangered endemic malagasy orchids and overpower the techniques of micropropagation.

Taking into account the results already obtained in our laboratory and based on the vitropropagation technology on exotic orchids and other ornamental plants, *in vitro* asymbiotic seeds germination of Madagascar orchids has been done. The aim of this study is the rapid multiplication through seeds. It was also used not to damage the mother plant during the reproduction and the conservation of these species.

1. MATERIAL

1.1. BIOLOGICAL MATERIALS

51 species of endangered endemic Orchids of Madagascar, listed in Appendix II of CITES have been tested. The list and maturity of capsules from plants cultivated into the orchidarium of horticulturists are given in the table below.

Table 1: List and capsules maturity of the species tested in the Laboratory.

Taxa	Maturity of capsules
Acampe pachyglossa	green
Aerangis citrata	ripe
Aerangis cryptodon	green
Aerangis curnowiana	green
Aerangis françoisii	ripe
Aerangis fuscata	ripe
Aerangis modesta	ripe
Aeranthes henricii	ripe
Aeranthes longipes	ripe
Aeranthes sp	ripe
Angraecum ankaranense	dehiscent
Angraecum compactum	ripe
Angraecum equitans	ripe
Angraecum filicornu	ripe
Angraecum finetiana	ripe
Angraecum germinianum	ripe
Angraecum leonis var. Diego	ripe
Angraecum leonis var.Maevatanana	ripe
Angraecum madagascariense	ripe
Angraecum magdalenae	ripe
Angraecum mahavavense	dehiscent
Angraecum pannicifolium	ripe
Angraecum sororium	ripe
Angraecum viguieri	green
Beclardia macrostachya	ripe
Beclardia sp	ripe
Bulbophyllum occlusum	ripe
Bulbophyllum sp	ripe
Bulbophyllum sp	ripe
Bulbophyllum sp	ripe
Calanthe sylvantica	ripe
Cymbidiella humlotii	green
Cymbidiella rhodochila	ripe
Eulophiella roempleriana	dehiscent
Grammangis ellisii	ripe
Grammangis spectabilis	ripe
Jumellea arborescens	ripe
Jumellea françoisii	ripe
Jumellea gladiator	green
Jumellea gracilipes	ripe
Jumellea maxillaroides	ripe
Jumellea teretifolia	ripe
Jumellea sp	ripe
Jumellea sp	ripe
Jumellea sp	ripe
Microcoelia gilpinae	ripe

Neobathiea perrieri	ripe
Oeonia volucris	green
Polystachya falcigera	ripe
Polystachya sp	ripe
Sobennikoffia robusta	dehiscent

These species are among the most exploited to satisfy the demand of national and/or international trade. The capsules bought at the market are of wild origin while those provided by horticultural societies are grown in orchidarium.

2. METHODS

The non-symbiotic germination is to germinate the seeds of orchids on culture medium containing all the nutrients necessary for their development to produce green protocorms without fungal infection.

A general test of *in vitro* asymbiotic seeds germination of Madagascar Orchids was performed on the basis medium Murashig and Skoog in half strength MS/2 (1962) supplemented with vitamins, myo-inositol and sucrose.

As any *in vitro* culture, asymbiotic germination requires prior control of sterilization. This sterilization technique is based on the combination of different types of disinfectants with different soaking time depending on the case.

2.1. Surface sterilization of plant material

Surface sterilization of plant material is performed before seed germination. Subsequently, the explants taken from in vitro condition didn’t require a new sterilization, but directly used depending on the objective. Seeds from dehiscent or indehiscent, green or ripe capsules have been used.

Before germination, all capsules or seeds are surface sterilized because they contain microorganisms adhered to the outer surface.

- **Indehiscent capsules**

They are rinsed with tap water and brushed lightly. Thereafter, they are sterilized by the fungicide solution "mancozeb" 1% added 2 drops of Tween 20 for 1 hour, then rinced 4 times with sterile distilled water. They are soaked in a sodium hypochlorite (NaOCl) to 70% added

2 drops of Tween 20 for 20 minutes. Finally rinced 4 times successively with sterile distilled water.

- **Dehiscent capsules**

The seeds are put into a test tube filled with mancozeb of 1% solution added with a drop of Tween 20 for 30 minutes then rinsed 4 times with sterile distilled water. Thereafter, they were soaked in sodium hypochlorite 10% for 15 minutes and rinsed 4 times successively with sterile distilled water.

