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EFFECTS OF EXTRINSIC FACTORS ON ASYMBIOTIC GERMINATION OF THREATENED ENDEMIC MALAGASY ORCHIDS: *EULOPHIELLA ROEMPLERIANA, GRAMMANGIS ELLISII* AND *GRAMMANGIS SPECTABILIS*

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ABSTRACT

The endemic Orchids of Madagascar are threatened with extinction because of the deforestation and their excessive collect. It's urgent to search the means of reproduction and the preservation in order to limit this danger. The control of the technics appropriated to the malagasy orchids especially for the species *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabilis*, was the main objective of this investigations. *In vitro* seeds germination of orchids depends on intrinsic factors related to the quality, viability, maturity of seed and on extrinsic factors such as physical and chemical conditions: temperature, light, culture medium. In order to obtain high rate of germination, some factors have been assessed successively. The interaction of pretreatment 26°C during 10 days followed by the treatment of 15 days in the dark condition, has improved the speed and capacity of germination of *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabilis*. While pretreatment 7°C delay the germination of these three species. The three basis media of MS/2 and those of Knudson, Vacin and Went each added with vitamin proved to be suitable for the germination of these 3 species. The sucrose failure in the medium inhibits their germination. The sucrose concentration to 30g/l has given a best result.

Keywords: Asymbiotic, germination, Madagascar, orchid, *Eulophiella roempleriana, Grammangis ellisii, Grammangis spectabilis.*

INTRODUCTION

Madagascar is characterized by its high floristic richness with a rate of endemism around 85%. But it is ranked among the 25 most critical areas for the preservation of life on earth "hot spots". Madagascar is third after the tropical Andes and the Mediterranean Basin, given the fast pace of environmental degradation (MITTERMEIER, et al. 1999). The family of orchids is one of the striking examples of this biodiversity loss. (ANGAP et al, 1998; OLIARIJAO, 2000, RAHELIVOLOLONA, 2001; BORIQUET, 1985).

Orchids are best known by the quality of their flowers and very popular with gardeners and collectors worldwide. They are an important source of currency. 150 malagasy Orchid species are estimated present on the international market (OLIARIJAO, 2000). Several species as *Aeranthes henricii, Cymbidiella humblotii, Cymbidiella rhodochila, Eulophiella roempleriana, Eulophiella elisabethae* ... are among the most wanted Orchids in the world. In addition to the worldwide reputation of endemic Malagasy orchids, in Madagascar, they are also used by man for various purposes, some of which are considered like food plants, others are used as medicinal plants.

For these reasons, it makes sense that they are the subject of active research both basic and applied.

Modern technological measures, focused on artificial propagation *in vivo* and *in vitro* method are a form of *ex situ* conservation of orchid species. The aim is to produce rapidly a high number of healthy, viable plants.

Since the famous experiment of MOREL (1960) on the *Cymbidium,* Orchid micropropagation was a huge success and considerable growth in developed countries. It was a revolution in Orchid propagation methods. Subsequently, many researchers have tried to improve the method (Eeyore, 1985). Orchids can be rapidly multiplied *in vitro* either by propagation using germination or vegetatively methods based on the technique of micropropagation or inducing protocorm regeneration.

After a general test of the asymbiotic seeds germination of 51 Malagasy Orchid species, further study on the impact of various external factors on the *in vitro* seeds germination of 3 species: *Eullophiella roempleriana, Grammangis ellisii, Grammangis spectabilis* was performed.

1. MATERIALS

Eulophiella roempleriana, Grammangis ellisii, Grammangis spectabilis are subject to more detailed study. The choice is based on the fact that they are rare when they were in high demand and are important in international trade since 1990 and in the domestic market (ANGAP, et al., 1998, OLIARIJAO, 2000).

The list and maturity of capsules from plants cultivated in orchidarium of horticulturists are given in the table below

Table 1: List and mature capsules of the three species tested.

Taxa	Capsule maturity
Eulophiella roempleriana	Ripe dehiscent
Grammangis ellisii	Ripe indehiscent
Grammangis spectabilis	Ripe indehiscent

2. METHODS

Various factors for *in vitro* asymbiotic seeds germination of *Eulophiella roempleriana, Grammangis ellisii and Grammangis spectabilis*, were studied. In this case, three series of experiments were performed.

- Pretreatment influences of temperature combined with the light / dark, followed by treatment in the dark.
- Comparison of three basal culture medium: MS/2, KN, VW
- Sucrose concentration effects.

Each treatment consists of 4 repetitions with 200 seeds per replication.

2.1. Influence of temperature, light and dark on the asymbiotic seeds germination

Mature seeds, milky white, apparently dry, from indehiscent ripe capsule are used. After surface sterilization, the seeds are divided into 3 groups (A, B, C) for 10 days pre-treatment. After, seeds of each lot are inoculated into 12 tubes containing MS/2 solid medium, supplemented with vitamins, sucrose and they will undergo the treatment (Table 2). The seeds of each lot in 12 tubes represent 3 treatments which are repeated 4 times. For each treatment in darkness, all culture is transferred in light at 16 hours a day.

