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IN VITRO AND *EX VITRO* GERMINATION OF THREE *HANDROANTHUS* SPECIES (BIGNONIACEAE)

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Summary: Apóstolo, N. M., E. E. Larraburu, M. N. Gil, M. A. Zapater & B. E. Llorente. 2016. *In vitro* and *ex vitro* germination of three *Handroanthus* species (Bignoniaceae). Bonplandia 25(1): 5-15.

Handroanthus impetiginosus, *H. ochraceus* and *H. lapacho*, "lapachos", are found in the Northwest of Argentina and have germination and conservation problems in their natural habitats. The germination of seeds under controlled conditions is an alternative that ensures the propagation. In this integrated study, *in vitro* and *ex vitro* germination, seed characteristics and seedling morphology of three species were analyzed. The length and width of the seeds, the width of the wings, the width and length of the seed body and embryo were measured. The germination capacity of the three species was determined during 12 months after seed harvesting, carrying out *ex vitro* and *in vitro* germination trials. The seeds and embryos of *H. impetiginosus* were significantly larger than those of other species. *Handroanthus impetiginosus* seeds maintained a high germination capacity over the 12 months (95%). Seed germination capacity of *H. ochraceus* gradually decreased four months after seed collection (63 to 47%), whereas that of *H. lapacho* decreased abruptly after eight months. No significant differences were observed between *ex vitro* and *in vitro* methods in *H. impetiginosus* and *H. ochraceus.* In the three species, the germination capacity is directly proportional to the size of seeds and seedlings.

Key words: *Handroanthus impetiginosus, H. lapacho, H. ochraceus,* seed, seedling.

Resumen: Apóstolo, N. M., E. E. Larraburu, M. N. Gil, M. A. Zapater & B. E. Llorente. 2016. Germinación *in vitro* and *ex vitro* de tres especies de *Handroanthus* (Bignoniaceae). Bonplandia 25(1): 5-15.

Handroanthus impetiginosus, *H.ochraceus* and *H. lapacho,* "lapachos", se distribuyen en el NO Argentino y presentan inconvenientes de germinación y conservación en su ambiente natural. La germinación de semillas bajo condiciones controladas es una alternativa para asegurar la propagación de especies con este tipo de problemáticas. En el presente estudio integral, se analizó la germinación *in vitro* y *ex vitro*, las características de las semillas y la morfología de las plántulas de las tres especies de *Handroanthus* mencionadas. Para ello, se midió el largo y ancho de las semillas, el ancho de las alas de la cubierta seminal, el ancho y largo del cuerpo seminal y del embrión. El poder germinativo de las tres especies fue determinado durante 12 meses luego de la cosecha de las semillas. Fueron determinados los parámetros de las plántulas obtenidas *in vitro* y *ex vitro*. El tamaño de la semilla y embrión de *H. impetiginosus*

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fue significativamente mayor que el de las otras especies estudiadas. *H. impetiginosus* mantuvo altos porcentajes de germinación hasta los 12 meses (95 %). *H. ochraceus* perdió gradualmente su poder germinativo a partir de los 4 meses posteriores a la cosecha de las semillas (63 a 47 %). Mientras que, en *H. lapacho* disminuyó abruptamente a los 8 meses luego de la cosecha. No se observaron diferencias significativas entre ambos tratamientos de germinación (*in vitro* y *ex vitro*) para *H. impetiginosus* y *H. ochraceus*. Las tres especies mostraron una relación directa entre la capacidad germinativa y las dimensiones de las semillas y características de las plántulas.

Palabras clave: *Handroanthus impetiginosus, H. lapacho, H. ochraceus,* plántula, semilla.

Introduction

The genus *Handroanthus* belongs to the Bignoniaceae family and arose from the segregation of the genus *Tabebuia* Gomes ex DC. by molecular phylogenetic studies (Grose & Olmstead, 2007a, b). Currently, the species included in *Tabebuia* (Gentry, 1972) are distributed in three genera: *Roseodendron* Miranda, *Handroanthus* Mattos and *Tabebuia* Gomes ex DC. In Argentina, there are only two species in *Tabebuia: T. aurea* and *T. nodosa,* and none in *Roseodendron*. Most of the native species belong to *Handroanthus*: *H. impetiginosus* (Mart. ex DC) Mattos, *H. lapacho* (K. Schum.) S. Grose and *H. ochraceus* (Cham.) Mattos are distributed in northwest Argentina, whereas *H. albus* (Cham.) Mattos, *H. pulcherrimus* (Sandw.) S. Grose and *H. heptaphyllus* (Vell.) Mattos are found in the northeast (Grose & Olmstead, 2007b; Lozano & Zapater, 2008; Zapater et al., 2009; Zuloaga et al., 2011).