The evaluation parameter was the rate of contamination which is defined by the number of contaminated cultures divided by the total number of inoculated tubes multiplied by 100.

2.3. Culture medium

Seeds from 51 species of Madagascar Orchids are sown on the basic medium of Murashige and Skoog (1962), in half strength (MS / 2), supplemented with vitamins 10 mg / L Thiamine-HCl + 1 mg / L Pyridoxine-HCl + 1 mg / L nicotinic acid + 0.01 + Biotin 100 mg / L myoinositol and 3% (w / v) sucrose.

PH of the medium was adjusted to 5.5 with 1N KOH or HCl (STENBERG.M E, 1998. Satinder Kaur, 1997; YANAGAWA, T, 1995; BENLINDEN 1980; DALLA ROSA M & Laneri, U., 1977).

All culture media were solidified with agar 1%. The culture media were sterilized by autoclaving at 121°C for 20 minutes and at a pressure of 1.1 bar.

After autoclaving, culture medium was distributed into test tubes each containing 5 ml of medium. Stock solutions were stored at + 5°C up to 3-4 weeks SMITH & MOÏSANDER J., 1991; AUGE, R., et al. 1989).

2.4. Culture Conditions

The cultures are incubated in a culture room at a temperature of 25°C, under a light intensity of 3,000 lux and a photoperiod of 16 h (light) / 8h (dark).

2.5. Monitoring and evaluation

Observations every 2 days were carried out regularly on all cultures. The evolution of seed color and obtaining white and green protocorms were noted. Germination capacity, defined as

the percentage of seeds capable of germination in specific conditions. (Como, 1970; CHAUSSAT & DEUNF, 1975), is evaluated by the number of green protocorms obtained divided by the number of seeds sown, multiplied by 100. Germination rate is the time taken for the seed to germinate. Time for a week after the emergence of first green protocorms was chosen.

2.6. 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 at the probability threshold of 5%.

3. RESULTS

3.1. Speed and germination capacity

The speed and capacity of germination of each species is given in Table 2.

Table 2: Speed and germination capacity of 51 endemic orchid species of Madagasca

Taxa	Maturity of capsules	Speed of germination (week)	Capacity of germination (%)
Acampe pachyglossa	Verte	11	84
Aerangis citrata	Mûre	10	68
Aerangis cryptodon	Verte	10	15
Aerangis curnowiana	Verte	12	59
Aerangis françoisii	Mûre	6	63
Aerangis fuscata	Mûre	6	60
Aerangis modesta	Mûre	8	78
Aeranthes henricii	Mûre	6	66
Aeranthes longipes	Mûre	5	92
Aeranthes sp	Mûre	5	81
Angraecum ankaranense	éclatée	0	0
Angraecum compactum	Mûre	8	67
Angraecum equitans	Mûre	7	92
Angraecum filicornu	Mûre	6	89
Angraecum finetiana	Mûre	8	72
Angraecum germinianum	Mûre	13	65
Angraecum leonis var. Diego	Mûre	6	90
Angraecum leonis var.Maevatanana	Mûre	5	87
Angraecum madagascariense	Mûre	9	65
Angraecum magdalenae	Mûre	5	88
Angraecum mahavavense	Eclatée	0	0
Angraecum pannicifolium	Mûre	8	70
Angraecum sororium	Mûre	4	85

Angraecum viguieri	Verte	12	77
Beclardia macrostachya	Mûre	10	65
Beclardia sp	Mûre	9	72
Bulbophyllum occlusum	Mûre	10	78
Bulbophyllum sp	Mûre	10	56
Bulbophyllum sp	Mûre	10	83
Bulbophyllum sp	Mûre	11	67
Calanthe sylvantica	Mûre	18	68
Cymbidiella humlotii	Verte	18	62
Cymbidiella rhodochila	Mûre	16	62
Eulophiella roempleriana	éclatée	10	71
Grammangis ellisii	Mûre	8	70
Grammangis spectabilis	Mûre	6	62
Jumellea arborescens	Mûre	8	58
Jumellea françoisii	Mûre	12	63
Jumellea gladiator	Verte	0	0
Jumellea gracilipes	Mûre	8	76
Jumellea maxillaroides	Mûre	11	69
Jumellea teretifolia	Mûre	8	85
Jumellea sp1	Mûre	13	60
Jumellea sp2	Mûre	9	67
Jumellea sp3	Mûre	12	66
Microcoelia gilpinae	Mûre	7	80
Neobathiea perrieri	Mûre	13	92
Oeonia volucris	Verte	8	48
Polystachya falcigera	Mûre	12	67
Polystachya sp	Mûre	13	85
Sobennikoffia robusta	Eclatée	6	50