Tableau 2: Study of the effect of pretreatment during 10 days and of different treatments on germination of Eulophiella roempleriana, Grammangis ellisii, Grammangis spectabilis.

Batch		**A**			**B**			**C**		
Pretreatment (before sowing)	Temperature (°C)	26	26	26	26	26	26	7	7	7
	Light or dark	Light	Light	Light	Dark	Dark	Dark	Dark	Dark	Dark
Treatment in darkness (day)	Day	0	15	30	0	15	30	0	15	30

2.2. Comparison of 3 basic culture medium on the asymbiotic germination of Eulophiella roemplariana, Grammangis ellisii and Grammangis spectabilis

After pretreatment at 26°C in the dark for 10 days, the seeds are sown on the surface of previously prepared media KN, MS / 2, VW, supplemented with vitamins and sucrose. These media are among the most used media in orchids (BROLY, 1982). Moreover, they are treated in complete darkness for 15 days before being transferred under an illuminance of 3000 lux for 16 hours a day and at the same temperature of 26°C.

2.3. Effect of sucrose concentration on the asymbiotic seeds germination

Germination of exotic orchids has shown that the sugar requirement varies according to the species (Arditti, J. et al. 1982). Different sucrose concentrations are studied: 0, 10, 20, 30, 40, 50 and 6g/L. The seeds were sown only on MS/2 medium, supplemented with vitamins and will undergo the dark treatment for 15 days. Thereafter, seeds were exposed to light 16 hours a day under a light intensity of 3000 lux at the temperature of 26 +/- 2°C.

2.4. Monitoring and evaluation

Observations every 2 days were carried out regularly on all cultures. The evolution of seed color and obtaining of white and green protocorms are noted.

Germination capacity, defined as the percentage of seeds capable of germination in specific conditions (Como, 1970; CHAUSSAT and DEUNF, 1975), is evaluated by the number of green protocorms obtained divided by the number of seeds sown, multiplied by 100. Speed of germination rate is the time taken for the seed to germinate. We used the time for a week after the emergence of first green protocorms.

2.5. 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

The germination process comprises imbibition and swelling of the seed still covered integument; the integument tear and the separation of the embryo, then progressive enlargement thereof, forming a spherule called "protocorm"; the formation of a growing point at the top and progressive greening of the protocorm with chlorophyll formation.

Green protocorm with a growing point, able to multiply or/and to differentiate thereafter mark an effective germination. The germination rate and speed of each species is given in Table 2.

Table 2: Germination capacity Speed *Eulophiella roempleriana; Grammangis ellisii; Grammangis spectabilis.*

Taxa	Capsule maturity	Speed of germination (week)	Germination capacity (%)
Eulophiella roempleriana	Ripe dehiscent	10	71
Grammangis ellisii	Ripe indehiscent	8	70
Grammangis spectabilis	Ripe indehiscent	6	62

The speed and the germination capacity vary from one species to another and also vary within the same genus. They are 8 weeks with 70% for *Grammangis ellisii*; while they are 6 weeks with 62% for *Grammangis spectabilis.*

3.1. Influence of Temperature And Light / Dark on Seed Germination

3.1.1. Influence of 10 days pretreatment before sowing

The results of speed and germination capacity are shown in Figures 1 and 2.

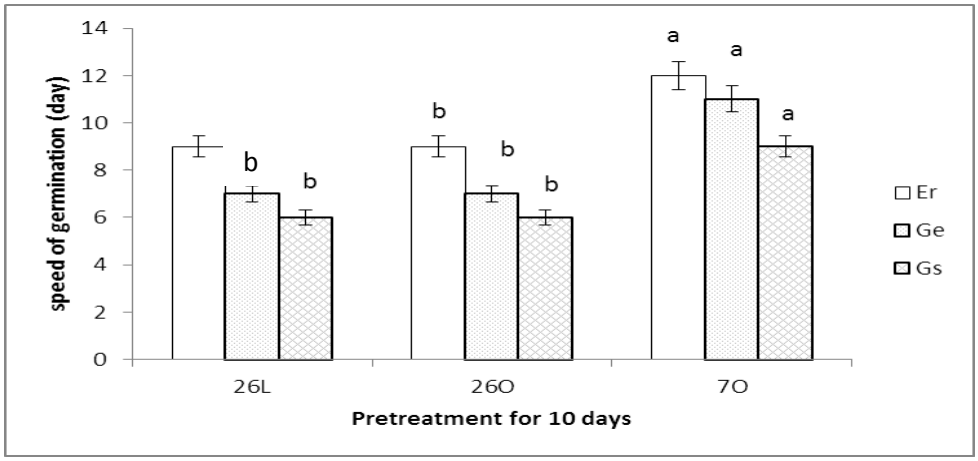

Figure 1: Speed of germination of *Eulophiella roempleriana, Grammangis ellisii, Grammangis spectabilis depending on 10 days* pretreatment.

