http://dx.doi.org/10.1590/2317-1545v36n31013

Morphological aspects of fruits, seeds, seedlings and *in vivo* and *in vitro* germination of species of the genus *Cleome*[1]

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ABSTRACT - The genus *Cleome* is widely distributed in drier areas of the tropics and subtropics. *Cleome dendroides* and *C. rosea* are Brazilian native species that occur mainly in Atlantic Forest and sandy coastal plains, respectively ecosystems negatively affected by human impacts. *Cleome spinosa* is frequently found in urban areas. Many *Cleome* species have been used in traditional medicine, as *C. spinosa*. In the present work, was investigated *C. dendroides*, *C. rosea* and *C. spinosa* germinative behavior under *in vivo* conditions, as well as was established suitable conditions to *in vitro* germination and seedling development. The *in vivo* germination was performed evaluating the influence of temperature, substrate and light. It was observed that only *C. spinosa* seeds presents physiological dormancy, which was overcome by using alternate temperatures. The substrate influenced significantly the germination of *C. rosea* and the seeds of *C. dendroides* showed the highest germination percentages in the different conditions evaluated. The post-seminal development stages under *in vivo* and *in vitro* conditions were defined. It was observed that the development was faster under *in vitro* than *in vivo* conditions. An effective methodology for *in vitro* germination, enabling the providing of material to experiment on plant tissue culture was established to *C. dendroides* and *C. spinosa*.

Index terms: *Cleome dendroides*, *Cleome rosea, Cleome spinosa,* post-seminal development.

Aspectos morfológicos de frutos, sementes, plântulas e germinação *in vivo* e *in vitro* de espécies do gênero *Cleome*

RESUMO - O gênero *Cleome* encontra-se distribuído em áreas tropicais e subtropicais do mundo. *Cleome dendroides* é uma espécie endêmica de Mata Atlântica do estado do Rio de Janeiro, enquanto *C. rosea* é uma espécie nativa que ocorre principalmente em restingas, ambos ecossistemas que sofrem constante impacto antrópico. *Cleome spinosa* é frequentemente encontrada em áreas urbanas. Muitas espécies de *Cleome* têm sido utilizadas na medicina tradicional, como *C. spinosa*. No presente trabalho, avaliou-se a influência do substrato, da temperatura e da luz sobre a germinação *in vivo* das três espécies, bem como determinaram-se as condições para germinação *in vitro* e as etapas do desenvolvimento pós-seminal. Observaram-se que apenas sementes de *C. spinosa* apresentam dormência fisiológica, a qual é superada utilizando-se temperaturas alternadas. O substrato influenciou significativamente a germinação de *C. rosea* e as sementes de *C. dendroides* apresentaram alta porcentagem de germinação nas diferentes condições avaliadas. As etapas do desenvolvimento pós-seminal sob condições *in vivo* e *in vitro* foram definidas. Observou-se que o desenvolvimento sob condições *in vitro* foi mais acelerado que *in vivo*. Uma eficiente metodologia para germinação *in vitro* foi estabelecida para *C. dendroides* e *C. spinosa*, fornecendo material juvenil em condições assépticas para futuros experimentos de cultura de tecidos vegetais.

Termos para indexação: *Cleome dendroides*, *Cleome rosea, Cleome spinosa,* desenvolvimento pós-seminal.

Introduction

Cleome is the largest genus from Cleomaceae family, with over 200 species distributed in drier areas of the tropics and subtropics (Iltis, 1960). It consists mainly of annual or perennial herbaceous plants and, rarely, shrubs. Several species of *Cleome* are used in traditional medicine and many of them have been subject of pharmacological and phytochemical studies (Aparadh et al., 2012). In Brazil, some of these species have been used in traditional medicine, such

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as *C. spinosa*, used in the treatment of diseases related to respiratory tract, and several inflammatory disorders (Agra et al., 2007). Several species may be employed as ornamental due to their attractive inflorescences.

Biotechnological studies have been conducted with *Cleome* species in order to develop efficient protocols for mass propagation and to enhance the production of bioactive molecules under *in vitro* conditions. Such studies have been performed with *C. rosea* Vahl, Brazilian native species mainly found in coastal sandy plains, ecosystems negatively affected by human impacts and *C. spinosa* Jacq., found in urban areas and highly vulnerable to human activities and insect infestations. *In vitro* protocols were established for these species (Simões et al., 2004; Albarello et al., 2006; Albarello et al., 2007; Simões et al., 2009a; Simões et al., 2010a; Simões-Gurgel et al., 2012), and the medicinal potential of *in vivo* and *in vitro* materials was also evaluated (Simões et al., 2006; Simões et al., 2009b; Simões et al., 2010b; Simões-Gurgel et al., 2012; Albarello et al., 2013).

Germination is a critical stage in plants the life cycle and tends to be highly unpredictable over space and time (Baskin and Baskin, 2001). Successful establishment of plants largely depends on successful germination. The germination process may be affected by environmental and internal seed factors and frequently, ideal conditions are species-specific and need to be determined through experimentation.