Handroanthus impetiginosus is a tree 20-30 m in height, with pink flowers, and it is found up to 1,300 m in the Tucumano-Oranense forest in the provinces of Jujuy, Salta, Tucumán and Catamarca, Argentina. It has the widest distribution in Argentina of the species in this genus and it is cultivated as an ornamental from northwest Argentina to the province of Buenos Aires. However, its natural area has been reduced by anthropogenic activities (Silva et al., 2004; Lozano & Zapater, 2008; Zapater et al., 2009).

Handroanthus ochraceus is a tree of 30-35 m in height, with yellow flowers, and its range of distribution covers Salta and Jujuy provinces in Argentina (from 300 to 1200 m). It suffers from irregular reproduction linked to unseasonal frosts (Zapater et al., 2009).

Handroanthus lapacho is a tree up to 20 m in height, with yellow flowers, and its distribution is restricted to a narrow altitudinal belt (1400-1750 m) in the north of Argentina (Jujuy and Salta). Even though it is naturally protected, it is considered as vulnerable by the International Union for Conservation of Nature (IUCN) as it occurs in a geographically fragmented area and its occurrence and quality of habitat are declining. It is cultivated sporadically in urban areas in Salta and Jujuy (IUCN, 2004; Zapater et al., 2009).

The seeds of *Handroanthus* are thin, exalbuminous and have a winged seed coat. The seed body is cordiform, flat and wide (Silva et al., 2004; Ferreira & Cunha, 2000; Zapater et al., 2009). Costa et al. (2004) and Sampaio et al. (2007) have studied the ontogeny of *H. ochraceus* seed. Morphological and physiological aspects of the germination of *H. impetiginosus* have been reported by Silva et al. (2004). Morphological studies of the fruit, seed and seedlings have been reported by different authors (Corner, 1976; Carvahlo et al., 2008; Ferreira & Cunha 2000; Souza et al., 2005).

Even though most species of *Handroanthus* produce large quantities of seed, many of them have germination and conservation problems in their natural habitats (Bocchese et al*.,* 2008; Carvahlo et al., 2008; Justiniano et al., 2000; Nery et al., 2008). *In vitro* germination has been little studied (Nery et al., 2008), however some studies have been carried out on the *ex situ* germination of several species of this genus (Silva et al., 2004; Ferreira & Cunha, 2000; Nery et al., 2008). *In vitro* cultivation of seeds is an appropriate method that allows the

propagation of species with recalcitrant seed, with preservation difficulties or that have problems with asexual reproduction. After *in vitro* germination the seedlings obtained can be acclimatized directly under greenhouse conditions or used as explants *in vitro* morphogenesis (Blakesley et al., 1996; Fay, 1994; Larraburu et al., 2012; Thorpe et al., 1991). Therefore this technique is appropriate for *ex situ* conservation plans for rare or threatened species and also for massive propagation of species of economic interest (Silva et al., 2005; Fay, 1994).

In this study the *in vitro* and *ex vitro* germination of three species of *Handroanthus* that occur in northwest Argentina (*H. impetiginosus, H. lapacho* and *H. ochraceus*) were evaluated. The morphology of the seed and seedlings was also analyzed so that their characteristics could be related to the germination rate in the three species.

Materials and Methods

Plant material

Seeds of *H. impetiginosus, H. lapacho* and *H. ochraceus* (Bignoniaceae) were collected from different habitats in the province of Salta, Argentina. The seed samples were stored in paper envelopes after being left to dry and then were kept in a fridge at 5ºC until their analysis. The seed used for data on morphology, germination energy and capacity were collected in the first season, whereas seed used for *in vitro* and *ex vitro* culture trials were collected in the second season.

Seed morphology

The parameters of 30 seeds of each species were recorded at the time of collection: length, width and thickness of the seed, width of the wings, width and length of the seed body and width, length, thickness and midsection of the embryo (Fig.1G, H).

Germination energy and capacity of the seed

For each species, 20 to 30 seeds, depending on their size, were germinated on damp paper in Petri dishes. Seeds and dishes were previously disinfected with sodium hypochlorite (NaClO) (55 $g.L^{-1}$ active chlorine) at 50% for 5 min and rinsed with sterilized distilled water. The paper was sterilized in an autoclave at 1 atm (120 ºC) for 20 min. Three repetitions were carried out for each species at 0, 4, 8 and 12 months since the seed collection. The seeds were stored in the fridge at 5ºC between these periods. The germination dishes were kept in the culture chamber at 25 ± 2ºC under continuous light. The germination percentage was weekly evaluated during 30 days (germination capacity). The germination energy was determined at five days after the beginning of the each test of germination.