Er: *Eulophiella roempleriana;* Ge: *Grammangis ellisii;* Gs: *Grammangis spectabilis*

26L: Temperature 26°C, 16h light /day;

26O: Température 26°C, in dark;

7O: Température 7°C, in dark

For 3 species, seeds having undergone a pretreatment of 26°C germinated faster than those treated with a temperature of 7°C. In the case of *Eulophiella roempleriana*, germination occurred in the ninth week against 12 weeks when the seeds are pretreated with a temperature of 7°C. For *Grammangis ellisii*, the seeds have germinated after 7 weeks instead of 11 and for *Grammangis spectabilis*, speed of germination is 6 weeks instead of 9. In other words, the cold pretreatment retards germination of these three species.

At 26°C, pretreatments in light and complete darkness have no influence on the speed of germination of these three species. They gave the same results.

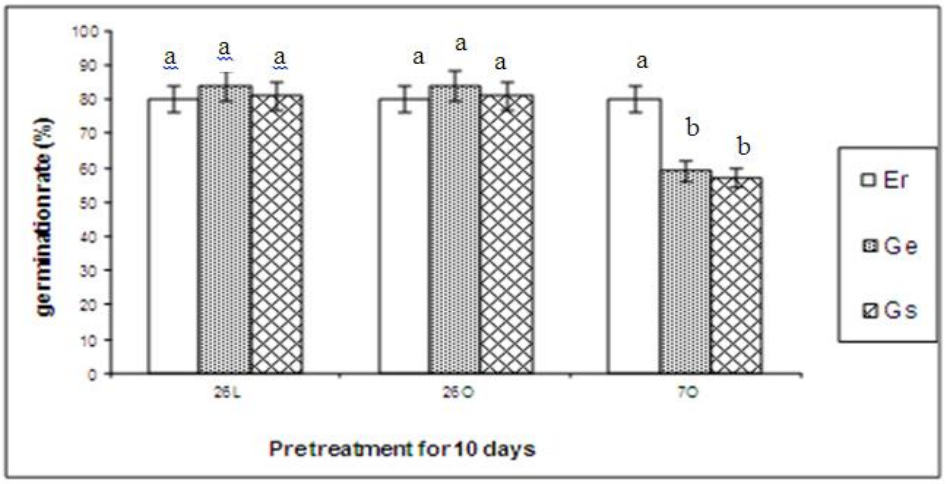

Figure 1: Germination capacity of *Eulophiella roempleriana, Grammangis ellisii* and *Grammangis spectabilis.*

Er: *Eulophiella roempleriana*; Ge: *Grammangis ellisii*; Gs: *Grammangis spectabilis*

26L: Temperature 26°C, 16h light /day;

26O: Température 26°C, in dark;

7O: Température 7°C, in dark

Pretreatment 26°C and the 7°C give the same germination rate 80% for *Eulophiella roempleriana* while for *Grammangis ellisii* and *Grammangis spectabilis*, germination rates of seeds treated with a temperature 7°C are lower, 59 and 57% over those pre-treated at the temperature 26°C at which germination rates are respectively 84 and 81%. The results show that the speed and the germination capacity depend on the species studied.

3.1.2. Influence of treatment in the dark

The results are shown in Figures 3 and 4

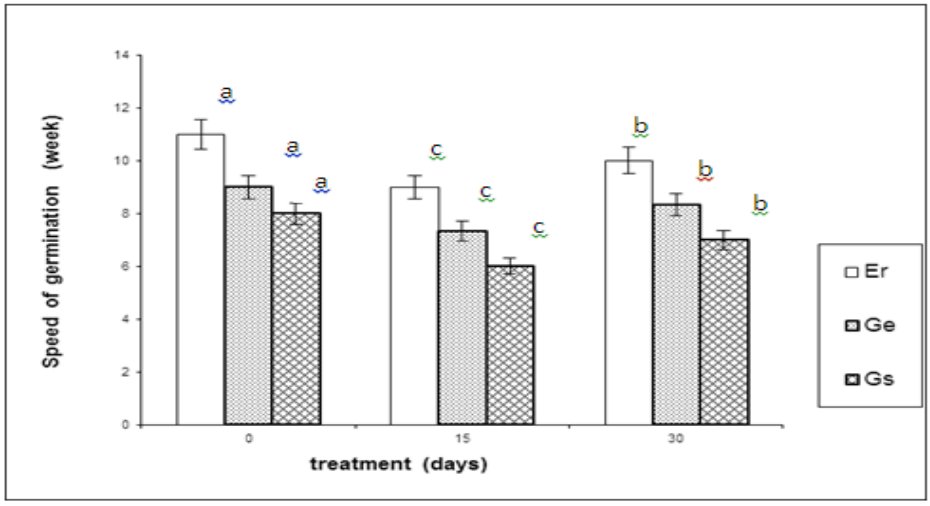

Figure 3: Speed of germination of *Eulophiella roempleriana*, *Grammangis ellisii and Grammangis spectabilis* depending on depending on duration of darkness treatment.

Er: *Eulophiella roempleriana;* Ge: *Grammangis ellisii;* Gs: *Grammangis spectabilis.*

For each species studied, the values obtained by these three treatments are significantly different from the statistical analysis at the 5% threshold, according to the test of Newman-Keuls.