There is little information available in literature about seed germination of *Cleome*. These studies report low and non-uniform germination, presence of seed dormancy and a significantly variation in germination rates depending on the seed lot used (Ochuodho and Modi, 2007; Raboteaux and Anderson, 2010; Tlig et al., 2012; K'Opondo, 2011). As seeds, in general, are more resistant to disinfection treatments required to obtain aseptic material, when compared to tissues of a developed plant, physiological knowledge of *in vitro* germination allows the establishment of efficient methods that ensure the supply of stock plants in excellent phytosanitary conditions to be used as source of explants for plant tissue culture. There are no studies related to *in vitro* germination of the three species selected.

The purpose of this research was to investigate *C. dendroides*, *C. rosea* and *C. spinosa* germinative behavior under *in vivo* conditions, as well as to establish suitable conditions to *in vitro* germination and seedling development.

Materials and Methods

Seeds of the three species selected were collected in Rio de Janeiro state, Brazil. Fruits were collected from populations located at 22°56'60" S and 43°10'0" W (*C. dendroides*), at 22º58´01" S and 42º58´36" W (*C. rosea*), and at 22°54'17" S and 43°15'52" W (*C. spinosa*). The authenticity of the specimens was previously confirmed and vouchers specimen were deposited in the Herbarium of the Rio the Janeiro State University, Rio de Janeiro, Brazil (*C. dendroides* / HRJ11.104, *C. rosea* / HRJ7185, *C. spinosa* / HRJ7639).

The variables evaluated for fruits were fruit length, thickness, shape and color, whereas for seeds, were seed length, thickness, shape, color, fresh and dry weight, moisture content, viability, coat permeability and mean number of seeds per fruit.

Analysis followed the Rules for Seed Testing (Brasil, 2009). Ten seed lots with one (*C. spinosa* and *C. rosea*) or five (*C. dendroides*) seeds each were weighed to determine the fresh weight (FW) and then subjected to drying in an oven at 105±3 ºC for 24 h to determine the dry weight (DW). The moisture content (MC) was defined as: MC = (FW-DW)/FW x 100.

Seed viability was determined through the triphenyl tetrazolium chloride (TTC) test using five lots of 20 seeds. Seeds were longitudinally sectioned and soaked in 2% TTC solution at 30 °C for 24h in the dark. The viability was assessed using a stereomicroscope (Olympus SD30).

To evaluate seed-coat permeability, eleven lots of five seeds were immersed in distilled water for 24 h at 26 ± 1 °C. During successive periods the fresh weight of each lot was measured to establish the imbibition curve.

Seeds were washed with detergent Fisoex® (10%), rinsed in tap water, and placed in sodium hypochlorite (NaOCl) at different concentrations (1.0; 2.0 or 2.5% w/v) containing Tween 80 (0.05% w/v) for different periods (5; 10 or 20 min). Seeds were rinsed thrice with sterile distilled water and were germinated in transparent plastic germination boxes (Gerbox) (11 x 11 x 3.5 cm). Different substrates were tested: vermiculite (average grain diameter 1.4 to 4.0 mm), sand and towel paper. Substrates were autoclaved at 121 °C for 45 min and moistened with sterile distilled water. The effect of constant (20 and 25 °C) and alternate (15-25 and 20-30 °C) temperatures was evaluated. The experiments were conducted in germination chamber (Eletrolab EL202), under a 16 h photoperiod (20 mmol $m^{-2}s^{-1}$) and relative humidity of 80%. Another set of experiments was conducted in the absence of light using the best conditions of temperature and substrate established for each species in the previous experiment.

Based on the method proposed by Labouriau (1983), germination rate (GR), mean germination time (GT), coefficient of velocity of germination (GV) and relative frequency of germination (GF) were measured and defined as:

Germination rate: GR = N/A x 100

where: N = total number of germinated seeds; A = total number of seeds;

Mean germination time: $GT = \Sigma n_i t_i / \Sigma n_i$

where: n_i = total number of germinated seeds per day; t_i = incubation time (days); Σn_i = total number of germinated seeds.

Coefficient of velocity of germination: $GV = GT^{-1}$

Relative frequency of germination related to the incubation time: $GF (\%) = n_i.100 / \Sigma n_i$

Evaluations were performed every two days during 50 days. When needed substrates were moistened to keep suitable conditions for germination and seedling development. Twelve treatments (three substrates x four temperatures) were considered with three replications of 20 seeds. Experiments were repeated twice.

Seeds were washed with detergent Fisoex® (10%), rinsed in tap water, and disinfected under aseptic conditions in laminar flow hood. Two protocols of decontamination were evaluated: exposure to atmosphere saturated with formaldehyde (paraformaldehyde 80%) for 1, 2 and 3 h, and immersion in a solution of NaOCl (1.0; 1.5; 2.0 and 2.5% w/v) containing Tween 80 (0.05% w/v) for 10, 15, 20 and 30 min. After that, the seeds were rinsed thrice with sterile distilled water and inoculated into flasks containing 30 mL of MS medium (Murashige and Skoog, 1962) added with 30 $g.L^{-1}$ sucrose and solidified with 8 $g.L^{-1}$ agar. The pH of media was adjusted to 5.8 prior to autoclaving (121 °C - 15 min).