This table shows that the seeds of 48 species of the 51 tested germinated on medium MS 2, supplemented with vitamins and without symbiotic way. While for the seeds of *Angraecum ankaranensis, Angraecum mahavavense and Jumellea gladiator*, no change was observed until the end of the experiment.

The speed and the germination capacity vary from one species to another. In this case, they are from 4 to 18 weeks with 15 to 92%. The reaction of the seeds is different even on the species of the same genus, for example:

**Angraecum sororium:* 4 weeks with a 85% germination capacity, whereas 7 semaines with 67% for *Angraecum compactum.*

**Grammangis ellisii:* 8 weeks with 70% while 6 weeks with 62% for *Grammangis spectabilis.*

Seed from green capsules of *Acampe curnowiana, Angraecum viguieri, Cymbidiella humblotii, Jumellea gladiator* and *Oeonia volucris* release phenolic compounds, resulting in browning or yellowing or reddening of culture medium. Their effect intensifies with time. Transferred every 15 days on new culture media, the seeds have sprouted, except those of *Jumellea gladiator.*

Seed from *Aerangis curnowiana* green capsule have not germinated. A month later, with seeds from unripe capsule, germination capacity of 59% was obtained.

According to Table 3, the observations show that the best seed germination rate (100%) was obtained with seeds from ripe and indehiscent capsules, and non-germinated seeds originate either from dehiscent capsules or from green capsules.

Table 3: *in vitro* germination rate of seeds depending on the capsules maturing stage.

Capsule state	Dehiscent	Green	Ripe and indehiscent
Germination rate (%)	33.33	85.71	100

DISCUSSION

The seeds of 48 species of Malagasy endemic Orchids tested, sprouted on the medium MS / 2, supplemented with vitamins and in asymbiotic way. However, the speed and germination capacity vary by species. Towards the end of the experiment, observations under the binocular of non-germinated seeds show the presence or absence of the embryo.

Seed quality is one of the factors in the success of the germination. Note that the majority of tested capsules at the laboratory are from natural pollination. In this case, the fertilization is random, produced by pollination vectors (DAVIS, R.W., 1986; BOWLES, M. L., 1983; Brownell, A. V. 1981). One of the causes of low germination capacity is the absence of pollination. Without pollination, flowers produce empty capsule or sterile seeds without embryo (parthenocarpic fruit).

- **Seeds from green capsule**

Jumellea gladiator seeds from green capsule have not germinated. The observation under a binocular microscope shows seeds with and without embryo. Once sown on germination medium, they do not develop. Firstly, the seeds without embryo are sterile; secondly,

embryogenesis can be incomplete resulting in an incompletely formed embryo incapable of germination for those with embryo (LUCKE, E., 1981, VEYRET, Y., 1969).

For this experiment, the seeds from green capsules have generally low germination. Table 4 shows some examples of species in which the germination rate is slow.

Table 4: Duration (week) of *in vitro* germination of same species.

Taxa	Time of *in vitro* germination (week)
Oeonia volucris	8
Aerangis cryptodon	10
Acampe pachyglossa	11
Aerangis curnowiana	12
Angraecum viguieri	12
Cymbidiella humblotii	18

It seems that the completion of embryogenesis takes time before germination. In such condition, the seeds from *Grammangis ellisii* ripe capsule germinated after 8 weeks. A subsequent experiment performed with the same experimental conditions, on immature seeds gave a delayed germination of 16 weeks. Another example, seeds from *Aerangis curnowiana* green capsules have not germinated. One month after the first experience of this kind, a second experiment was made, but on older green capsule, germination occurred with a germination capacity of 59%.