For the three species, the light treatment (control) shows a slower germination speed relative to the dark for 15 days and those for 30 days. Germination speeds are respectively 11 weeks against 9 and 10 for *Eulophiella roempleriana*, of 9 weeks against 7.33 and 8.33 for *Grammangis ellisii* and 8 weeks against 6 and 7 for *Grammangis spectabilis*. Treatments in the dark accelerate seed germination with an optimum for 15 days.

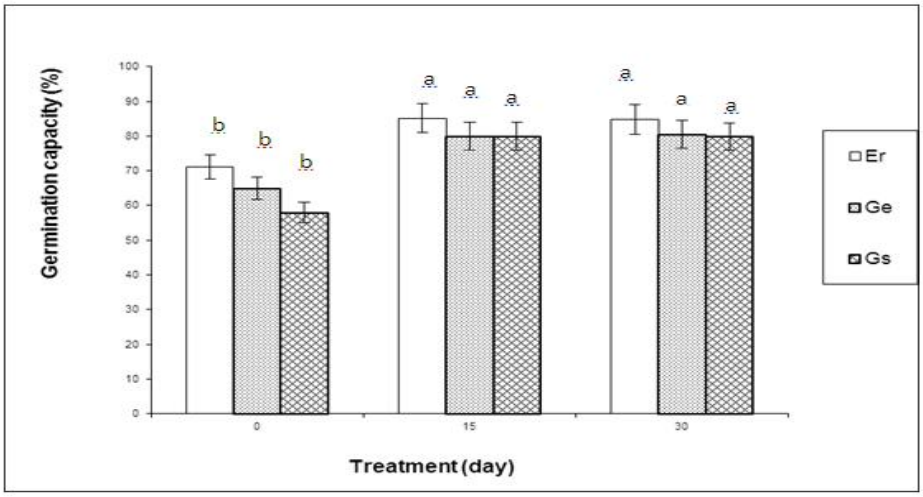

Figure 4: Germination capacity depending on duration of treatment in the dark.

Er: Eulophiella roempleriana; Ge: Grammangis ellisii; Gs: Grammangis spectabilis.

For each species studied, the difference in value to treatment for 15 and 30 days is not significant after statistical analysis at the 5% threshold, according to the test of Newman-Keuls. But the germination capacity of seeds in control condition (without darkness) is significantly different from those of 15 and 30 days in the dark, according to the statistical analysis.

Thus, treatments in the dark during15 days and the 30 days give the same capacity for germination for the three species. This capacity is higher compared to no treatment in the dark:

For *Eulophiella roempleriana,* capacities of germination are 85,16 and 84,83% respectively for 15 and 30days in dark (> 71,16 for the control); in *Grammangis ellisii*: 80 and 80, 5% (> 65 for the control) and for *Grammangis spectabilis*, capacities of germination are 80 and 79,80% against 58% for the control condition.

3. 1.3. Pretreatment / treatment interaction

The results are shown in Figure 5 and 6.

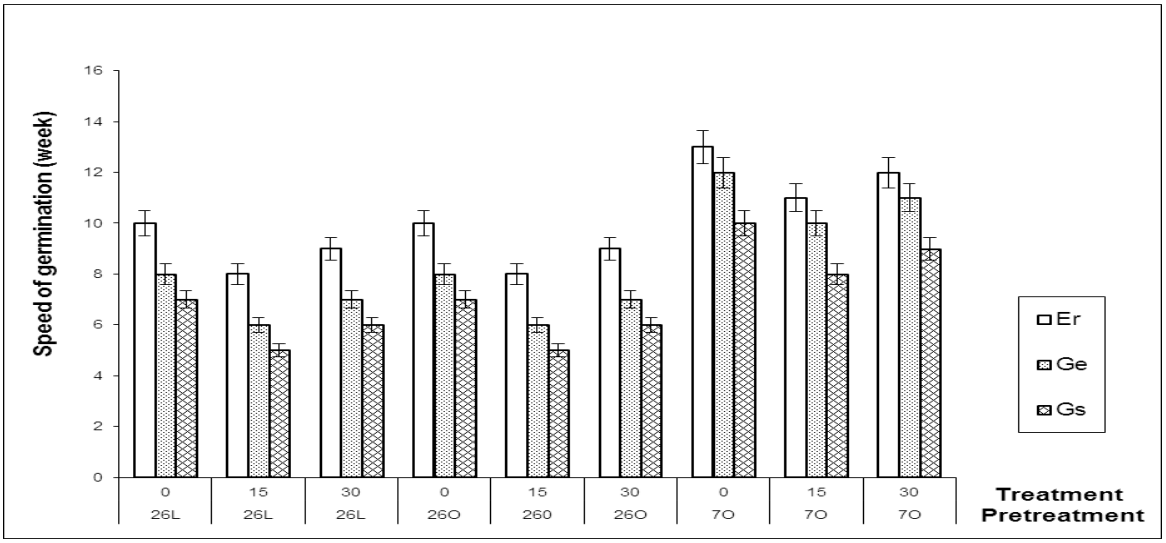

Figure 5: Speed of germination of *Eulophiella roempleriana, Grammangis ellisii* and *Grammangis spectabilis* depending on the interaction of pretreatment –treatment.