Flasks were closed with polypropylene caps and maintained in a growth chamber at 26 ± 2 °C, under a 16 h photoperiod provided by cool white fluorescent tubes (45 mmol $m^{-2}s^{-1}$) or in germination chambers (Eletrolab EL202) under alternate temperatures (20-30 °C), a 16 h photoperiod (20 mmol $m^{-2}s^{-1}$) and relative humidity of 80%. Four seeds were inoculated in each flask in a total of 30 flasks per treatment. Experiments were repeated twice.

The germination was carried out on MS medium with reduced salt concentrations (MS1/2, MS1/3 and MS1/4). Seeds of *C. rosea* were also inoculated on MS medium containing glucose (30 $g.L^{-1}$) as source of carbohydrate and phytagel (2 $g.L^{-1}$) as solidified agent. The influence of aeration was evaluated using aerated caps (Sigma B-3031) to close the flasks.

Morphological aspects were recorded since root protrusion to primary leaves expansion (Brasil, 2009). Root, hypocotyl and epicotyl length as well as the number of leaves, were measured. Seedlings were considered normal when essential structures were perfectly developed (root system well developed, hypocotyl, two cotyledons, epicotyl with apical bud and primary leaves expanded), according to Rules for Seed Testing (Brasil, 2009). The external morphology of seeds, fruits and seedlings were described and illustrated. Graphic records were obtained from fresh material using a stereomicroscope (Olympus SD30) and photographs were taken using Sony H9 camera.

Data were subjected to analysis of variance (ANOVA). Data from germination percentage, when necessary, were transformed into arcsine prior to analysis to normalize their distribution. Means were compared by Tukey test at 5% probability ($p \leqslant 0.05$). The analyses were performed using the GraphPad Prism version 5.0 software.

Results and Discussion

Although the three species under study presented morphological similarities, some traits showed to be species-specific (Table 1). Fruits are siliqua-shaped capsules, dry and dehiscent, and in *C. dendroides* they are also inflated. In *C. rosea* the capsules are greenish (Figure 1A), while in *C. dendroides* (Figure 1B), they are greenish and turn pale brown when riped and in *C. spinosa* capsules are pale yellow turning brown when riped (Figure 1C). The fruit dehiscence occurs from base to apex separating the two valves, to release the seeds. Some seeds may remain attached to the *replum*, a strip holding the valves edges (Figure 1D).

Seeds of three species are small and present particular morphological traits in each species. In *C. spinosa* the seeds are pale yellow to dark brown with kidney-like to cochlear shape with exarillate seed coat, having open terminations and crests on the surface (Figure 1E). *Cleome rosea* seeds are also pale yellow to dark brown and cochlear. They present vesicular aril and tegument with crusts and transversal ribs (Figure 1F). *Cleome dendroides* has brown orbicular seeds. They are applanate, with papilose and muricate surface with finger-shape extensions (Figure 1G). Figure 1H shows *C. dendroides* seed internal structure. Embryos are cotyledon-type with flat, folded and overlapping cotyledons. They present slightly larger thickness than the radicle-hypocotyl axis.

All species had viability higher than 80% and moisture content ranged from 6% to 15% (Table 1). Imbibition curves showed a fast imbibition phase up to the first two hours, with seeds water content reaching around 55%-60% (data not shown), demonstrating high seed-coat permeability.

The substrate and the use of constant or alternate temperatures had no effect on GR in *C. dendroides* (Table 2). Lowest GT values and highest GV values were reached on sand and paper. *Cleome rosea* germination was significantly influenced by the different substrates. Highest values of GR were obtained in vermiculite, although no statistical differences were observed on GT and GV. The use of alternate temperatures was an essential condition to seed germination

in *C. spinosa*. As observed in *C. dendroides*, lowest GT values and highest GV values were reached in sand and paper. In the three species, the germination was observed both in the presence and absence of light.

When considering all physical conditions evaluated, it was found that *C. dendroides* and *C. spinosa* seeds had the highest germination capacity, showing GR values above 90%, while GR values for *C. rosea* stayed below 80%. In addition, in all species, seedling development was superior in vermiculite (Figures 2A-C). No abnormal seedlings were observed in all tested conditions.

Table 1. Characterization of fruits and seeds in different species of the genus *Cleome*.

Plant Material	Variables	Species		
		C. dendroides	*C. rosea*	*C. spinosa*
Fruits	Length (cm)	6.5 ± 2.1	9.0 ± 2.5	17.0 ± 2.0
	Thickness (cm)	1.5 ± 0.5	0.8 ± 0.4	1.35 ± 0.2
	Shape	Siliqua-shaped capsules	Siliqua-shaped capsules	Siliqua-shaped capsules
	Color	Greenish to pale brown	Greenish	Pale yellow to brown
Seeds	Length (mm)	2.04 ± 0.07	1.49 ± 0.32**	2.34 ± 0.24**
	Thickness (mm)	1.19 ± 0.09	1.09 ± 0.30**	1.35 ± 0.22**
	Shape	Orbicularis	Cochlear	Kidney-like to cochlear
	Color	Brown	Pale yellow to dark brown	Pale yellow to dark brown
	Fresh weight (mg)	2.73 ± 0.15*	1.00 ± 0.20**	2.00 ± 0.04**
	Dry weight (mg)	2.34 ± 0.21*	0.94 ± 0.15**	1.70 ± 0.03**
	Moisture (%)	12.17 ± 4.20	6.00 ± 2.40**	15.00 ± 1.30**
	Viability (%)	90 - 100	80 - 90**	90 - 100**
	Coat permeability	High	High	High
	Number per fruit	380 ± 55	90 ± 15	139 ± 20

*average weight of five seeds.
** dark brown seeds.

