

GERMINATION *IN VITRO* OF SEEDS OF A THREATENED ARBOREAL SPECIE IN THE MUNICIPAL DISTRICT OF ABAÍRA (BA)

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ABSTRACT — *The aim of the present study was to evaluate the effect of culture media and disinfection treatments on germination and the contamination in vitro of Zeyhera montana seeds. The trial was comprised of two experiments: in the first, the seeds were surface sterilized with alcohol 70% (1 min) + NaOCl 0.25, 0.5 or 1% active chlorine (5 or 10 min). The seeds were placed in flasks containing MS, MS rooting, Knudson B, Knudson C, Agar or WPM culture media. In the second experiment, the seeds were inoculated on the culture media which showed the best results in the previous experiment. Germination was slow and disuniform. The best results were 75% and 40% germination on Knudson C and MS rooting media, respectively.*

KEY WORDS: *Zeyhera montana; in vitro germination; micropropagation.*

RESUMO — *Objetivou-se avaliar o efeito de meios de cultura e tratamentos de desinfestação sobre a germinação e a contaminação in vitro de sementes de Zeyhera montana. O estudo foi composto por dois experimentos: no primeiro os tratamentos constaram de imersão em álcool 70% (1 min) + imersão em hipoclorito de sódio a 0,25, 0,5 ou 1% (5 ou 10 min). As sementes foram colocadas para germinar em frascos contendo como*

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substrato meios MS, MS enraizamento, Knudson B, Knudson C, Ágar e WPM. No segundo experimento, as sementes foram germinadas nos meios de cultura que apresentaram os melhores resultados no experimento 1. As sementes apresentaram germinação lenta e desuniforme ao longo do período de avaliação, sendo que os melhores índices de germinação encontrados foram 75 e 40%, para os meios Knudson C e MS enraizamento, respectivamente.

PALAVRAS-CHAVE: *Zeyhera montana*; germinação in vitro; micropropagação.

INTRODUCTION

The arboreal flora in Catolés, municipal district of Abaíra, Bahia, is economically important. At least nine species are used for lumber. Thus, the forests are increasingly threatened by the predatory exploitation of several woody species. Of these species, *Zeyhera montana* Mart., commonly known in the area as “pau d’arco de cuia”, has been greatly reduced in terms of population density and distribution.

Seed germination and the propagation by cuttings of this specie would be of great utility for the local population, however, there are some problems with the germination of the seeds. According to LORENZI (1992) *Z. montana* possesses recalcitrant seeds and CREPALDI (unpublished data) observed a great irregularity in the germination behavior.

Propagation *in vitro* is an alternative to uniformize and optimize the germination process to produce plants. This practice has been used for woody species with economic importance for some time in regions of temperate climate (ABBOTT, 1977; 1978; GREENWOOD et al., 1991).

In Brazil, these techniques have had limited application especially with species of Cerrado (Savanna Forest) (CALDAS, 1996). Example of *in vitro* propagation include *Kielmeyera coriacea* Martius (ARELLO & PINTO, 1993) and *Annona squamosa* L. (LEMOS & BLAKE, 1996), both economically valuable.

CALDAS (1996) mentioned the advantage of the techniques mainly in the cases in which the plants are distant and/or rare or slow to germinate. These arguments justify the attempts to propagate *Z. montana in vitro*.

The present study was designed to test different culturing media and disinfestation procedures and to define the best combination (medium x time disinfestation) for the germination *in vitro* of *Z. montana* seeds.

MATERIAL AND METHODS

1. Local of seed collections and laboratory studies.

The experiments were carried out in the Laboratory of Plant Tissue Culture of the State University of Feira de Santana, Bahia, Brazil. The fruits of *Z. montana* were collected in the district of Catolés in September, 1996. The fruits were opened and the seeds were removed; the wings were eliminated to decrease the source of contamination and to improve the disinfestation.