Er: *Eulophiella roempleriana;* Ge: *Grammangis ellisii;* Gs: *Grammangis spectabilis;* Pretreatment (day); Treatment (day); Speed of germination (week)

26L: Temperature 26°C, 16h light /day;

26O: Température 26°C, in dark;

7O: Température 7°C, in dark

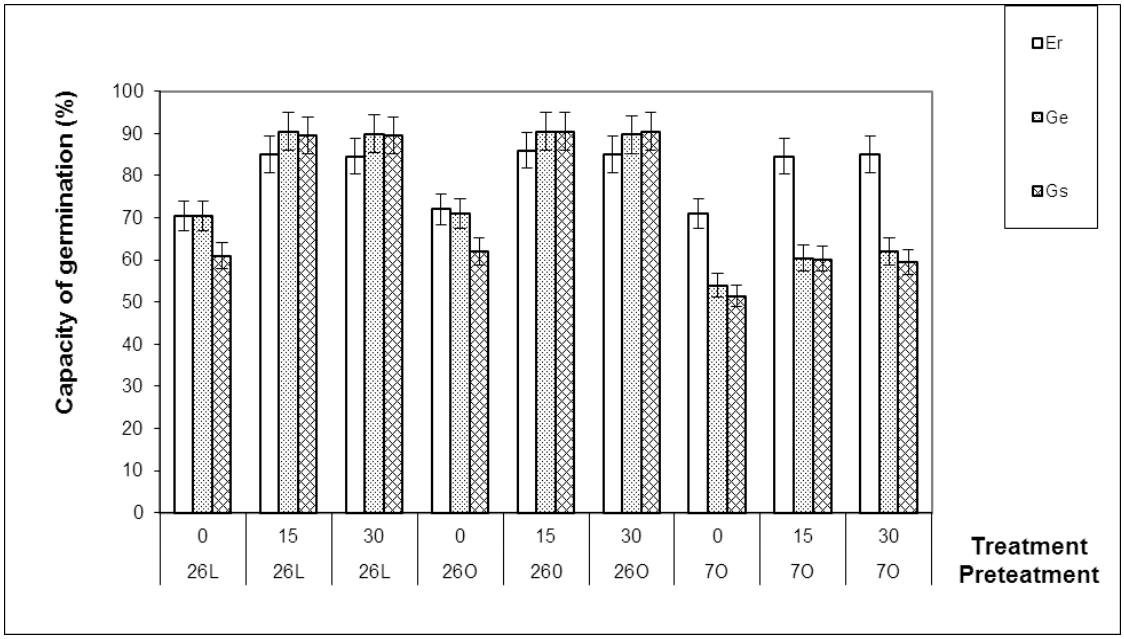

Figure 6: Germination capacity of *Eulophiella roempleriana, Grammangis ellisii* and *Grammangis spectabilis* depending on the interaction of the pretreatment – treatment.

Er: *Eulophiella roempleriana;* Ge: *Grammangis ellisii;* Gs: *Grammangis spectabilis;* Pretreatment (day); Treatment (day); Capacity of germination (%)
26L: Temperature 26°C, 16h light /day;
26O: Température 26°C, in dark
7O: Température 7°C, in dark

These figures show that *Grammangis spectabilis* has a faster germination compared to the other two species and then comes *Grammangis ellisii* and finally *Eulophiella roempleriana*. In addition, the results show that there is interaction between pretreatment and treatment. Thus, the fastest germination is achieved by interaction of pretreatment at 26°C, in complete darkness or in the light 16 hours a day, with treatment 15 days in the dark. This interaction gives the highest germination capacity.

The slowest germination results from the interaction of pre-treatment 7°C in the dark without treatment in the dark. These conditions give the lowest capacity for these three species.
Pretreatment 7°C produces the same effect as 26°C on the germination capacity of *Eulophiella roempleriana*, unlike the other two species that have low germination capacity.

In summary, the seeds of *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabilis* do not require cold pretreatment (7°C) to germinate.

Pretreatments in the light and that in the darkness have no influence neither on the speed nor on the germination capacity of these 3 species.

The complete dark treatment accelerates the germination and gives higher germination capacity for these species.

Germination of *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabilis* needs incubation in the dark. The period of 15 days is sufficient for obtaining a higher capacity in a relatively short time.

3. 2. Comparison of The Three Basal Culture Media on Asymbiotic Germination

The results are shown in the following figures 7 and 8. For all three species, statistical analysis showed that the difference observed in the 3 different basic media is not significant; it means that the seeds sown have an identical behavior towards 3 media used.

The seed germination rate of *Eulophiella roempleriana* was 8 weeks. As the species *Eulophiella roempleriana*, seed of *Grammangis ellisii* and *Grammangis spectabilis* germinate indifferently on these 3 basic media. The germination rates of these two species are respectively 6 and 5 weeks.

The significant difference in outcome was observed between species. The seeds of the species *Grammangis spectabilis* germinated faster compared to those of *Grammangis ellisii* and *Eulophiella roempleriana*.