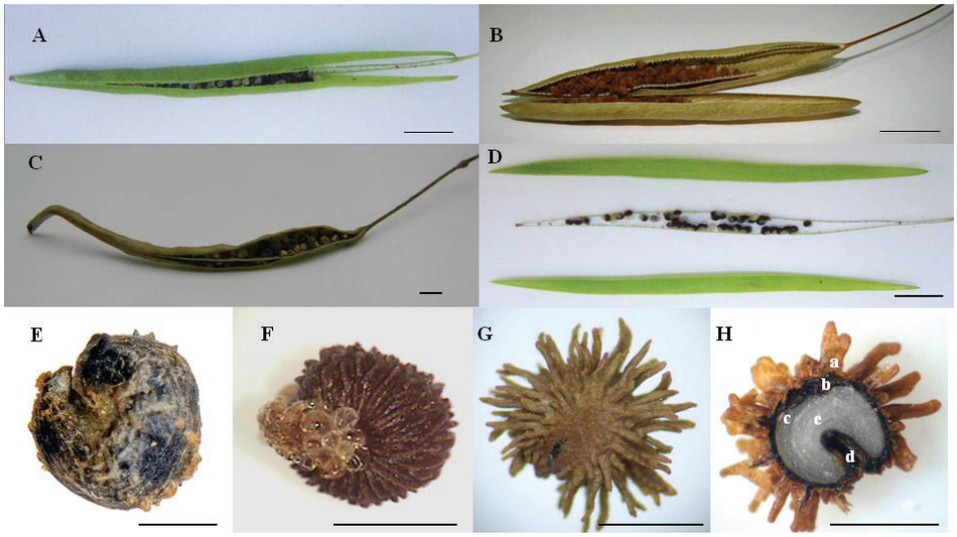

Figure 1. Morphological aspects of fruits and seeds of the genus *Cleome*: A - fruit of *C. rosea*; B - fruit of *C. dendroides*; C - fruit of *C. spinosa*; D - seeds of *C. rosea* still attached to replum; E - seed of *C. spinosa*; F - seed of *C. rosea*; G - seed of *C. dendroides*; H - the internal structure of *C. dendroides* seed (a - extensions of testa; b – testa; c - endosperm; d - hilum; e - hypocotyl-radicle axis). Scale bars: 1 cm.

Table 2. Effect of temperature and substrate on germination rate (GR), mean germination time (GT), and coefficient of velocity of germination (GV) in species of the genus *Cleome*.

Parameters	Substratum	*C. dendroides*			*C. rosea*				*C. spinosa*			
		Temperature (ºC)										
		20	25	20-30	20	25	15-25	20-30	20	25	15-25	20-30
GR (%)	Paper	96.7a	96.7a	96.7a	60.0b	33.3bc	66.6b	26.6c	0b	0b	100a	96.7a
	Sand	93.3a	100a	93.3a	66.6b	40.0bc	46.6bc	53.3b	0b	0b	96.7a	96.7a
	Vermiculite	96.7a	100a	96.7a	80.0a	33.3bc	73.3a	80.0a	6.6b	0b	93.3a	90.0a
GT (days)	Paper	9.8b	7.3c	7.4c	13.1b	16.4b	15.7b	23.8a	-	-	2.9c	3.1c
	Sand	9.3b	6.8c	7.3c	18.2b	17.2b	27.6a	20.2a	-	-	2.5c	2.2c
	Vermiculite	12.9b	10.2b	15.2a	14.7b	13.6b	22.9a	28.8a	11.5a	-	5.7b	4.0b
GV (days)	Paper	0.10b	0.14a	0.13a	0.08a	0.06a	0.06a	0.04b	-	-	0.35a	0.32a
	Sand	0.11b	0.15a	0.14a	0.06a	0.06a	0.04b	0.05b	-	-	0.40a	0.45a
	Vermiculite	0.08b	0.10b	0.06c	0.07a	0.07a	0.04b	0.04b	0.10c	-	0.18b	0.25b

Means of each parameter evaluated for each species followed by the same letter do not differ by Tukey test (5%).
- no germination.