2. Experiment 1: In this preliminary experiment, were used the culture media MS (MURASHIGE & SKOOG, 1962), MS rooting (supplying with 5 mg of Thiamine-HCl, 25 mg of Pyridoxine-HCl, 25 mg of Nicotinic acid, 100 mg of Glicine and 5 mg of Inositol), Knudson C (KNUDSON, 1946), Knudson B, WPM (LLOYD & McCOWN, 1980) and agar, distributed 20 ml per flask. The pH was adjusted to 5.7 ± 0.1 with KOH 0.1 N before autoclaving for 20 min at 0.1 MPa. One seed was inoculated in each flask, four days after collecting the material. Cultures were incubated in a growth chamber at $25 \pm 2^\circ\text{C}$ with 16 h photoperiod using cool-white fluorescent tube lights (13 W.m^{-2}).

The seed disinfestation process consisted of immersion in alcohol 70% for 1 min., then NaOCl (sodium hypochlorite), varying the concentration (0.5% or 1% active chlorine) and immersion time (5 or 10 min.) and finally three rinses with sterile distilled water.

3. Experiment 2: The Knudson C medium was used since it presented the best results in the preliminary experiment and the MS rooting medium since it is used commonly, with 5 disinfection treatments as follows:

- T1- 1 min in alcohol 70% + NaOCl 0.25% for 10 min;
- T2- 1 min in alcohol 70% + NaOCl 0.5% for 5min;
- T3- 1 min in alcohol 70% + NaOCl 0.5% for 10 min;
- T4- 1 min in alcohol 70% + NaOCl 1.0% for 5 min;
- T5- 1 min in alcohol 70% + NaOCl 1.0% for 10 min.

The incubation conditions were the same as those in Experiment 1.

4. Statistical Design.

The design was entirely randomized, and each treatment had 4 replications. Each experimental unit was composed of 15 flasks, for both experiment 1 and experiment 2.

For analysis of variance, the original data in percentage were transformed to arc sin of the square root of the germination percentage ($Y = \arcsin \sqrt{X/100}$), according to HEATH (1981). The means of the treatments were compared by Tukey Multiple Range Test at the level of 5% probability.

5. Evaluation parameters.

Evaluations were made periodically, starting from the first day after the introduction of the seeds, with observations of the level of contamination and the number of germinated seeds (seeds that had emitted the radicle).

RESULTS

Experiment 1.

It was observed, in the first experiment, that the contamination was more intense in the first 7 days on MS, MS rooting, WPM and Knudson B media. On the Knudson C and Agar media contamination was delayed (Figure 1). It was also observed that the latter media had the lowest levels of contamination (6% for Knudson C medium, followed by 38% for Agar medium) and both

Knudson C and Agar media differ significantly from the other medias.

The WPM, Knudson B, MS and MS rooting media had high levels of contamination, reaching 100% in some cases, but in all media there was some germination, except for the MS medium (Table 1).

The germination of *Z. montana* was very slow and non-uniform, extending up to 60 days after the introduction of the seed in the medium (Figure 2). The best germination was seen on the Knudson C and Agar media (84% and 60% respectively), and Knudson C medium differed significantly from the WPM, MS rooting and MS media.

Experiment 2

In the second experiment, the best combination between medium and disinfestation treatment was Knudson C with 0.25% of NaOCl during 10 min (treatment 1), with 75% germination, differing significantly from the other combinations, except for the combination with treatment 3. MS rooting medium combined with treatment 4 showed 40% rate of germination.

TABLE 1. Effect of standard disinfestation treatment⁽²⁾ on contamination (after 28 days) and germination (after 60 days) of *Z. montana* seeds in different culture medium *in vitro*.

Culture Medium	Contamination (%)	Germination (%)
Knudson C	6 b ^(Y)	84 a ^(Y)
Agar	38 b	60 ab
Knudson B	78 a	20 abc
WPM	90 a	10 bc
MS rooting	94 a	6 c
MS	100 a	0 c
Tukey 5%	36.43	32.17

⁽²⁾ – Seeds were surface sterilized with alcohol 70% for 1 min + NaOCl 0.25% for 10 min.

^(Y) – Means followed by the same letter, in each column, are not significantly different at $P \leq 0.05$ level by Tukey Multiple Range Test.