Regarding germination rate, regardless of the basic medium used, the two species, *Grammangis ellisii* and *Grammangis spectabilis* have the same germination capacity that are the highest, 90% and 91% respectively compared to that of *Eulophiella roempleriana*. For the latter, the germination ability is 85%.

In all cases, the three basal media used were found to be favorable for the germination of these three species.

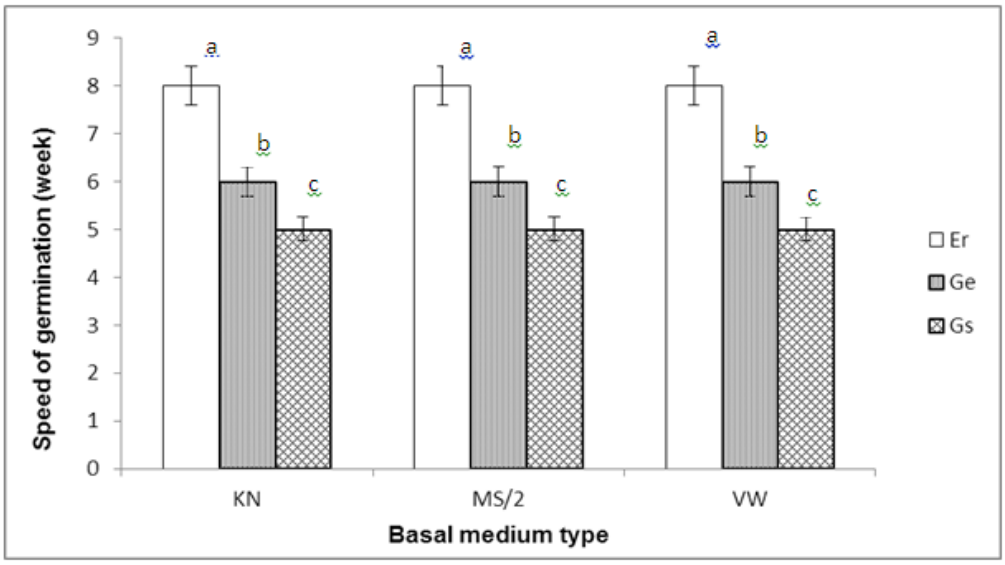

Figure 7: Germination speed of *Eulophiella roempleriana, Grammangis ellisii* and *Grammangis spectabilis* depending on the three basal media, each added of sucrose and vitamins.

Er: *Eulophiella roempleriana;* Ge: *Grammangis ellisii; Gs* : *Grammangis spectabilis*

KN: Knudson; MS/2: Murashige and Skoog in half strenght; VW: Vacin and Went.

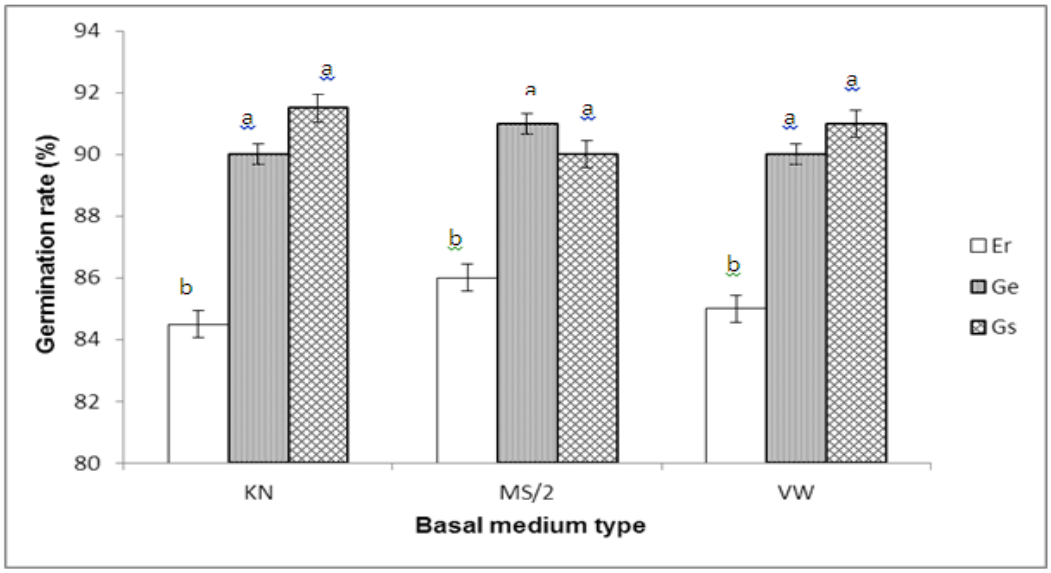

Figure 8: Germination capacity of *Eulophiella roempleriana, Grammangis ellisii* and *Grammangis spectabilis* depending on the three basal media.

Er: *Eulophiella roempleriana;* Ge: *Grammangis ellisii; Gs*: *Grammangis spectabilis*
KN: Knudson; MS/2: Murashige and Skoog in half strenght; VW: Vacin and Went.