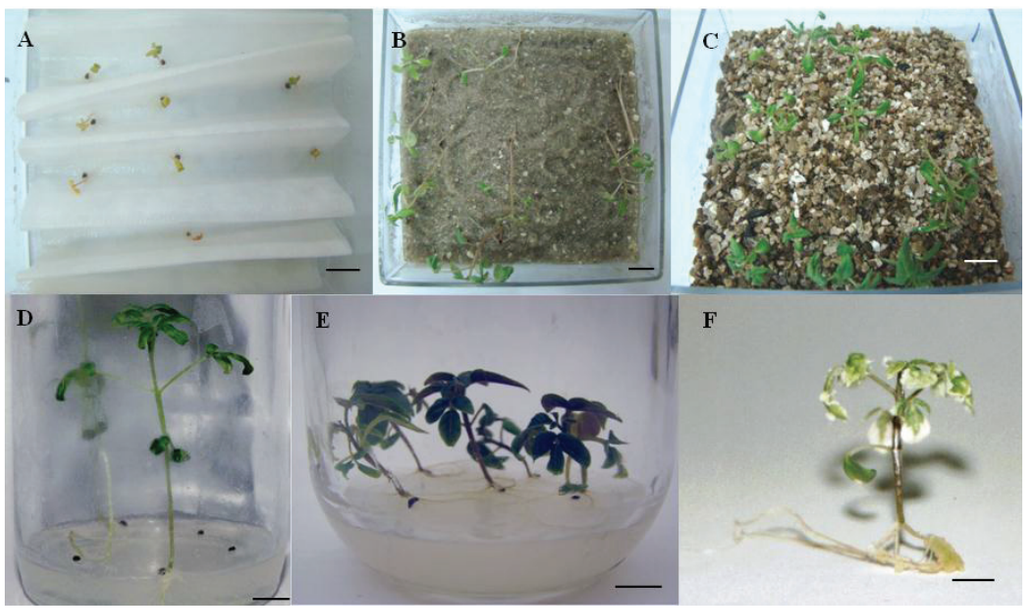

Figure 2. *In vivo* and *in vitro* germination of *Cleome*. *In vivo* germination of *C. dendroides*, at 25 ºC, on different substrates: A - paper; B - sand; C - vermiculite. *In vitro* germination: D - *C. dendroides*; E - *C. spinosa*; F - *C. rosea.* Scale bars: 1 cm.

The relative frequency polygons for germination, which determine the proportion of seeds germinated daily (Figure 3), showed that *C. spinosa* seeds presented the fastest germination when kept in alternate temperatures (Figure 3A). In this species, radicle protrusion took place at 1 - 3 days, on paper and sand. Maintenance of *C. dendroides* seeds in alternate temperatures of 20-30 ºC or in constant temperature of 25 ºC, accelerated radicle protrusion, which took place around the seventh day, on paper and sand (Figure 3B). These results showed that both *C. spinosa* and *C. dendroides* have a synchronic germination process. *Cleome rosea* showed a delayed and intermittent germination, in some cases, the beginning of the process occurred after 40 days (Figure 3C).

Germination under *in vitro* conditions was successfully established to *C. dendroides* and *C. spinosa*, but it was not efficient to *C. rosea*. The exposure of seeds to formaldehyde saturated atmosphere was more efficient protocol of decontamination when compared to the immersion in sodium hypochlorite solution. To *C. dendroides*, three hours exposure to formaldehyde resulted in an efficient decontamination level (100%) and in GR of 100%. The use of sodium hypochlorite (2.5% for 20 min) allowed low contamination (10%), seeds showed lower GR (90-95%) and delayed the

start of germination process. Similar results were observed in *C. spinosa*. Decontamination in formaldehyde atmosphere for 1 h resulted in GR above 80%, while the best results obtained with sodium hypochlorite (1.5%, 20 min) were 48%. To *C. rosea* the highest GR (85%) was reached with sodium hypochlorite at 1% for 10 min.