It is therefore very important to know the optimal date for the capsule collection. The capsule optimal age for embryo cultivation varies with the genus, species, hybrid and local growing conditions. (ARDITII, J., et al. 1982). This time could be determined experimentally (ARDITI, J., et al. 1982). Some examples of exotic species are shown on Table 4).

Table 4: Time interval (in days) between pollination and *in vitro* embryo culture (SOURCE:. ARDITTI and al 1982; FAST, 1980; FROSH 1980; SAGAWA and WALMAYAR 1966; SAULEDA 1976).

Species	Time interval (days)
Aerides odoratum	150 – 180
Ansellia species et hybrides	150 – 180
Ascocenda	120 –150
Brassovola cucullata	75 - 80
Broughtonia sanguinea	32-34
Cattleya bifoliate	110-150
Cattleya labiata	130-180
Cirrhopetalum	140-180

Cymbidium	280-360
Cypripedium	30
Cyrtopodium	150-270
Dendrobium devonianum	160-250
Doritaenopis	90
Encyclia	130-180
Epidendrum atropurpureum	150-160
Laelia harpophylla	110-120
Laeliocattleya hybrides	120-180
Maxillaria	120-140
Miltonia candida	120-140
Odontoglossum	80-90
Oncidium altissimum	110-140
Paphiopedilum	240-300
Phalaenopsis species	110-120
Renanthera	150-180
Sophrocattleya	110-150
Sophrolaelia	110-150
Sophronitis	75-100
Vanda cv Patricia lee x Ascocenda cv Mem. Jim. Wilkins.	90-150

The capsule harvest time after pollination has a significant effect on germination. For endemic Orchids of Madagascar, scientific experiments should be conducted on this.

Seeds from green capsules release of phenolic compounds which may cause explants death. The use of antioxidant products such as activated carbon, polyvinilpyrolidone (PVP) or ascorbic acid may provide an effective solution (ERNEST R., et al. 1971). However, the charcoal stimulates the hydrolysis of sucrose, which is normally 10% during autoclaving. This rate increases to 95% in the presence of activated carbon which leads to a pH lowering and increased osmolarity, resulting in rigidity of the agar medium (DRUART & DEWLF, 1993). In addition, activated carbon, because of its ability to adsorb different products distributed in the culture medium, is no exception with the elements the seedling needs so that a growth retardation can occur (DE PAUW, MA et al. 1993). Therefore, to avoid poisoning explants, they are transferred every 15 days, on fresh media. Not to waste too much medium, the amount per test tube is approximately of 5 ml.

- **Seeds from mature indehiscent capsules**

All species from these indehiscent ripe capsules germinated. The speed and germination capacity vary widely depending on the species, from 4 to 18 weeks with 56 to 92%. These changes appear to be caused by:

- The genetic character of the species or variety;
- a seed quality: the number of fertilized seeds by the total number of seeds inside capsules, knowing that each ovary contains thousands or even millions of eggs (ARDITTI, J, 1967; KOOPOWITZ, H, 2001);
- In the culture conditions. It seems that each species has its own requirement. In our experimental conditions, seeds from indehiscent ripe capsules give the best results compared to those from green capsules and those already dehiscent capsules. This can be attributed to the fact that mature non-dormant seeds produced by a non-dehiscent fruit still retain moisture within their structure (BEN LINDEN 1980). Besides this moisture, embryogenesis is fully completed. It is immediately able to resume the development cycle as soon as the seeds are in contact with the culture medium.

- **Seeds from dehiscent capsules**

The seeds of *Angraecum mahavense and Angraecum ankaranensis* did not sprout. They emerge from the already broken capsules like those of *Eullophiella roempleriana* and *Sobennikoffia robusta.* However, those of the last two species germinated, giving respectively 71 and 50%.

Observations of non-germinated seeds of *Angraecum mahavense* and *Angraecum ankaranensis* show the presence of embryo. Unlike seeds from indehiscent capsule, those from broken capsules have a greater state of dehydration and permeability of their wall is lower (BEN LINDEN 1980), making it more difficult soaking seeds (BALLARD, W.W, 1987). On the other hand, the Orchid seeds become dormant once they complete their embryonic development (BALLARD, W.W, 1987). To have success in germination, dormancy should be broken.

Another cause of this failure could be the loss of germination capacity (HICKS, J.A, 2001) by the capsule harvest delay. These reasons confirm the importance of knowing the optimum capsule harvest time.

