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Germination of *Cochlospermum regium* Seeds: Influence of Seed Size, Vials, Vial Sealing *In vitro*, and Substrate *In vivo*

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Authors' contributions

All authors designed the study, performed the statistical analysis, wrote the protocol, and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This work aimed to assess how seed size, vials, vial sealing (*in vitro*), and substrate (*in vivo*) affect *C. regium* germination and emergence. This study shall contribute to the viable production of *C. regium* seedlings.
Study Design: The experimental design used in these experiments was randomized.
Place and Duration of Study: Department of Plant Biotechnology, Universidade de RibeirãoPreto, between March 2010 and December 2010.
Methodology: This work has evaluated how seed size, vials, vial sealing (*in vitro*), and substrate (*in vivo*) influence the germination and emergence of *C. regium*.
Results: The results showed that cultivation of *C. regium* seedlings from seeds is viable,

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irrespective of seed size. Vial oxygenation is an important parameter to consider *in vitro*, to obtain a larger number of normal seedlings. As for *in vivo* conditions, germination should be conducted in sand, to ensure a greater amount of young seedlings.

Conclusion: The results presented here attested that it is possible to produce *C. regium* seedlings from seeds of any size both *in vivo* and *in vitro* conditions. *In vitro*, it is important to consider vial oxygenation, in order to obtain a greater amount of normal seedlings. *In vivo*, germination should be conducted in sand, to ensure production of a large quantity of seedlings.

Keywords: Bixaceae; Cochlospermum regium; germination; emergence; in vitro; in vivo.

1. INTRODUCTION

Cochlospermum regium (Schrank) Pilg. (Bixaceae), popularly known as algodãozinho-do-campo, is a species native to the Brazilian savanna, or Cerrado. It preferentially occurs in anthropized environments, such as highways and railways, among others [1]. Because it can emerge in both undisturbed and disturbed areas, *C. regium*is considered a pioneering species [2]. The extract from its roots has traditional uses in various fields. In particular, this extract helps to treat infections of the ovary and the female reproductive system [3,4].

C. regium seeds display orthodox features. For example, they tolerate desiccation at -20ºC. In addition, the presence of a hard integument hinders water permeability, making the seeds dormant [5]. The latter characteristic is probably related to the usual water deficit in the Cerrado during autumn and winter. Indeed, the seeds of many species endemic to this biome present dormancy during the dry period, but they germinate during the wet season, when the environmental conditions favor plant survival. Moreover, *C. regium* seeds are neutral photoblastic, with optimal germination at 25ºC [2].

The local population collects this plant species by extractivism. However, the number of agronomical investigations that could make the large-scale cultivation of this plant feasible is small. Hence, the industrial production of phytotherapeutics from *C. regium* is not yet viable.

In this scenario, this work aimed to assess how seed size, vials, vial sealing (*in vitro*), and substrate (*in vivo*) affect *C. regium* germination and emergence. This study shall contribute to the viable production of *C. regium* seedlings.

2. MATERIALS AND METHODS

2.1 General

C. regium seeds, *in situ* in the savannah region and stored at ambient temperature (≈26ºC) for approximately 13 months in Kraft paper bags, were immersed in sulfuric acid 98% for 140 min, to overcome the integument dormancy [5]. The seeds were then washed with distilled water three times and placed in an autoclave system, or in a laminar flow chamber for *in vitro* inoculation.

In vitro, seeds were inoculated in MS [6] medium supplemented with sucrose 30 g L^{-1} and transformed into a gel with Phytagel® 2.5 g L^{-1}. All the seeds were kept in a growth room at a temperature of (25±2)ºC, under light intensity of 25 µMol m^{-2} s^{-1}, with photoperiod of 16:8 h.

The specimen was identified by Dr Lin Chau Ming (Departamento de Produção Vegetal, UNESP, Botucatu, SP, Brazil). A voucher specimen (No. 1463) has been deposited at the Herbarium of Medicinal Plants of the University of Ribeirão Preto (HPM-UNAERP, Ribeirão Preto, SP, Brazil).

2.2 Influence of *C. regium* Seed Size on Germination

C. regium seeds were separated into large, medium and small [5] seeds and were inoculated in test tube (height of 10 cm and diameter of 2 cm) containing MS medium. Growth was evaluated after 60 days. The following parameters were assessed: percentage of germination; percentage of normal young seedlings; percentage of seedlings types 1, 2, and 3; and percentage of seedlings with reddish or greenish coloration in the hypocotyl. Seedlings with cotyledon opening were considered germinated. The experimental design was totally randomized, with three repetitions and 20 replicates, which amounted to 60 seeds per treatment.