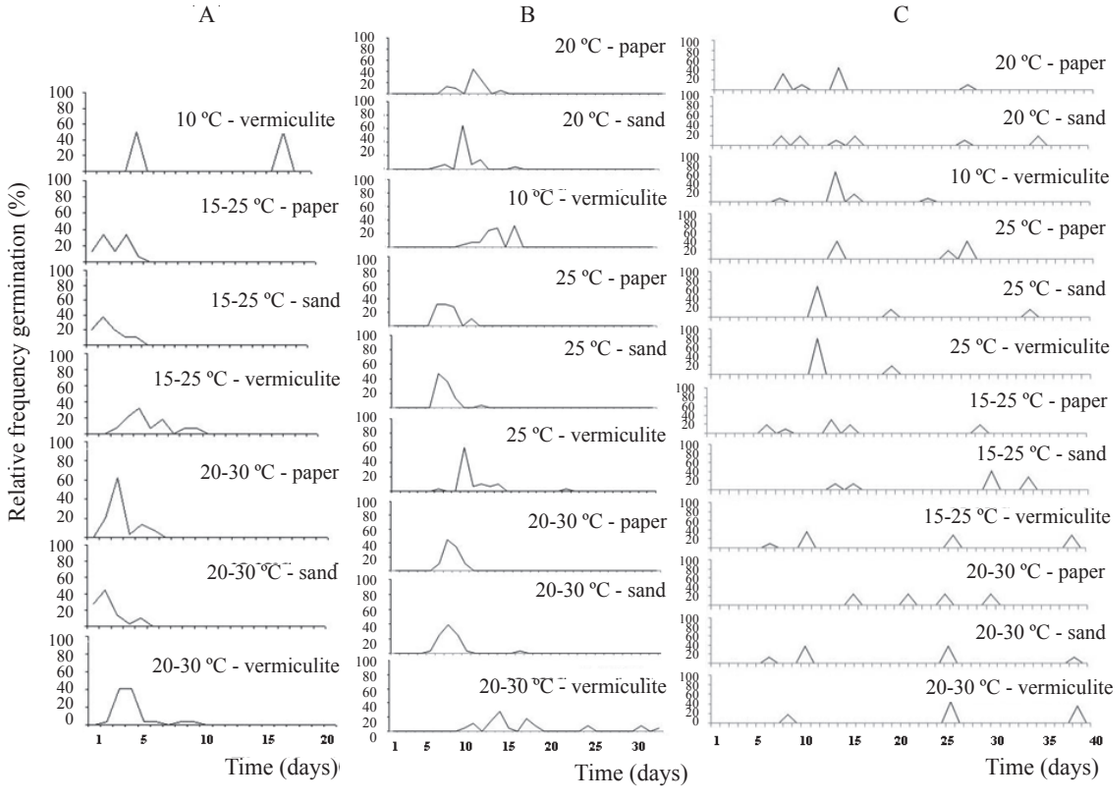

Figure 3. Relative frequency polygon from the germination of *C. spinosa* (A), *C. dendroides* (B) and *C. rosea* (C) under different temperatures and substrates.

After decontamination, seeds where inoculated on MS medium in regular or with reduced salt concentration (MS1/2 and MS1/4). *Cleome dendroides* seedlings showed hyperhydricity on MS medium, but developed normally on MS1/4 (Figure 2D). Germination occurred both in constant (26 ± 2 ºC) and alternate (20-30 ºC) temperatures. As for *C. spinosa,* salt concentration had no influence on seedling development (Figure 2E) and alternate temperatures were required for germination.

In vitro germination of *C. rosea* resulted in seedlings presenting an intense callusing in folioles, cotyledons and stem (Figure 2F). To establish healthy seedlings new assays were performed with modifications on culture conditions, however the new conditions did not prevent callus formation and, in addition, glucose caused a decrease in GR (60%) and seedlings size.

Cleome dendroides in vivo germination started after around seven days, with radicle protrusion, which breaks the tegument in the hilum (Figure 4B). After nine days, primary root was white, cylindrical, thin, and reached 8 mm (Figure 4C). On the day 11, hypocotyl growth was faster than the root, showing cylindrical shape and bright green color. In this stage, the cotyledons are carried above the soil surface by the elongating hypocotyl (Figure 4D) characterizing the epigeal germination. Around day 12, cotyledons were free from the tegument, which is typical of phanerocotyledonous germination. The cotyledons were orbicularis (diameter 0.5 cm), opposite, glabrous, dark green on the adaxial surface and bright green on the abaxial surface, entire margin and 1 cm petiole. Cotyledon expansion was observed on the day 13 (Figure 4E). At this stage, hypocotyl elongation ceased (1.5 - 2.0 cm) and it was observed the development of a root system of the axial type, with cylindrical, thin, hairy and white secondary roots (Figure 4F). Epicotyl was recorded around day 25. It was cylindrical and bright green. Around day 30, the pair of primary leaves was formed. Cotyledons abscission occurred after about 35 days. The young plant, after 40 (Figure 4G), presented the pair of opposite primary leaves, trifoliate, membranous, and oblong-elliptical leaflets with

attenuated base, obtusely acuminate at apex, smooth edges, pubescent adaxially and slightly pubescent on the abaxial surface with printed rib on the adaxial and prominent on the abaxial surface, dark green on the adaxial and bright green on the abaxial surface, but more clear than the cotyledons. Central leaflets were larger than lateral ones. The cylindrical petioles had about 0.8 cm, leaflets were sessile and the axial root system become fully established.

