CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Institute of Tropics and Subtropics

Department of Crop Sciences and Agroforestry in Tropics and Subtropics

In vitro propagation of selected Amazonian tree species

M.Sc. thesis

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

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DIPLOMA THESIS ASSIGNMENT

Reyes Valera Dina Gianina

Tropical Crop Management and Ecology

Thesis title

In-vitro propagation of selected Amazonian tree species

Objectives of thesis

The objective of the thesis is to find out suitable methods for in-vitro propagation of two Amazonian tree species Dipteryx micrantha, and Swietenia macrophylla King. The main aim is to verify the influences of various concentrations of cytokinins and auxins on shoot and root induction.

Methodology

Data will be collected during July - September 2010 in provinces of Coronel Portillo, in Ucayali region. Seeds were collected from selected trees. Then the teguments will be removed manually and the seeds will have been surface sterilized before being cultivated in-vitro. After sterilization, seeds will be sown on solidified half strength MS medium (Murashige&Skoog, 1962) containing 30 g sucrose, 8 g agar and 100 mg l-1 myo-inositol (pH 5.7) per one liter, into Erlenmayer`s flasks (100 ml), under sterile condition of flow box, accomplished with sterile instruments.

The grow regulators effect will be tested on 1 MS media enriched by cytokinins. About nutrient concentration, will be investigate influence of following concentrations of nutrients in MS,1 MS, and 1/4 MS media in the experiment. These have been supplemented by 3%, of sucrose as the source of carbon and energy for the explants. Value of pH have been regulated at 5.7 by KOH 1M. Medium used for all experiments was supplemented by 8 g l-1 of agar.

Rooted plants will be transplanted ex vitro. Survival percentages will be determined 4 weeks after transplantation. The success of acclimatization will be determined as the survival rate (%). Significance will be determined by analysis of variance (ANOVA) and the leastsignificant ($p < 0.05$) differences among mean values.

Schedule for processing

Oct 2010 – Jan 2011 – literature review

Feb – May 2011 – methodology preparation

Jun – Sep 2011 – data collection in Peruvian Amazon

Oct 2010 – Jan 2012 – data evaluation

Feb – Mar 2012 – completing the thesis

The proposed extent of the thesis

40 - 80 pages

Keywords

Dipteryx micrantha Harms, micropropagation, tropical species, cytokinis, Swietenia macrophylla King.

Recommended information sources

Gamborg, O.L and Phillips, G.C. 1995. Plant Cell, Tissue and Organ Culture-Fundamental Methods. Berlin-Germany. pp 358

Pena et al. 2010. Multiple adventitious shoot formation in Spanish Red Cedar (Cedrela odorata L.) cultured in vitro juvenile and mature tissues: an improved micropropagation protocol for a highly valuable tropical tree species. In vitro Cell. Dev. Biol.-Plant 46:149-160.

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Declaration

I hereby declare that the M.Sc. Thesis "*In vitro* propagation of selected Amazonian tree species" has been written by myself indepently, only with the expert guidance of my thesis supervisor doc. Ing. Bohdan Lojka, Ph.D., co-supervisor Ing. Iva Viehmanová, Ph. D. and Ing. Jan Vítamvás Ph. D. without any external unauthorized help.

I further declare that all data, figures, tables and information I have used in this thesis come from initiate sources stated in the references.

Dina Reyes

Prague, April 10, 2012 ……..

Signature

Acknowledgement

I would like to sincerely thank to my thesis supervisor doc. Ing. Bohdan Lojka, Ph.D. Head of Department of Crop Sciences and Agroforestry in Tropics and Subtropics, Institute of Tropics and Subtropics at Czech University of Life Sciences Prague, for his professional help, comments, suggestions and valuable information during the guidance of my thesis, also for accept to continue the master studies until I finish.

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Abstract

This study was focused on optimization of *in vitro* propagation of two Amazonian tree species *Schizolobium parahyba* (Vellozo) Blake var. *amazonicum* (Huber ex Ducke) Barneby and *Swietenia macrophylla* G. King. Nine MS-based media and five WPM-based media, containing different concentrations of BAP (0.1, 0.5, 1.5 and 3.0 mg l^{-1}) alone or in combination with IBA (0.1 mg l^{-1}) were tested for multiplication of axillary and apical segments in *Schizolobium parahyba*. Also, five MS-based media and five WPM-based media supplemented with different concentrations of BAP (0.1, 0.5, 1.5 and 3.0 mg l^{-1}) were used for multiplication of *Swietenia macrophylla*. The plant height, callus formation, number of roots and number of shoots per explant were measured in both species. After one-month cultivation of plants on these media, one half of all plants was transferred to rooting medium containing for NAA (0.1 mg l^{-1}) and the other half to MS without any plant growth regulators, and we assessed number of roots and callus growth. The best results in *Schizolobium parahyba* were achieved on MS medium supplemented with 0.5 mg l^{-1} BAP or WPM medium with addition of 0.1 mg l^{-1} BAP. Lower concentrations of BAP provided satisfactory results for both axillary and apical meristems. On the contrary, in *Swietenia macrophylla*, MS medium without any plant growth regulators provided the highest increase of plant height and can be recommended for multiplication of the species. On WPM medium plants were smaller compared to MS medium. The formation of roots has not been achieved for both species and must be optimized in further research. It can be concluded that *in vitro* cultures of Amazonian tree species can be suitable for the propagation of both species.