Seed culture

The seeds were stored at 5ºC for a year after their collection. They were disinfected after the removal of the wing portion of the seed coat. The disinfection was done by washing with tap water for 1 h, immersion in 20% NaClO for 30 min with agitation and, finally, three rinses with sterilized distilled water.

Ex vitro culture. The disinfected seed was grown in seedling trays previously sterilized with 20% NaClO. The substrate used was soil:peat:perlite (1:1:1), which was autoclaved at 120ºC (1 atm) for 45 min.

In vitro culture. For the *in vitro* germination of each species the disinfected seeds were grown in basic Woody Plant Medium (WPM) (Lloyd and McCown 1980) suplemented with 5 mM bencylaminopurine (BA), 5 $g.L^{-1}$ activated carbón, 100 $mg.L^{-1}$ myo-inositol, 20 $g.L^{-1}$ sucrose and 6 g L^{-1} agar, at pH 5.8. The culture medium (15 ml) was divided into flat bottomed tubes 50 cm^3 tubes and sterilized in an autoclave at 1 atm for 20 min.

Both the *ex vitro* and the *in vitro* cultures were maintained in a controlled chamber at 24± 2° C, with a photoperiod of 16 h light and 25-26 mmol $m^{-2}s^{-1}$ relative humidity. After 30 days from the start of the cultures, the germination percentage was recorded as well as the morphological parameters of the seedlings obtained (lengths of the epicotyl and hypocotyl and the width of the cotyledons).

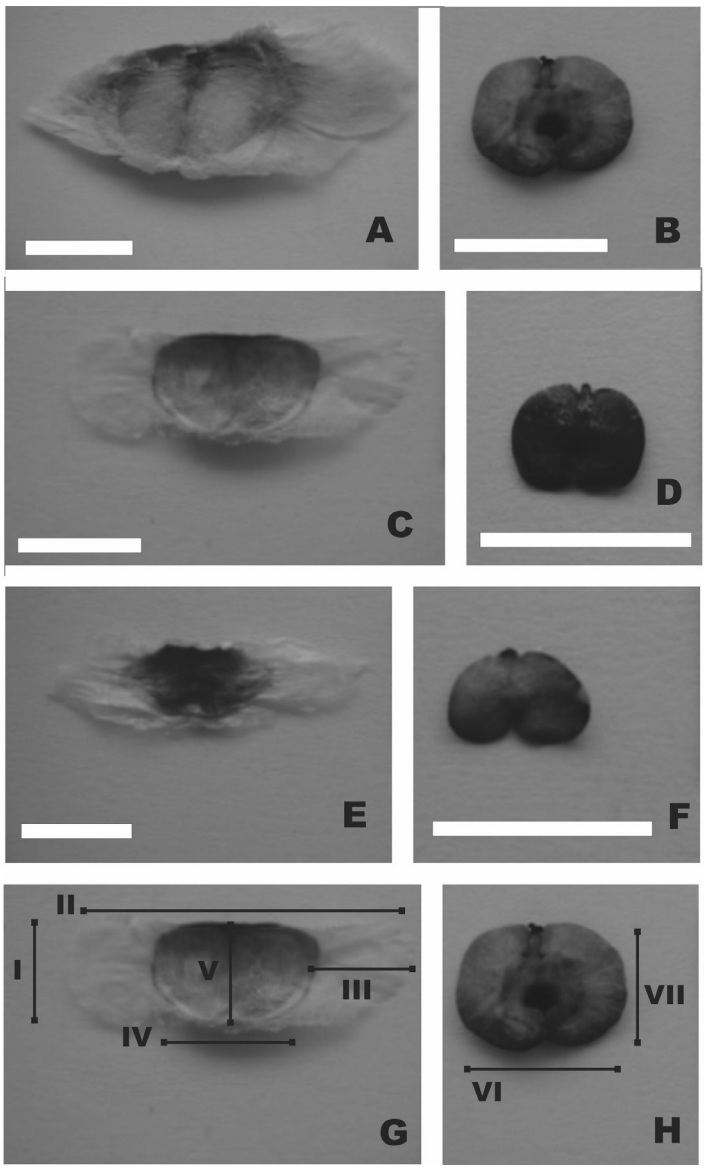

Fig. 1. Seed of *Handroanthus* species. A: General aspect of the seed of *H. impetiginosus*. B: Embryo of *H. impetiginosus*. C: General aspect of the seed of *H. ochraceus*. D: Embryo of *H. ochraceus*. E: General aspect of the seed of *H. lapacho*. F: Embryo of *H. lapacho.* G-H: Parameters measured in the seeds and embryo of the species: I, width of the seed; II, length of the seed; III, width of the wings of the seed; IV, length of the seed body; V, width of the seed body; VI, width of the embryo; VII, length of the embryo. Bars: A-F: 1 cm.