3.3. INFLUENCE OF SUCROSE CONCENTRATION ON SEEDS GERMINATION OF *Eulophiella roempleriana, Grammangis ellisii* and *Grammangis spectabilis*

The asymbiotic seeds germination of *Eulophiella roempleriana, Grammangis ellisii* and *Grammangis spectabilis,* is inhibited in the absence of sucrose. The seeds were grown after 4 weeks in *Eulophiella roempleriana*; after 3 weeks for *Grammangis ellisii* and after 2 weeks for *Grammangis spectabilis*. However, no further evolution was observed.

The addition of sucrose in the culture medium stimulates germination of these three species. Greenish white of bright green, green, yellowish green, light yellow and yellow protocorms were obtained respectively on media containing 10 respectively, 20, 30, 40, 50 and 60g/L of sucrose. In the other words, there has been progressive greening from the concentration of 10g/L to 30g/L of sucrose. Above this, the protocorms gradually turn yellow.

Sucrose, at certain concentrations, promotes secretion of polyphenols in the medium. When the seeds were sown in media containing 40, 50 and 60g/L, a gradual darkening of the medium was observed, respectively, from the 4^{th}, 3^{rd} and 2^{nd} week. Their effect intensifies with time of sowing. Without transfer to the new environment, there is a progressive death of protocorms obtained.

If the protocorms were kept in the same culture medium without transfer, with concentrations of 10, 20, and 30g/L of sucrose, root formation is favored. The optimal concentration is 30g/L for the three species. However, the time of seeds emergence varies from one species to another: 5 weeks after obtaining green protocorms for *Eulophiella roempleriana;* 6 weeks for *Grammangis ellisii* and 5 weeks for *Grammangis spectabilis*.

4. DISCUSSION

4. 1. Influence of 10 Days Pretreatment

Le prétraitement à froid 7°C a été utilisé dans le but d'améliorer la capacité et d'accélérer la vitesse de germination d'*Eulophiella roempleriana,* de *Grammangis ellisii* de *Grammangis spectabilis.* Néanmoins, des résultats contraires ont été obtenus. 7°C cold pretreatment was used in order to improve the capacity and accelerate the speed of germination of the three species. However, contrary results were because this condition deteriorates the germination of *Grammangis ellisii* of *Grammangis spectabilis*. It delays the speed and reduces the germination capacity of these two species. Conversely, for *Eulophiella roempleriana*, this cold pretreatment at 7°C retards germination but it gives the same capacity with the 26°C.

The seeds of orchids become dormant as soon as they complete their embryonic development (FAST, 1982; BALLARD, W. W, 1987). For this study, tested seeds are ripe. However, the opposite results were obtained indicating that the seeds of *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabilis* do not require cold pretreatment to germinate. VAN WAES and DEBERGH (1986 b) and LIGHT (1989) reported that the cold pretreatment decreases the germination of *Cypripedium calceolus and Cypripedium pubescens* var. While *Cypripedium reginae* needs cold pretreatment to germinate. DE PAUW, M. A. and REMPHREY, W.R. (1993) reported that the cold pretreatment effects on the germination of *Cypripedium candidum* are variable and therefore inconclusive. Contrariwise, cold pretreatment before seeding explants could be favorable to other systematic groups. Thus, 13°C cold pretreatment of rice anthers during 10 days before sowing increase the percentage of callus formation (FOLLOWING, C.S., 1979).

On the other hand, the pretreatment at a temperature of 26°C improved the results. It accelerated the germination of seeds of these 3 species and increased the germination capacity of *Grammangis ellisii* and *Grammangis spectabilis*.

4. 2. Effect of Treatment in The Dark

Treatments in the dark accelerate and improve the germination capacity of these three species studied. The difference between the treatments of 15 days to the 30 days resides on germination rate. Germination of *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabilis* needs darkness. Similar results are observed with *Angraecum compactum*, *Angraecum magdalenae* and *Sobennikoffia robusta*. But one could not generalize, since previous experiments show good development with high germination capacity of some species carried germination without undergoing pretreatment or treatment in the dark, but directly exposed under illumination 3,000 lux for 16 hours per day. Examples include: *Aeranthes longipes*: 92%; *Angraecum leonis*: 90%; *Neobathiea perrieri*: 92%.

For exotic orchids, seeds of several genus like *Cymbidium*, *Phalaenopsi*s and *Paphiopedilum* have developed well in the dark (ARDITTI, J., 1979). *Paphiopedilum ciliolare* have sprouted as if they were incubated in the dark for the first three months (SINGH, F., 1993). Some species may germinate under two conditions, light and darkness but the development of the seedlings in the light is different from those in the dark (ARDITTI. J, 1967). These results confirm that each species has its own requirements. The need for light or dark can be determined experimentally.

4. 3. Influence of Basal Media

The seeds of *Eulophiella roempleriana*, *Grammangis ellisii* and those of *Grammangis spectabilis* have well developed on KN culture medium or MS/2 or VW, added vitamins and sucrose.