In addition, the storage conditions may result in the germination capacity decrease or seeds death (HICKS, J. A 2001). The presence of well-formed embryo does not mean that the seeds are able to germinate.

Information on the duration and conditions of storage of samples, the viability of the embryo and seed germination capacity on the germination medium used is insufficient or absent. Furthermore, the seeds of orchids are devoid of reserves to ensure the survival of the embryo and each species has its own requirement. It is therefore necessary to use techniques to verify the viability of seeds, such as using the triphenyl tetrazolium chloride (TTC) and to determine the different factors involved for maximum germination of each species.

According to these results, we recommend using seeds from indehiscent ripe capsules. In our experimental conditions, the speed of germination of Malagasy orchids species varies widely from one species to another. Some have a relatively fast rate unlike some exotic orchid species that take months or even years to germinate (KATO, M., & TAHARA, M, 2001; CLEMENTS & ELLYARD, 1979). Some exhibit a relatively slow rate but does not exceed 18 months.

CONCLUSION

The study on asymbiotic seeds germination of 51 species of Madagascar orchids showed that 48 sprout on the basic medium Murashig and Skoog in half strength (MS/2), supplemented with vitamins after 4 to 18 weeks with a germination capacity of 15 to 92%. These results indicate that the reaction of the seeds varies from one species to another.

The asymbiotic seeds germination of orchids depends on the intrinsic and extrinsic factors. The first factor is related to the quality, reliability, maturity and seed dormancy. Knowledge of optimal capsules harvest time, on of the condition for the successful asymbiotic seeds germination should be studied in detail later. Likewise, we recommend the use of verification techniques of seed viability as one with the TTC (Triphenyl tetrazolium chloride) which ensures the desired results and saving time and materials. The symbiosis problem does not arise during our experiments on *in vitro* germination of these 48 species of endemic malagasy Orchids tested in the laboratory.

Nevertheless, this work has to implement the conservation of threatened endemic species. Positive results were obtained. This work largely contributes to the conservation of valuable species of Malagasy biodiversity.

REFERENCES

1. ARDITTI, J., CLEMENTS, M., FAST, G., HADLEY, G., NISHIMURA, G. And ERNEST, R., 1982. Orchid seed germination and seedling culture – a manual. In Orchid biology – reviews and perspectives, II. Edited by J. ARDITTI. Cornell University Press, New York.
2. ARDITTI, J., 1967. Factors affecting the germination of orchid seeds. The Botanical Review, 33(1): 1-97.
3. AUGE, R., 1989. Les phénomènes physiologiques liés à la réalisation des cultures *in vitro*. Dans la culture *in vitro* et ses applications horticoles. J. B. Baillère, éd., Lavoisier, Paris, 17-39.
4. BAJAJ, Y.P.S., FURMANOWA. M., OLSZOWSKA, O., 1988. Biotechnology of micropropagation of medicinal and aromatic plants. In: Bajaj Y.P.S (ed) Biotechnology in agriculture and forestry, vol 4. Medicinal and aromatic plants I. Springer, Berlin Heidelberg New York, 60-103.
5. BAJAJ, Y.P.S., 1991. Biotechnology in agriculture and forestry, vol 17. High. Technology and micropropagation (eds) YPS Bajaj. Springer, Berlin. Heidelberg New York.
6. BALLARD, W. W., 1987. Sterile propagation of *Cypripedium reginae* from weeds AOS Bull, 56: 935-946.
7. BEN LINDEN, 1980. Aseptic germination of seeds of northern terrestrial orchids Ann. Bot; Fennici, 17: 174-182.
8. BOURRIQUET, R., 1985. La culture "*in vitro*" des Orchidées. CHARLEVAL Journées des Orchidées 13-15 Avril 1985. Association Culturelle Municipale 27380 Charleval. 10p.
9. BOWLES, M.L., 1983. The tall grass prairie orchids *Plantanthera leucophaea* (Nutt.) Lindl. and *Cypripedium candidum* Muhl. ex Willd. Some aspects of their status, biology and ecology and implications toward management. Nat. Areas J., 3: 14-37.
10. BROWNELL, V. R., 1981. The small white lady's-slipper *Cypripedium candidum* Muhl. Ex Wild.) in Canada : a status report. Wildlife Branch, Ontario Ministry of Resources, TORONTO, Ontario.
11. CHAUSSAT, R. & Le DEUFN, Y., 1975. La germination des semences. Gauthier Villars: 232p.
12. CLEMENTS M. A. and ELLYARD R. K., 1979. The symbiotic germination of Australian Terrestrial Orchids American Orchid Society Bulletin, 48(8): 810-816.