Statistical analysis

The experimental design was totally randomized in all the trials. Unifactorial analysis was applied for the seed characteristics. Bifactorial analysis was used for the germination percentage, the epicotyl and hypocotyl lengths and the cotyledon width. The factors considered were the species (with three levels: *H. lapacho, H. impetiginosus, H. ochraceus*) and culture method (with two levels: *in vitro, ex vitro*).

The data was analyzed with ANOVA and a multiple mean comparison (Tukey Test, $p< 0.05$) using SPSS® 12.0 (SPSS Inc. Chicago, Illinois, USA).

Results

The seeds of the studied species showed morphological characteristics of the Bignoniaceae family: winged seed coat, cordiform seed body and embryo, and exalbuminous seed (Fig. 1 A-H).

The dimensions of the seed and embryo of *Handroanthus impetiginosus* were significantly larger than those measured in *H. ochraceus* and *H. lapacho*, except for the width of the seed body (Table 1, Fig. 1 A-F). Intermediate values of the seeds were recorded for *H. ochraceus,* although they showed few significant differences with those determined for *H. lapacho* (Table 1, Fig. 1 C-F). Only *H. ochraceus* has polyembryonic seed.

Handroanthus impetiginosus showed the greatest germination energy during the 12 months of storage (Fig. 2). *H. lapacho* and *H. impetiginosus* showed germination energy similar to that at the time of collection (33%). However, as from the fourth month of storage, *H. lapacho* showed an abrupt decrease in this parameter, whereas *H. impetiginosus* increased its values (Fig. 3). At eight months after storage the germination energy of *H. ochraceus* was lower in comparison with the other species studied (Fig. 2).

Similar germination percentages for *H. impetiginosus, H. lapacho* and *H. ochraceus* (85% to 98%) were seen at the time of collection (Fig. 3). However, seeds of *H. ochraceus* gradually decreased their germination capacity to 63% after 4 months, reaching 43% at 12 months after harvest (Fig. 3). The results for *H. lapacho* were similar but there was an abrupt decrease in the germination capacity at eight months after harvest (47%) (Fig. 3). The seeds of the species studied that were stored at 5ºC for one year, mainly *H. impetiginosus*, showed germination percentages of 82-100% (Table 2; Fig. 3).

No significant differences were seen between the *ex vitro and in vitro* methods of germination in *H. impetiginosus* and *H. ochraceus*, but germination of *H. lapacho* was better in the *in vitro* culture (Table 2).

Handroanthus impetiginosus, H. lapacho and *H. ochraceus* showed appropriate *in vitro* development in WPM (Table 2, Fig. 4 A-C). The percentages of *in vitro* germination varied according to the species (49% to 82%) and the highest values were obtained in *H. impetiginosus* (Table 2, Fig. 4 D, E, G). The WPM supplemented with cytokinins allowed the elongation of the stem of the seedling in all three species studied (Fig. 4 A-C, F).

The *ex vitro* germination substrate (peat:perlite:soil, 1:1:1) showed percentages between 38% and 85%, and *H. impetiginosus* had the highest under these conditions (Table 2, Fig. 4 D-F).

The species studied have epigeal germination (Fig. 4 A-E). The morphology of the seedlings obtained in the different trials was similar: stem with short internodes, cotyledons with a central notch, epicotyls with simple and trifoliate leaves (protophylls or transitional leaves) and a curved root with little branching (Fig. 4 E-H).

The factorial analysis showed that "species" is the principal factor affected, significantly all the parameters evaluated ($p < 0.001$), whereas the "culture method" did not affect the epicotyl length and the germination percentage. Moreover, the "species x culture methods" interaction was significant ($p < 0.01$) for the hypocotyl length, cotyledon width and the germination percentage (Table 3).

When analyzing the significant differences ($p < 0.05$) in the epicotyl length it was seen that *H. impetiginosus* showed the highest values, followed by *H. ochraceus* and then *H. lapacho*, in agreement with findings in the parameters of the seed and embryo previously described (Tables 1, 2).

Even though the differences found in the hypocotyl length and cotyledon width are due to the effect of the two independent factors and their interaction (Tables 2, 3), it was seen that the hypocotyl length was greater in the seedlings in *ex vitro* culture; however this difference was not statistically significant in *H. lapacho* (Table 2; Fig. 4 E-H). The cotyledon width of the seedlings of *H. ochraceus* germinated *in vitro* showed significant differences ($p < 0.05$) in respect to the cotyledons of the other species (Table 2).

The dimensions of the seeds and embryos of the three species studied exhibited a relationship directly proportional to the size of the seedling. The larger seeds produce more vigorous seedlings, such as in *H. impetiginosus* (Tables 1, 2; Fig. 1 A-F; 4 A-F). Furthermore, this species is the one that showed the greatest germination energy and percentage compared with the other species studied.