The *Eulophiella roempleriana* seeds germinated on medium with very different composition of microelements: poor medium as V W with a single micro-element (Mn SO_4 $4H_2O$); moderately rich medium like the KN medium with 4 microelements (H_3BO_3; MoO_4; Cu SO_4; $ZnSO_4$, $7H_2O$); rich medium as the medium MS/2 with 9 microelements (KI; H_3BO_3; $MnSO_4$ $2H_2O$; Na_2MoO_4, $2H_2O$, $CuSO_4$, $5H_2O$; $CoCl_5H_2O$; $ZnCl_2$; NaEDTA; $FeSO_4$, $7H_2O$). It seems that the microelements are not critical for germination of Eulophiella roempleriana. According to Singh, F., (1993), almost all Orchids can germinate on 2 types of media KN and VW. From the results of *Cypripedium reginae* on Harvais media (1982), those of VAN WAES. J. and DEBERGH (1986 b) and that of NORSTOG changed (1973), the culture medium composition is not a limiting factor for germination (DE PAUW, W. R, and REMPHREY, 1993).

Contrariwise, the results on *Cymbidiella flabellate* and *Aerangis platyphylla*, 3 other culture medium those are the Anderson, Lorenzen and Lindemann showed influence of the basal medium. The best result offered by the Lindemann culture medium was its high content of NH_4 + (RABE ANDRIANANRASANA.H. 2004). This is consistent with assertions STENBERG and KANE (1998) that the high ammonium ion concentration in the culture medium improves the orchid seeds germination. These results lead to assume that the germination depends on the species which has its own requirements.

4. 4. Effect of Sucrose Concentration

The asymbiotic seeds germination of *Eulophiella roempleriana*, *Grammangis ellisii* and those of *Grammangis spectabilis* was inhibited in the absence of sucrose. Similar results were obtained on *Angraecum compactum*, *Angraecum magdalanae* and *Sobennikoffia robusta.*

These results are comparable to Harrison (1973), indicating that the embryos cultured on medium without sugar reach the stage of protocorms but are unable to produce leaves or roots.

The addition of sugar in the culture medium stimulates the germination of *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabilis*. But the high concentration or concentration equal to 40 g/L causes the progressive yellowing of the obtained protocorms. KNUDSON (1922) observed that the *Cattleya mossiae* seeds germinate and differentiate on medium containing sucrose while without it, the embryos do not develop beyond small spherules. A similar situation was observed in *Cypripedium acaule* where protocorms produced leaves and roots in the presence of carbohydrates. However, they have remained at the stage of young protocorm without even producing promeristem if there is no supply of external carbohydrates (Leroux, G., et al. 1995).

It seems that the majority of reserves in the orchid seeds are lipids (PODDUBNAYA-ARNOLDI & ZINGER 1961; ARDITTI.J., 1992). Orchid Seed has no organs which can transform lipids to carbohydrates (albumen, perisperm or cotyledon) through glyoxysomes, so it cannot perform the glucogenesis. This explains the need for the seeds of orchids, to use an external source of carbohydrate. The primary role is played by the fungus responsible of mycorrhiza until the seedling becomes autotrophic (ARDITTI.J, 1967; 1979; ARDITTI.J, et al ARDITTI.J 1982, 1990 et al.).

Finally, the problem of symbiosis during germination does not occur for these species studied. Germination provides tens of thousands or even more than one million viable and healthy plants from seeds from a capsule even without protocorms multiplication (ARDITTI. J and GHANI. A.k.a., 2001).

CONCLUSION

This study has helped to develop biotechnology *in vitro* culture from the viewpoint of the conservation of endemic Malagasy orchids, plants reflect the incredible wealth of Madagascar.

The asymbiotic germination of orchids depends on the intrinsic and extrinsic factors. The first factor is related to the quality, viability, maturity and seed dormancy. Knowing optimal time capsules harvest, one of the conditions of successful germination asymbiotic 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 biological material.

The second factor is the culture conditions, such as temperature, light or darkness and the culture medium.

The combined pretreatment study of temperature and light/dark with treatment in the dark allowed to infer that the 26°C pretreatment interaction for 10 days with treatment in the dark for 15 days improved the speed and germination capacity of *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabili*. While 7°C pretreatment delay the germination of these 3 species and decreased *Grammangis ellisii* and *Grammangis spectabilis* germination capacity whereas it is not the case for *Eulophiella roempleriana*. Pretreatments to darkness and light have no influence on either the rate or the germination capacity of these three species.

Comparing the asymbiotic germination of *Eulophiella roempleriana*, *Grammangis ellisii* and *Grammangis spectabilis*, on MS/2, KN and the VW basal media showed that microcells are not decisive for the germination of these species. The medium containing macronutrients, vitamins B and sucrose sufficient for asymbiotic germination of these three species.

Sugar is a limiting factor of the asymbiotic germination of these three species. Without sucrose, no seed is germinated; concentration 30g/L gave the best result. The high

concentration or the concentration equal to 40 g/L promoted the secretion of phenolic product and gave light yellow protocorms.

Tests of different parameters including culture medium composition allowed to highlight the differential behavior of these three species. These experiments could thus be extended to other plant species for their conservation and their valorization.

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