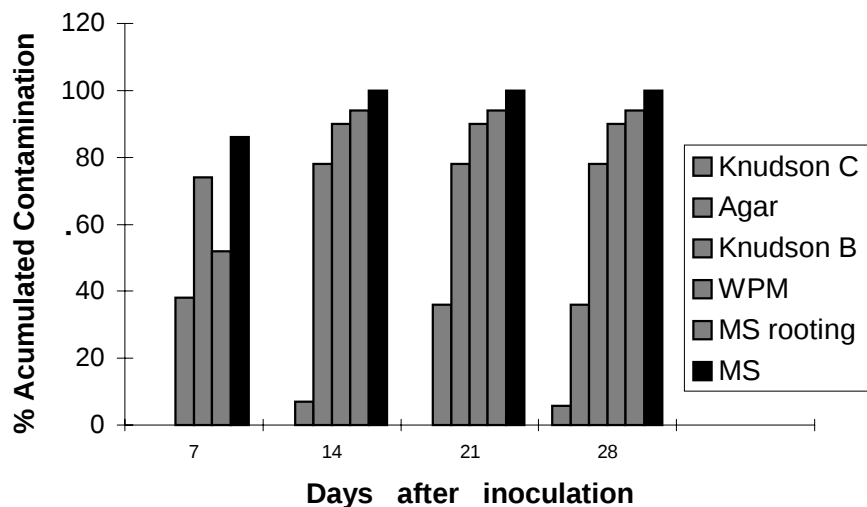


Figure 1. Evolution of contamination *in vitro* of *Z. montana* seeds on different culture media.

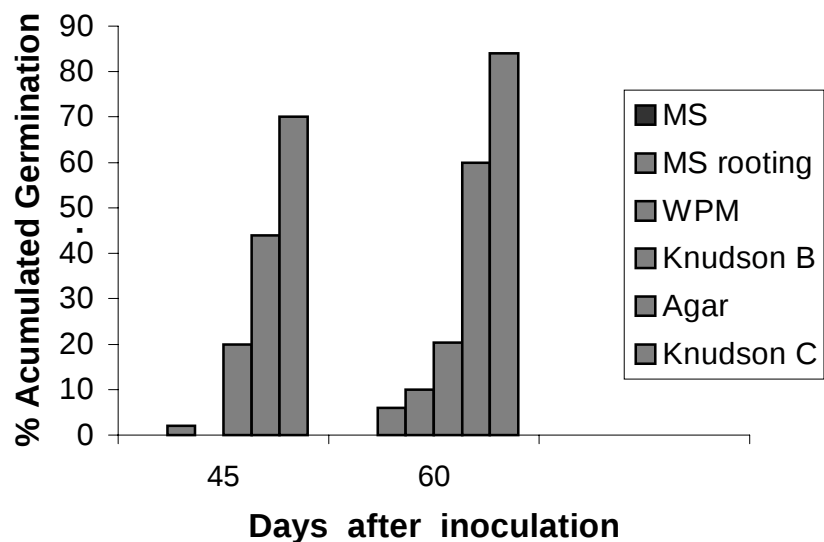


Figure 2. Evolution of germination *in vitro* of *Z. montana* seeds on different culture media.

TABLE 2. Effect of different disinfestation treatments on contamination of *Z. montana* seeds *in vitro* (after 28 days) and germination (60 days) on MS rooting and Knudson C media.

Culture Medium	Treatment ^(Z)	% Contamination	% Germination
MS rooting	1	90,0 ab ^(Y)	5,0 bc ^(Y)
	2	100,0 a	0,0 c
	3	65,0 abc	15,0 abc
	4	15,0 c	40,0 a
	5	45,7 bc	25,0 ab
	Tukey 5%	46,27	29,69
Knudson C	1	5,0 b ^(Y)	75,0 a ^(Y)
	2	57,5 a	20,0 b
	3	47,5 a	35,0 ab
	4	40,0 a	27,5 b
	5	35,0 a	12,5 b
	Tukey 5%	26,92	27,69

- ^(Z) 1- Seeds surface sterilized with alcohol 70% for 1 min + NaOCl 0,25% for 10 min.
 2- Seeds surface sterilized with alcohol 70% for 1 min + NaOCl 0,50% for 5 min.
 3- Seeds surface sterilized with alcohol 70% for 1 min + NaOCl 0,50% for 10 min.
 4- Seeds surface sterilized with alcohol 70% for 1 min + NaOCl 1,0% for 5 min.
 5- Seeds surface sterilized with alcohol 70% for 1 min + NaOCl 1,0% for 10 min.