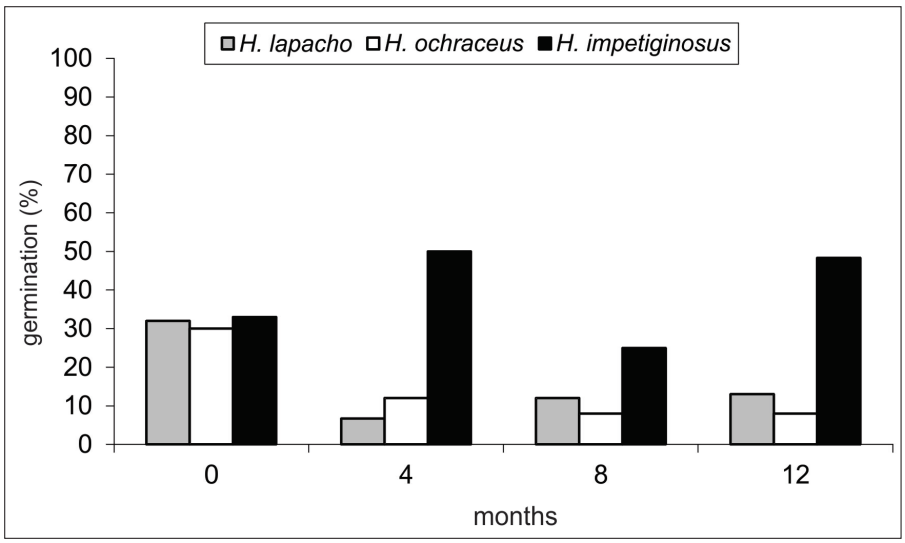

Fig. 2. Germination energy of *Handroanthus impetiginosus*, *H. lapacho* and *H. ochraceus*. Seeds cultivated in Petri dishes at the time of collection in the first season.

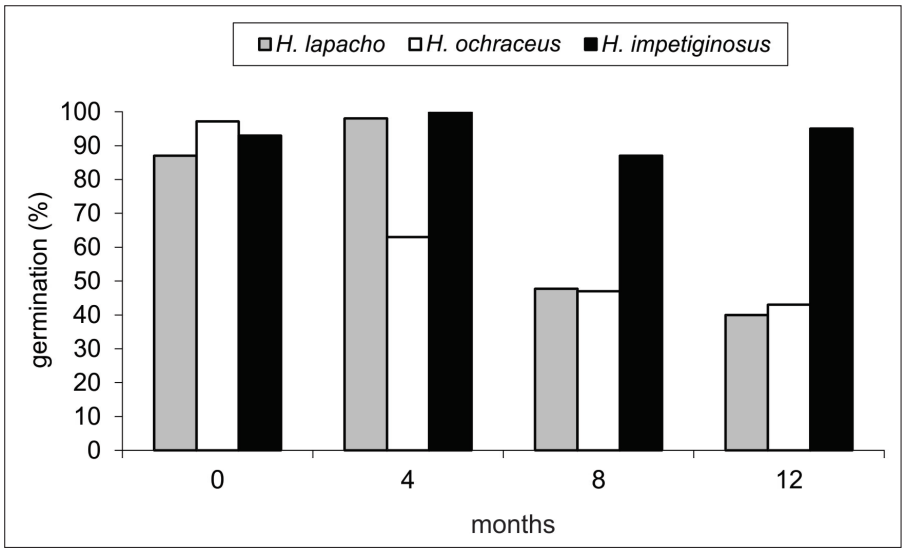

Fig. 3. Germination capacity of *Handroanthus impetiginosus, H. lapacho* and *H. ochraceus*. Seeds cultivated in Petri dishes at the time of collection in the first season.

Discussion

Several species of *Handroanthus* have germination problems in natural habitats. The process is extremely variable and differs between species and with the site and conditions of seed formation (climate, phytosanitary status and physiological aspects) (Justiniano et al., 2000; Bocchese et al., 2008; Carvahlo et al., 2008; Nery et al., 2008). Sakai (2010) reports that most recalcitrant species are associated with Dicotyledons primitive or ancestral characters, such as large exalbuminous seeds and a tropical woody habit.

Some authors show that in the mature seed of representatives of the Bignoniaceae it is common to see that the membranous cover,

Table 1. Morphometric characteristics of the seeds of *Handroanthus impetiginosus*. *H. lapacho* and *H. ochraceus* from northwest Argentina (province of Salta). Different letters indicate significant differences between species at P> 0.05 (Tukey).