2.3 Influence of Vial and Vial Sealing on *C. regium* Seed Germination Rate *In vitro*

C. regium seeds were inoculated in different vials, sealed with distinct materials, as follows: (1) SIGMA® flask sealed with membrane - Phytatray II/number P5929 (height: 10 cm, width: 10.5 cm, and depth: 9 cm); (2) test tube sealed with plastic lid (height: 10 cm and diameter: 2 cm); (3) test tube sealed with cotton; (4) test tube sealed with PVC film; (5) flask sealed with plastic lid (height: 8 cm and diameter: 5.5 cm); and (6) flask sealed with plastic and membrane. All the seeds were kept in the growth room. The experimental design was totally randomized, with three repetitions and ten replicates, which amounted to 30 seeds per treatment. Growth was analyzed on a daily basis, for 60 days. The following parameters were assessed: percentages of germination and emergence, speed of germination and emergence index [7]. Seeds with root protrusion and seedlings with cotyledon opening were considered germinated and emerging, respectively.

2.4 Influence of Substrate on *C. regium* Germination Rate

In a greenhouse, the seeds were deposited on styrofoam trays containing the following substrates: (1) commercial substrate Bioplant®; (2) sand; (3) Cerrado soil (dusky red latosols); and (4) Cerrado soil + cattle manure (1:1). The greenhouse was kept at ambient temperature (≈ 26ºC). The plants were watered manually, on a daily basis. The experimental design was totally randomized, with four repetitions and 25 replicates, which amounted to 100 seeds per treatment.

The following parameters were assessed on a daily basis, for 60 days: percentage of emergence and speed of emergence index. Seedlings with cotyledon opening were considered emerging.

2.5 Statistical Analysis

The experimental results were submitted to ANOVA and means compared by Scott Knott test at the 5% significance level; the program SISVAR was employed for this purpose [8].

3. RESULTS AND DISCUSSION

3.1 Influence of *C. regium* Seed Size on Germination

Small, medium, and large *C. regium* seeds (Fig. 1A) presented low percentage of germination (31.67%, 21.67%, and 36.67%, respectively; Table 1). This may have resulted from the non-longevity of the seeds, which had been stored at ambient temperature (~ 26ºC) for a period of 13 months prior to the study. The longevity of seeds is an important characteristic from both the agricultural and ecological viewpoints [9]. However, low longevity is a relatively common feature of non-domesticated native species, and the cell maintenance capacity varies according to the family and the storage conditions (artificial storage or seeds bank) [10,11,12]. Some factors underlie the long longevity of seeds; for example, the presence of vitamin E and higher levels of gibberellins (GA_1 and GA_4). Vitamin E may limit non-enzymatic lipidic oxidation during storage, germination, and early development of seedlings [9]; gibberellins probably help to reinforce the integument of seeds [13]. In the case of *C. regium* seeds, these factors require better evaluation, and determination of the ideal storage conditions is necessary. Seed size did not significantly affect the percentage of normal young seedlings that the seeds produced. In other words, seeds of any size could produce seedlings.

Table 1. Percentage of germination and normal seedlings germinated from large, medium and small seeds of *Cochlospermum regium*

Seed size	Germination (%)	Normal seedlings (%)
Small	31.67a	58.33a
Medium	21.67a	57.14a
Large	36.67a	82.28a

Means followed by the same letters within each column did not significantly differ by Scott–Knott Test at P = .05

Apart from distinct seed sizes, [5] verified that the resulting seedlings were different with respect to the aerial part/root ratio, which led to the following classification: seedlings type 1 (4:1 aerial part/root ratio), seedlings type 2 (1:1 aerial part/root ratio), and seedlings type 3 (1:4 aerial part/root ratio). Hence, seed size could directly influence seedling size. Except for medium seeds, which afforded seedlings types 1, 2, and 3, seedlings type 1 were the most frequent among small seeds (78.06%) and large seeds (77.06%) (Table 2). Relating the seedling type with the production of normal seedlings, seedlings type 3, with higher root ratio, were the ones with the smallest number of normal seedlings (12.5%). Seedlings types 1 and 2 produced statistically similar percentage of normal seedlings (78.95% and 85.71%, respectively).

Another feature of *C. regium* seedlings is the presence of a reddish color in the hypocotyl of some seedlings (Figs. 1B and 1C) [5]. Small seeds produced a larger number of seedlings with reddish coloration (73.89%; Table 3) as compared with medium and large seeds, which produced seeds with no reddish color. This reddish color probably stemmed from anthocyanins, a compound that may occur in *C. regium* leaves during senescence in their natural habitat. Because small seeds are less vigorous in plants and also in the *C. regium* species, anthocyanins could exist in seedlings originating from small seeds [5]. In general, large seeds are advantageous over small seeds: large seeds present higher germination rate, improved survival during the growth of seedlings, shorter germination time, and enhanced seedling development [14,15]. Anthocyanins are also known to play a role in cell protection in plants. These substances act in several ways: they adjust osmosis in the event of stress caused by drought and frost; they serve as an antioxidant; and they protect the plant against the effects of UV and visible light [16,17]. In addition, during early plant development, deciduous tropical species, like *C. regium*, produce red leaves in response to droughts; such red leaves also confer these plants photoprotection and protection against fungi [18]. Taking all these factors into account, small *C. regium* seeds produce reddish seedlings that should allow the young plant to develop resistance against environmental stress, thereby enhancing seedling survival in nature.