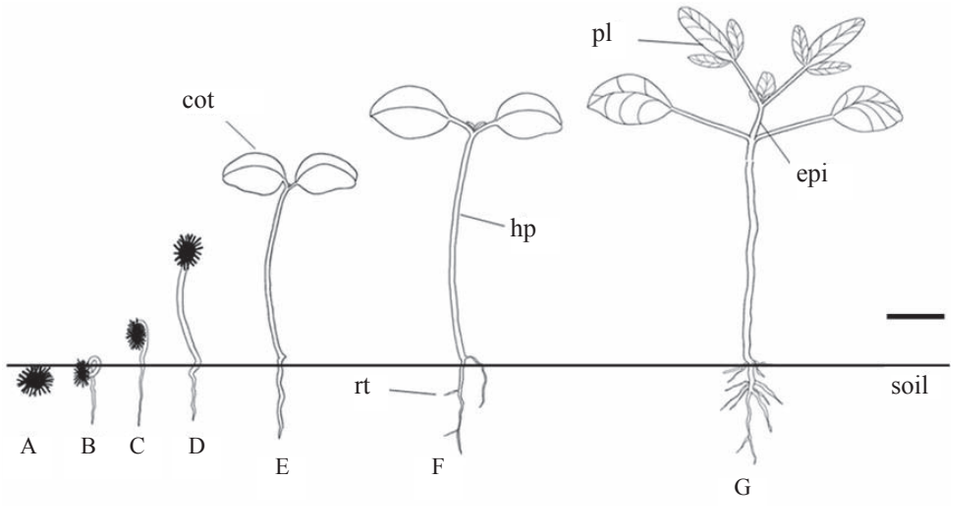

Figure 4. Morphological aspects of *in vivo* germination of *C. dendroides*, in sand, at 25 ºC: A - seed; B - 7 days after primary root protrusion; C - 9 days; D -11 days; E - 13 days; F - 20 days; G - 40 days. cot - cotyledon; hp - hypocotyl; rt - root; pl - primary leaves; epi-epicotyl. Scale bars: 4 mm.

Cleome spinosa in vivo germination took place around day 3. On day 5, primary root reached 0.5 cm and hypocotyl with the same dimensions. Hypocotyl was bright green and cylindrical. During its development, elevated foliar cotyledons already released the tegument, characterizing the epigeal germination. Around the day 15, the epicotyl (0.2 cm) was formed. On day 20, the first pair of membranous and trifoliate leaves was established and after 30 days the second pair of leaves with alternate phyllotaxy was formed. The compound leaves presented the central leaflets larger than the lateral ones.

Cleome rosea in vivo germination was observed 7 - 9 days after sowing. From day 10 hypocotyl entered a period of active growth, stretching and elevating the cotyledons above the substrate characterizing the epigeal germination. On day 15 the early development of the epicotyl and first pair of leaves were observed. Between 18 and 20 day, first pair of trifoliate leaves was formed and after 40 days they possessed a bright-green, glabrous stem axis consisting of the hypocotyl (3.5 cm) and epicotyl (3.8 cm) and two pairs of trifoliate leaves. At day 60, plants had an axial root system, well developed, with many secondary roots and three pairs of compound leaves, arranged in alternate phyllotaxy, with membranous and hairy leaflets, with entire margin and bright green color. Central leaflets were larger than the laterals.

Cleome dendroides in vitro germination took place after 7-8 days of culture, by the rupture of the tegument in the hilum and root protrusion. After 8 days, hypocotyl differentiation and primary root elongation occurred. After 9 days, the tegument remained adhered to the cotyledons and hypocotyl elongation occurred. The emergence and expansion of cotyledons was observed after 9 - 10 days. The pair of primary leaves emerged around day 17 and epicotyl and secondary roots around the day 20. After 25 days, the seedling had all the essential structures: roots, hypocotyls, cotyledons and epicotyl with the pair of primary leaves expanded.

The start of *C. spinosa in vitro* germination took place after 1 - 2 days after inoculation. Hypocotyl and primary root developed after three days. The elevation of foliaceous cotyledons occurred after 5 days and epicotyl was formed after 7 days. The first pair of membranous and trifoliolate leaves was observed after 12 days and the visualization of the second pair of leaves occurred after 18 days.

Mature fruits of both *C. rosea* and *C. spinosa* contained seeds of varying color and size, prevailing dark brown seeds with larger dimensions, higher dry weight, and lower moisture. Considering *C. dendroides* seeds they are only brown. The analyzed lots showed moisture content ranging from 6 to 15%. Tegument color, along with these other characteristics,

may provide data on the quality of the seed lot (Ochuodho and Modi, 2010). Physiological maturity of seeds is associated with low moisture content and at this stage, seeds reach maximum germination (Black et al., 2006). Thus, dark brown seeds of all species evaluated were considered fully mature.

The topographical tetrazolium test enables fast and efficient determination of the percentage of viable seeds, especially those that present dormancy, the recalcitrant and species that require long time to germinate in laboratory (Brasil, 2009). This test is routinely used in quality control programs for various species (Carvalho et al., 2013). The TTC test results showed seed viability ranging from 80 to 100% and the high viability of the lots was confirmed in germination tests.

Considering the tegument permeability, the results indicated that *Cleome* seeds do not exhibit dormancy in the tegument (physical dormancy) since it presented no resistance to water entry. Physical dormant seeds restrict imbibition by different mechanisms, such as the presence of one or more layers of lignified palisade cells on the tegument, making it impermeable (Baskin and Baskin, 2001).

The influence of substrate and temperature on the germination process showed that the optimum conditions varies among the species evaluated. The substrate influenced most significantly the germination in *C. rosea,* while for *C. spinosa* the alternating temperature was an essential condition. On the other hand, *C. dendroides* reached high germination rates in all conditions tested. Some species of the genus *Cleome* present no dormancy and germinate without the need of pretreatment. *Cleome amblyocarpa* germinates in the absence of light, and 25 °C was considered the ideal temperature, with germination percentage of 81% (Tlig et al., 2012). However, several species of *Cleome* exhibit physiological dormancy. Ekpong (2009) studying *C. gynandra* observed germination rate of 17.3% in freshly harvested seeds, at alternate temperatures (20 - 30 °C), without any pre-germination treatment. According to the author, the species requires a post-maturation period of three months, at environment temperature in order to break dormancy, after what germination rates were at 90%. Physiological dormancy was also reported for *C. lutea* (Cane, 2008), *C. serrulata* (Cane, 2008) and *C. hassleriana* (Raboteaux and Anderson, 2010).