Species	Seed			Wings	Seed body		Embryo			
	Length (mm)	Width (mm)	Thickness (mm)	Width (mm)	Length (mm)	Width (mm)	Length (mm)	Width (mm)	Thickness (mm)	Midsection (mm)
Handroanthus lapacho	5.5±0.5c	23.2±2.1c	0.6±0.1b	7.0±0.9b	4.8±0.4c	8.8 ±1.0a	4.4±0.2c	6.6±0.2c	0.5±0.1b	2.6±0.1c
Handroanthus ochraceus	7.6±0.9b	27.4±2.5b	0.6±0.1b	7.6±1.3b	7.0±1.0b	9.9±4.5a	6.1±0.3b	8.8±0.4b	0.4±0.1b	3.4±0.4b
Handroanthus impetiginosus	10.6±0.7a	37.6±2.9a	1.8+0.3a	13.0±1.6a	9.2±0.8a	11.8±1.0a	8.4±0.6a	10.3±0.8a	1.1±0.1a	4.5±0.5a

Table 2. Germination percentage and morphometric characteristics of the seedlings of *Handroanthus impetiginosus. H. lapacho* and *H. ochraceus* from *in vitro* and *ex vitro* cultivated seed. Seed of the second season grown after being preserved for a year at 5ºC. *In vitro* medium: WPM with 5 µm BA and activated carbon (5 $g.L^{-1}$). *Ex vitro substrate*: soil:peat:perlite (1:1:1). Different letters indicate significant differences between species and treatments (P≥ 0.05).

		Epicotyl Total Length (mm)	Hypocotyl Total Length (mm)	Cotyledon Width (mm)	Germination (%)
Handroanthus lapacho	*in vitro*	3.4±1.4 c	12.6±1.7 b	9.8±2.7 c	57.8 b
	ex vitro	2.7±1.2 c	14.5±1.7 b	13.9±0.2 ab	38.1 c
Handroanthus ochraceus	*in vitro*	9.1±1.9 b	13.8±1.5 b	17.8± 2.7 a	49.1 bc
	ex vitro	8.5±1.0 b	41.4±1.1 a	16.3±0.4 a	58.7 b
Handroanthus impetiginosus	*in vitro*	41.1±8.0 a	15.1± 1.4 b	12.1±3.3 bc	81.7 a
	ex vitro	42.5±6.5 a	31.7±1.05 a	14.5±0.6 ab	85.0 a

Table 3. *Ex vitro* and *in vitro* germination. Analysis of variance for the effect of the factors (species and culture method) and their interaction with the morpho-physiological parameters of seedlings of *Handroanthus impetiginosus*. *H. lapacho* and *H. ochraceus* (P< 0.01).

		Epicotyl Total Length (mm)		Hypocotyl Total Length (mm)		Cotyledon Width (mm)		Germination %	
	GI	F	Sig.	F	Sig.	F	Sig.	F	Sig.
Species (S)*	2	176.10	0.000	6.87	0.001	23.83	0.000	99.67	0.000
Methods (M)**	1	0.43	0.535	17.76	0.000	7.04	0.009	0.02	0.892
S x M	2	1.28	0.280	17.14	0.000	6.80	0.000	12.27	0.00

Referencia: GI: Grados de libertad; F: Estadístico F de Fisher; Sig.: Significancia para P< 0.05.
* *Handroanthus impetiginosus. H. ochraceus* or *H. lapacho*
** *ex vitro* or *in vitro*