3.2 Influence of Vial and Vial Sealing on the Germination Rate of *C. regium* Seeds *In vitro*

Vial sealing impacted the percentage of *C. regium* germination and the speed of germination index *in vitro*. In the case of test tube sealed with plastic lid, the percentage of germination was higher as compared with test tube sealed with a PVC film or cotton (80%, 63.33%, and 13.33%, respectively; Table 4). The speed of germination index was significantly lower for seeds stored in test tube sealed with cotton—in this condition, no seedling emerged because a large amount of culture medium evaporated during the experiment.

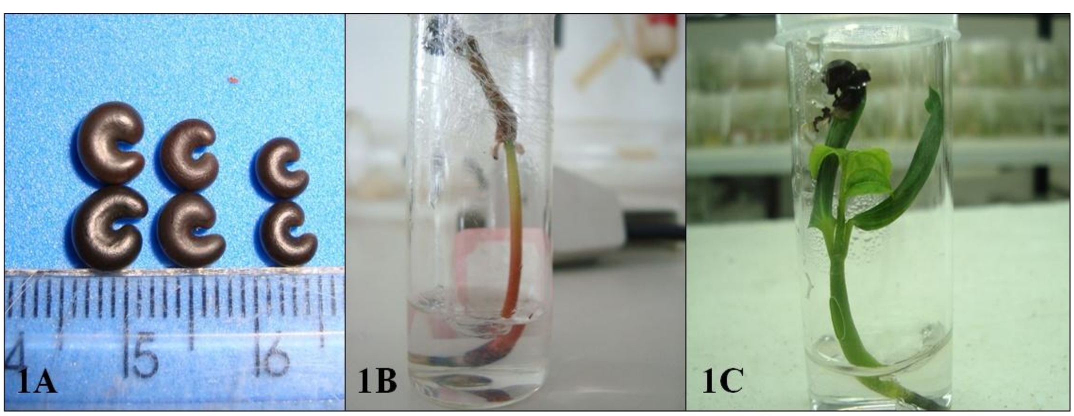

Fig. 1. (A) *Cochlospermum regium seeds* classified as large, medium, and small; (B) *C. regium* with reddish coloring hypocotyl and (C) *C. regium* with greenish coloring hypocotyl

Table 2. Percentage of type 1, 2, and 3 seedlings of *Cochlospermum regium* from large, medium and small seeds

Seed size	Type 1 seedling (%)*	Type 2 seedling (%)**	Type 3 seedling (%)***
Small	78.06a	6.67b	15.28b
Medium	52.38a	26.98a	20.63a
Large	77.06a	17.86b	4.76b

Means followed by the same letters within each column did not significantly differ by Scott–Knott Test at P = .05.
** 4:1 4:1 aerial part/root ratio; ** 1:1 aerial part/root ratio; *** 1:4 aerial part/root ratio*

Table 3. Influence of *Cochlospermum regium* seed size on hypocotyl coloring

Seed size	Reddish coloring hypocotyl (%)	Greenish coloring hypocotyl (%)
Small	73.89aA	26.11bB
Medium	4.76bB	26.11bB
Large	4.76bB	95.24aA

Means followed by the same lowercase within each column and uppercase within each line did not significantly differ by Scott–Knott Test at P = .05

Except for the cotton seal, sealing did not affect the emergence of seedlings. Nevertheless, the percentage of abnormal seedlings was higher among seeds stored in test tube sealed with plastic lid: most of the seedlings did not display chlorophyll (Table 4). The absence of chlorophyll was the ultimate outcome (Table 4), probably because less oxygen was available. Oxygen, temperature, and water are crucial for the germination of any seed. In the particular case of oxygen, it promotes organic matter oxidation in the seed, to produce energy and other compounds that are essential during the anabolic process of germination. In other words, oxygen is implicated in embryonic respiration [19]. Low oxygen levels might suppress plant growth and inhibit metabolism in many plant species. As a result, the respiratory rate and the amount of adenosine-5'-triphosphate (ATP) decreases, and abnormal seedlings arise [20]. In the short term, this phenomenon may elicit adaptive responses that will reduce oxygen consumption by the seed, consequently diminishing the biosynthetic flow of storage compounds [21].