13. COME, D., 1970. Les obstacles à la germination. Ed. Masson et Cie, Paris: 162p.
14. DALLA ROSA, and M., LANERI, U., 1977. Modificationn of nutrient solutions for germination and growth in vitro of some cultivated orchids and for the vegetative propagation *Cymbidium* cultivars. Amer. Orchid Soc. Bull., 46(9): 813-820.
15. DAVIS, R W., 1986. The pollination biology of *Cypripedium acaule* (Orchidées). Rhodora, 88: 445-450.
16. DE PAUW, M. A. and REMPHREY, W. R., 1993. *In vitro* germination of three *Cypripedium* species in relation to time of seed collection, media and edd treatment. Can. J. Bot., 71: 879-885.
17. DRUART,P. and DEWULF,O., 1993. Activated charcoal catalyses sucrose hydrolysis during autoclaving. Plant Cell Tissue Organ Cult., 32: 97-99.
18. ERNEST, R., ARDITTI, J., & HEALEY, P., 1971. Carbohydrate physiology of orchid seedling. II. Hydrolysis and effects of oligosaccharides. Am. J., Bot., 58: 827-835.
19. FAST G., 1982. European terrestrial Orchids Symbiotic and asymbiotique methods. In Orchid biology – reviews and prospective. II Edited by J. ARDITTI Cornell University Press, Ithaca., 309-326.
20. FROSCH, W., 1980. Asymbiotische Aussaat von *Orchis morio orchidee* (*Hamburg*), 31: 123-124.
21. HICKS A. J., 2001. Asymbiotic Technique of Orchid Seeds Germination. Ed. Robert Huber. In United States of America., 134p.
22. KATO M. AND TAHARA M., 2001. Embryogenic callus formation in Calanthe APOC7 Poster session 2B-5-3 PS 6 March 2001, Japan.
23. KOOPOWITZ. H., 2001. Orchids and their conservation, Batsford Ltd. London 175p.
24. LUCKE E., 1981. Samensteuktur und Samenkeimung europaïcher Orchideen nach Veyret sowie weitere Untersuchungen (Teil 1). Orchidee (Hamberg), 32: 183-188.
25. MURASHIGE, T. and SKOOG, F., 1962. A revised medium for rapid growth and bioassays with *tobacco* tissue cultures. Physiol. Plant., 15: 472-497.
26. MURASHIGE, T. 1974. Plant propagation through tissue culture. Ann. Rev. Plant. Physiol., 25: 135-166.
27. RAHELIVOLOLONA, R., 1999. Contribution à l'étude de « conservation » des orchidaceae de Madagascar, mise au point de techniques de vitropropagation, Mem. D.E.A., Physiol. Vég. Dép. B. E. V. Fac; Sci. ANTANANARIVO. 64p.
28. SAGAWA, Y., SHOJI, T., 1966. Clonal propagation of *Cymbidium* though shoot meristem culture. Amer. Orchid Soc. Bull., 35: 118-122.

29. SATINDER KAUR., SARMA, C. M., 1997 selection of best medium for *in vitro* propagation of *Dendrobium lindleyi* stend Ad. Plant Sci., 10(1): 1-5 ISSN 0970-3586 ED BY Prof. Metha P. M.
30. SMITH D. R. M., and MOISANDER J., 1991. Plant tissue culture general principles. Information series Q 1899002 VIII-31pp.IO=O-2742-321.
31. STENBERG M. L. and KAN M. E. 1998. In vitro seed germination and green house cultivation of *Encyclia boothiana* var *erythronioïdes* An endangered floroda orchid. Lyndleyana, 13(2): 101-112.
32. VEYRET, Y, 1969. La structure des semences des Orchidées et leur aptitude à la germination *in vitro* en cultures pures. Mus. Hist. Nat. Paris. Traw. Lab. La Jaysinia, 3: 89-98.