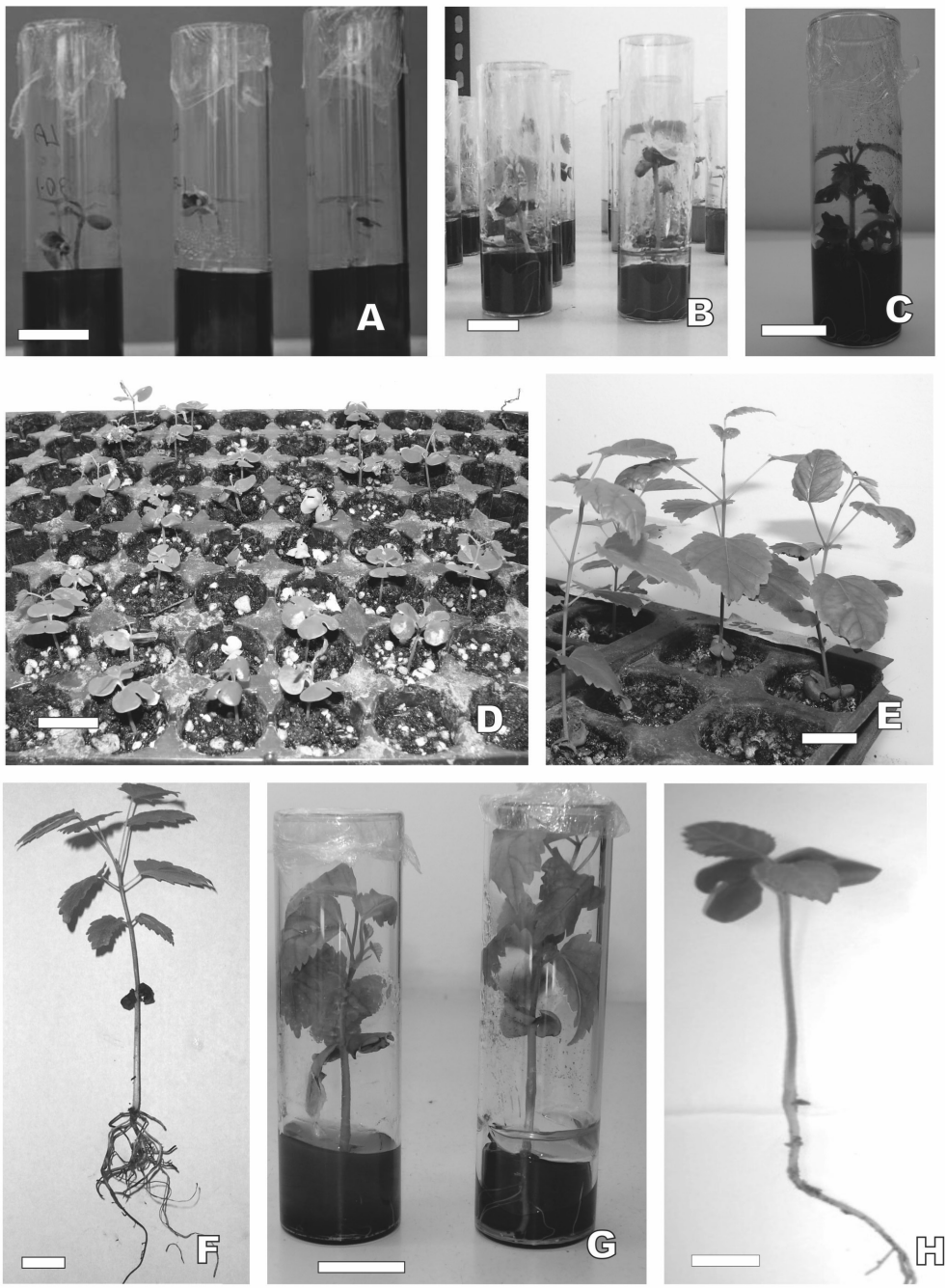

Fig. 4. Seedlings from *ex vitro* and *in vitro* germination. A-C: Iniciation of *in vitro* germination: A: *Handroanthus ochraceus.* B: *H. impetiginosus.* C: *H. lapacho.* D-E: *Ex vitro* germination of *H. impetiginosus*: D: Emergence of cotyledons. E: Developed seedling. F: *Ex vitro* seedling of *H. ochraceus.* G: *In vitro* seedling of *H. impetiginosus* after 30 days culture. H: Detail of the hypocotyl in seedling of *H. ochraceus* in *in vitro* culture. Bars: A-E, G: 2 cm; F, H: 1 cm.

composed of endothelium and the remains of the endosperm, is attached to the surface of the embryo in a unique cordiform structure (Souza et al., 2005; Sampaio et al., 2007; Souza et al., 2008). Studies on seed ontogeny in *Tabebuia rosea, T. impetiginosa* (= *H. impetiginosus*), *T. chrysotricha* (= *H. chrysotrichus*), *T. serratifolia* (= *H. serratifolius*), *T. ochracea*

(= *H. ochraceus*) and *T. pulcherima* (= *H. pulcherrimus*) have indicated that the seeds are considered exalbuminous because the cotyledons absorb most of the endosperm through the endothelium before maturity. This explains why embryos in seed of *Tabebuia* and *Handroanthus* increase in size considerably (Silva et al., 2005; Sampaio et al., 2007; Sakai, 2010).

Moreover, von Teichman & Wyk (1994) indicate that the large embryo in exalbuminous seed is an advantage for germination. The larger dimensions of the seed and embryo in *H. impetiginosus* coincide with high germination percentages over the 12 months of trials. According to Quinto et al. (2009), seed dormancy in the seed of *Handroanthus* species, mainly in cultivated *H. impetiginosus*, allows a high germination capacity over time.

The presence of germination inhibitors might be the cause of the gradual decrease in germination capacity over a long time. *T. serratifolia* showed a lower germination percentage during the time when the seed had the highest content of polyphenols, since the oxidation products of these compounds affect germination as they reduce oxygen availability in the seed (Carvahlo et al., 2008).