An investigation into *Arabdopsis thaliana* revealed that seedlings had smaller apoplast air volume in hyperhydric conditions; i.e., low oxygen availability [22]. Furthermore, saturation of the apoplast with water decreased the oxygen supply, which abated gas exchange between the symplast and its surroundings. As a consequence, gases (e.g., ethylene and methyl jasmonate) are accumulated in the symplast. Hence, deficient oxygen supply in this phase may impair the development of seedlings.

As for the flask sealed with plastic lid, with or without membrane, none of the emergence parameters were affected (Table 4), not even when different vials, like test tube and SIGMA® flask were compared.

3.3 Influence of Substrate on the Germination Rate of *C. regium* Seeds

Placing the seeds in Cerrado soil + manure negatively affected the percentage of emergence (6%; Table 5). In the substrate storing conditions (high temperature and low humidity), water retention in the Cerrado soil + manure was low, which made the germination of seeds difficult.

The substrate conditions affected the speed of emergence index. Emergence uniformity of the seedlings kept in sand was larger as compared with seedlings maintained in Bioplant®, soil, and soil + manure (SE = 0.72 – table 5). *C. regium* seeds maintained in Cerrado soil + manure presented low percentage of emergence (Table 5), especially because the substrate retained little water in the assessed conditions. The speed of emergence rate increased for seeds placed in sand, which constituted a more porous substrate and may have facilitated air and water flow to the seeds [23]. Viu et al. [24] and Coelho et al. [2] evaluated the best conditions for the germination and development of *C. regium* seedlings. These authors verified that the commercial substrate vermiculite provided the highest germination rate and the tallest seedlings; they attributed these findings to the physical properties of the substrate—it is highly porous and aerated, and it retains water satisfactorily.

Table 4. Percentage of germination and seedling emergence, speed of germination (SG) and seedling emergence (SE) index and percentage of abnormal seedlings according to the type of vial and seal

Vials/seals*	Germination (%)	Emergence (%)	SG	SE	Abnormal seedlings (%)
Test tube/plastic lid	80.0a	30.0a	15.28b	0.11a	20.0a
Test tube/PVC film	63.33b	33.33a	20.63a	0.12a	3.33b
Test tube /cotton	13.33c	0.0b	4.76b	0.00b	0.0b
Vials/seals*	**Germination (%)**	**Emergence (%)**	**SG**	**SE**	**Abnormal seedlings (%)**
Flask/plastic lid	73.33a	53.33a	0.67a	0.17a	13.33a
Flask/membrane	50.0a	40.0a	0.47a	0.21a	3.33a
Vials/seals*	**Germination (%)**	**Emergence (%)**	**SG**	**SE**	**Abnormal seedlings (%)**
Test tube/plastic lid	80.0a	30.0a	0.57a	0.11a	20.0a
Flask/plastic lid	73.33a	30.0a	0.67a	0.17a	13.33a
Vials/seals*	**Germination (%)**	**Emergence (%)**	**SG**	**SE**	**Abnormal seedlings (%)**
SIGMA® flask/membrane	76.67a	46.67a	0.19a	0.19a	3.33a
Flask/membrane	50.0a	40.0a	0.21a	0.21a	3.33a

*Means followed by the same letters within each column did not significantly differ by Scott–Knott Test at P = .05. *Test tube/plastic lid: test tube sealed with plastic lid (height: 10 cm and diameter: 2 cm); Test tube/PVC film: test tube sealed with PVC film; Test tube/cotton: test tube sealed with cotton; Flask/plastic lid: flask sealed with plastic lid (length: 8 cm and diameter: 5.5 cm); Flask/membrane: flask sealed with plastic lid and membrane; SIGMA® flask/membrane: SIGMA® flask sealed with membrane - Phytatray II/number P5929 (height: 10 cm, width: 10.5 cm, and depth: 9 cm)*

Table 5. Percentage of emergence and speed of emergence index (SE) of *Cochlospermum regium* seeds depending on the substrate

	Emergence (%)	SE
Sand	50.0a	0.72a
Bioplant®	43.0a	0.51b
Cerrado soil	30.0a	0.31c
Cerrado soil + cattle manure	6.0b	0.10d

Means followed by the same letters within each column did not significantly differ by Scott–Knott Test at P = .05

4. CONCLUSION

Taken together, the results presented here attested that it is possible to produce *C. regium* seedlings from seeds of any size both *in vivo* and *in vitro* conditions. *In vitro* conditions, it is important to consider vial oxygenation, in order to obtain a greater amount of normal seedlings. *In vivo* conditions, germination should be conducted in sand, to ensure production of a large quantity of seedlings.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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