In the present study the germination process was not influenced by light, characterizing the *Cleome* seeds as neutral photoblastic. However, experiments carried out to evaluate the influence of temperature and light on germination of *C. gynandra* from Africa (Ochuodho and Modi, 2007) concluded that the species showed photoinhibition. The authors recommend that germination should be performed in the absence of light and/or alternate temperature of 20-30 ºC.

Presented results show that *C. spinosa* needs the breaking of physiological dormancy in order to germinate, and this can be achieved with the use of alternate temperatures. This is in contrast to what was observed in *C. dendroides* and *C. rosea* that showed no dormancy. Alternate temperatures may favor dormancy breaking, by modulating hormone synthesis, altering endogenous levels and influencing seed germination (Black et al., 2006).

Germination was synchronic in *C. spinosa* and *C. dendroides* and asynchronous in *C. rosea*. Asynchronous germination leads to a wider distribution in seed germination time, which enhances the species' chances of survival under natural conditions by favoring the formation of seed banks (Baskin and Baskin, 2001). Among the studied species, *C. rosea* is the only that occurs in restinga, coastal ecosystems characterized by elevated temperatures and saline sandy soils. These abiotic features may require longer germination periods to ensure the species' establishment success. Asynchrony may be attributed to environmental oscillations during seed formation, as well as between individual intrinsic genetic variability within populations. Zamith and Scarano (2004), analyzing seedling production in species from Rio de Janeiro's restingas, observed large amplitude in germination time requirement.

In vitro conditions promote continuous physiological stress, which sometimes leads to hyperhydricity. Hyperhydric plants or buds present turgid, thick, wrinkled, twisted, translucent, with glass appearance, pale green stems and leaves, rigid and easily breakable. Shoots with shortened internodes and this morphological appearance suggest that water is in excess. The type of sealing may reduce or control the water on the atmosphere of culture flasks (Vasconcelos et al., 2012). In this present study, hyperhydricity was observed in plants of *C. dendroides* kept on MS medium, and the type of sealing showed no influence on its prevention. *In vitro* germination of *C. dendroides* was achieved on MS¼ medium with high GR yields. Besides the relative humidity, the concentration and type of gelling agent, and growth regulators, must be controlled to avoid hyperhydricity (Vasconcelos et al., 2012). The decrease in salt concentration, in particular of ammonium ions, may also be effective in preventing hyperhydricity (Guerra and Nodari, 2006), as seen in *C. dendroides*. The germination process under *in vitro* conditions of *C. rosea* seeds was not efficient. Despite the high percentage (85%) of seed germination in MS medium, plants presented low development of stem axis when compared to plants germinated *in vivo*. Callus formation was observed over the entire surface of the leaves.

The use of glucose as carbon source reduced germination

efficiency in comparison to sucrose. Carbohydrates play an important role in maintaining proper osmolarity on culture medium, as well as serving as nutrient source. The low germination percentage of *C. rosea* seeds inoculated in medium supplemented with glucose could be the result of an inadequate osmolarity to allow proper water exchange between the medium and seeds.

Calli observed in *C. rosea* leaves may be related to the presence of ethylene in the culture flasks. Under *in vitro* conditions ethylene may be produced after the buckling as a byproduct of combustion accumulating in vials. George (2008) reported the formation of callus-like structures on leaves and stems of intact plants kept on *in vitro* conditions, when they were maintained in ethylene enriched atmosphere for a prolonged time. The stimulation of cell division observed in *C. rosea* leaves and consequent callus formation may also have been due to auxin accumulation in their synthesis sites or in close regions, caused by the antagonistic action of ethylene on polar auxin transport (George, 2008). No reduction in leaf callogenesis was observed when flasks sealed with lids that allow greater gas exchange. According to Armstrong et al. (1997), only a forced ventilation system was able to significantly reduce ethylene levels inside culture flasks. Considering the possibility that *C. rosea* has a high sensitivity to ethylene, new methods to decrease its concentration in culture flasks must be employed in order to develop an effective protocol for germination the species under *in vitro* conditions.

During post-seminal development of *C. dendroides* and *C. spinosa*, it was observed faster development *in vitro* in comparison to *in vivo* conditions, which may be due to carbon supply (sucrose) and other nutrients present in culture medium, in addition to natural seed reserves.

Conclusions

The present study shows that optimal conditions for the germination are different for each of the species. *Cleome spinosa* has physiological dormancy, while *C. dendroides* and *C. rosea* do not exhibit dormancy under *in vivo* conditions. The different stages of *in vivo* post-seminal development of the three species are defined. In addition, an effective methodology for *in vitro* germination, enabling the providing of material to experiment on plant tissue culture is established to *C. dendroides* and *C. spinosa*.

Acknowledgements

The authors thank the technical support of Maria Francisca Santoro de Assunção (PROATEC/UERJ) and Adriana Maria Lanziotti (Qualitec/UERJ). The authors also thank Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for providing financial support.

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