^(Y) Means followed by the same letter, in each column, are not significantly different at $P \leq 0.05$ level by Tukey Multiple Range Test, to MS rooting and Knudson C media.

The combination of treatment 2 with the MS rooting and Knudson C media led to the highest levels of contamination (100% and 57,5%) respectively. But when the seeds were treated with 0,25% of NaOCl for 10 minutes and inoculated in Knudson C medium the contamination was only 5%.

DISCUSSION

The contamination reached high levels, with 100% of the flasks infested in some treatments. In agreement with GRATTAPAGLIA & MACHADO (1990), even though all precautions are taken with asepsis, microorganism growth (including bacterias, fungi and yeasts) in the culture medium can occur, affecting the germination of the seeds. The contamination of the tissue cultures can originate with the microorganisms that are naturally present in the plant material, or through flaws in the technical procedures used for surface sterilization (CASSELLS, 1994) and inoculation. GRATTAPAGLIA & MACHADO (1990) and GRATTAPAGLIA et al. (1995) emphasize that disinfestation pre-treatments, when endogenous microorganisms are present, are decisive for the success of the whole process. That is one of the problems presented by *Z. montana*, in that at least one type of endophytic fungus in the seed was already detected (CREPALDI, unpublished data).

The high levels of contamination should not be attributed only to endophytic fungi. Even though the wings of the seeds of *Z. montana* were removed, the remaining surface is still relatively large, which possibly explains the lack of success in some disinfestation treatments. On the other hand, the percentage of germination is inversely proportional to the time of immersion of the seeds in NaOCl. Very concentrated solutions of disinfestant or prolonged exposure times lead to seed death. Besides, the nature of the culture medium (pH close to neutral, high concentration of salts) can facilitate the growth of microorganisms. The decrease of the concentration of salts of the medium, combined with an appropriate treatment, can contribute to the inhibition of the formation of colonies.

To control the endogenous contamination of the seed, GILLADI et al. (1979), CASSELLS, 1994 suggest the incorporation of antibacterial and antifungal agents in the culture medium, taking precautions with the use of chemicals which can lead to reduction of the vigor of the explant. The use of these antimicrobial agents could be tested to improve the results.

The positive results verified in the first stage on the Knudson C and Agar media in relation to the other media indicated that these media are better adapted to the establishment of the seedlings of *Z. montana*, which was confirmed in the second stage with Knudson C medium. Different results were obtained by ARELLO & PINTO (1993) with seeds of *Kielmeyera coriacea*, in which the MS rooting medium promoted germination.

The germination *in vitro*, except for the uniformity of the process and of the high percentage of germination, did not differ in terms of time from the germination of the seeds placed in sacks in greenhouse (CREPALDI, unpublished data). No germination was obtained in less than 45 days. Therefore, modifications in the concentrations of salts of Knudson C media are suggested, to improve the minimum time required for germination.

The data suggest that satisfactory results with seed germination *in vitro* can be achieved.

The plants obtained *in vitro* (originating from the germination of seeds on the Knudson C medium) will provide healthier explants, such as meristems. The methods more commonly employed for the micropropagation of forest species consist of regenerating plants from pre-existent meristems (PASTUR & ARENA, 1996; ARELLO & PINTO, 1993). The problem of contamination can be minimized.

CONCLUSION

In the species *Z. montana* the percentage of seed germination *in vitro* is quite high (75% to 84%) on Knudson C medium; the germination rate can possibly be optimized with adjustments to the medium. That is the first step for obtaining plantlets in the laboratory to initiate mass micropropagation. The establishment of mother-plants in the laboratory is necessary due to the distance that separates the municipal district of Catolés from the city of Feira de Santana, and to the fact that individuals in the field have a high incidence of contaminating microorganisms, including endophytes.

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