Moreover, *H. ochraceus* has polyembrionic seed, as does *H. chrysotrichus,* due to the formation of one to four adventitious embryos by apomixis (Costa et al., 2004; Souza et al., 2005; Sampaio et al., 2007). Apomitic species have a great ability to colonize habitats, leading to wide distribution compared with non-apomitic species (Goldenberg & Shepherd, 1998). The dimensions observed in seed of *H. ochraceus,* together with the presence of polyembryony, ensure the continuation of the species in natural and exotic habitats.

According to Carvahlo et al. (2008) the seeds of *T. serratifolia* are mature enough to germinate 53 days after anthesis which coincides with the dehiscence of the mature fruit for dispersal. Therefore, the seeds used in this study were mature when collected, as the fruits showed clear evidence of the opening of the pericarp, enabling the analysis of germination energy and capacity.

One of the most serious problems in germination studies is fungal contamination of the seed. Different authors have proposed desinfection processes of seed of *T. roseo-alba, T. impetiginosa* and *T. serratifolia* by using (NaOCl) in concentrations of 2 to 10% active chlorine. However, they indicate that high concentrations of this disinfectant reduce the germination percentage (Carvahlo et al., 2008; Nery et al., 2008). The use of NaOCl in the germination trials of the three species of *Handroanthus* ensured adequate disinfection of the seed and significant germination percentages.

Metabolism is slower under low temperature so conservation under those conditions results in a better germination capacity and lengthens the life of the seed (Doria, 2010). Seed of *Handroanthus*, mainly *H. impetiginosus,* conserved at 5ºC for one year showed germination percentages up to 82-100% in both seasons of collection.

No differences were seen between the *in vitro* and *ex vitro* germination treatments, except in *H. lapacho*. Nery et al. (2008) carried out studies comparing the *in vitro* and *ex vitro* germination of *T. serratifolia* and they observed that the *in vitro* cultures gave higher germination percentages. On the other hand, Silva et al. (2005) determined that the optimum temperature for the germination of *T. impetiginosa* is 25ºC to 30ºC, coincident with the range used in the *in vitro* and *ex vitro* germination trials of the three *Handroanthus* species studied.

The use of WPM for *in vitro* germination of *H. impetiginosus, H. ochraceus* and *H. lapacho* was appropriate for seedling development. The solution of macronutrients alternative known as WPM (Lloyd & McCown, 1980) has been formulated for woody species and has a 25% concentration of nitrate and ammonium ions and a higher concentration of potassium and sulphate than the Murashige-Skoog (MS) formulation that is often used. Nery et al. (2008) showed that *T. serratifolia* had a higher germination percentage when the seeds were cultivated in media with the WPM formulation. However, Carvahlo et al. (2008) did not find any significant differences between WPM and MS when cultivating embryos of the same species.

The use of a substrate rich in organic matter

for *ex vitro* trials ensured good germination percentages, especially in *H. impetiginosus*. In the *ex vitro* germination of *T. heptaphylla* (= *H. hepatphyllus*), the clay substrate with additional organic matter gave a greater quantity of germinated seed (42%), the radicles starting to appear between 6 to 10 days after the start of the culture (Borchesse et al., 2008). Nevertheless, Fowler et al. (1998) found a higher germination percentage of *T. cassinoides* in sandy substrates compared with clay soils.

The species studied have epigeal germination and the morphological characteristics of the seedling are similar to those observed by other authors in representatives of the genus. Ferreira & Cunha (2000) reported that the seedling of *T. caraiba* have sinuous roots with little ramification, a glabrous cylindrical hypocotyl, persistent opposite cotyledons with a central notch, epicotyls with short pedicels simple, rarely trifoliate. Lozano & Zapater (2008) showed that the seedlings of *H. impetiginosus* had six unifoliate protophylls and two trifoliate transitional leaves with serrated borders at 60 days after *ex situ* germination.

The length of the hypocotyl was greater in *ex vitro* cultivated plants, although this difference was only statistically significant in *H. impetiginosus* and *H. ochraceus*. The elongation of the hypocotyls may be associated with the process of photomorphogenesis (Doria, 2010; Zhang et al., 2010). However, the light intensity in both culture methodologies analyzed (*in vitro* and *ex vitro*) was the same. It is possible that the differences observed might be related to the composition of the culture substrate. The *in vitro* substrate contains 2% sucrose and there is evidence that an increase in the levels of this substance suppresses hypocotyl elongation (Zhang et al., 2010).

On account of the seed characteristics and rapid loss of germination capacity together with a restricted distribution, *H. lapacho* is a species with considerable conservation problems in its natural habitat. *In vitro* germination is a useful tool that could solve this. On the other hand, *H. ochraceus* has seed of a size intermediate between the other species studied and its germination capacity gradually decreased. However, it is a seed that shows polyembryony, ensuring efficiency in the preservation of native specimens. Finally, the results for *H. impetiginosus* demonstrated its capacity to perpetuate itself whether cultivated or as a native tree.

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