Keywords: auxins, cytokinins, *in vitro* cultures, micropropagation, organogenesis, *Schizolobium parahyba*, *Swietenia macrophylla*, tropical woody species.

Abstrakt

Cílem diplomové práce byla optimalizace *in vitro* mikropropagace dvou amazonských dřevin *Schizolobium parahyba* (Vellozo) Blake var. *amazonicum* (Huber ex Ducke) Barneby and *Swietenia macrophylla* G. King. Množitelský potenciál byl u druhu *Schizolobium parahyba* testován na devíti variantách MS media a pěti variantách WPM media s různými obsahy BAP (0.1, 0.5, 1.5 and 3.0 mg l^{-1}) buď samotnými, nebo v kombinaci s IBA (0.1 mg l^{-1}). Rovněž u druhu *Swietenia macrophylla* bylo testováno pět variant MS media a pět variant WPM media s různými obsahy BAP (0.1, 0.5, 1.5 and 3.0 mg l^{-1}). Výška rostlin, tvorba kalusu, počet kořenů a počet výhonů byl měřen u obou druhů. Po jednom měsíci kultivace byla jedna polovina rostlin přenesena na zakořeňovací medium s obsahem NAA (0.1 mg l^{-1}) a druhá na MS medium bez přídavku růstových regulátorů. Nejlepších výsledků při množení *Schizolobium parahyba* bylo dosaženo na MS mediu s 0.5 mg l^{-1} BAP nebo WPM mediu s 0.1 mg l^{-1} BAP. Nižší koncentrace BAP poskytly uspokojivé výsledky jak pro axilární, tak apikální meristémy. U druhu *Swietenia macrophylla* bylo největších přírůstků dosaženo na MS mediu bez růstových regulátorů a toto medium může být doporučeno k mikropropagaci druhu. Na WPM mediu rostliny dosahovaly menší výšky v porovnání s MS mediem. Tvorba kořenů nebyla zaznamenána ani u jednoho druhu a tento krok bude třeba optimalizovat v dalším studiu. Na základě získaných výsledků lze doporučit *in vitro* technologie pro efektivní množení obou studovaných druhů.

Klíčová slova: auxiny, cytokininy, *in vitro* kultury, mikropropagace, organogeneze, *Schizolobium parahyba*, *Swietenia macrophylla*, tropické dřeviny.

Resumen

El presente estudio esta enfocado a la optimización de propagación *in vitro* de dos especies forestales amazónicas *Schizolobium parahyba* (Vellozo) Blake var. *amazonicum* (Huber ex Ducke) Barneby and *Swietenia macrophylla* G. King. Nueve MS-basal media y cinco WPM-basal media, conteniendo diferentes concentraciones de BAP (0.1, 0.5, 1.5 and 3.0 mg l^{-1}) solo o en combinación con IBA (0.1 mg l^{-1}) fueron probados para la multiplicación de segmentos apicales y axilares en *Schizolobium parahyba*. También, cinco MS-basal media y cinco WPM-basal media suplementado con diferentes concentraciones de BAP (0.1, 0.5, 1.5 and 3.0 mg l^{-1}) fueron usados para la multiplicación de *Swietenia macrophylla*. La altura de la planta, formación de callus, numero de raíces y numero de brotes por explante fueron evaluados en ambas especies. Después de un mes de cultivar las plantas en esos medios, la mitad de todas las plantas fueron transferidas al medio de enraizamiento conteniendo NAA (0.1 mg l^{-1}) y la otra mitad al MS sin ningún regulador de crecimiento de plantas. Los mejores resultados en *Schizolobium parahyba* fueron logrados en MS medio suplementado con 0.5 mg l^{-1} BAP o WPM medio suplementado con 0.1 mg l^{-1} BAP. Bajas concentraciones de BAP proporciona mejores resultados para ambos meristemas, apical o axilar. Por el contrario, en *Swietenia macrophylla*, MS media sin ningún regulador de crecimiento de plantas proporciona mayor aumento en el crecimiento de las plantas y puede ser recomendado para la multiplicación de esta especie. En WPM medio, las plantas fueron pequeñas en comparación con MS medio. La formación de raíces no ha sido lograda para ambas especies y debe ser optimizado en una posterior investigación. Se puede concluir que la cultura *in vitro* de especies forestales amazónicas puede ser adecuado para la propagación de ambas especies.

Palabras clave: auxinas, citoquininas, cultura *in vitro*, micropropagación, organogénesis, *Schizolobium parahyba*, *Swietenia macrophylla*, especies forestales tropicales.

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List of Abbrevation

ANOVA-Analysis of Variance

BAP- 6-benzylaminopurine

CULS-Czech University of Life Sciences Prague

EtOH-ethanol

GA_3-gibberellic acid

IBA-indole-3-butyric acid

KIN-kinetin

MS-Murashige and Skoog (1962) medium

NAA-naphthalene acetic acid

NaClO-sodium hypoclorite

PGRs-Plant growth regulators

SD-standard deviation

TDZ-thidiazuron

WPM-woody plant medium (Lloyd and McCown, 1981)

2- iP-isopentenyl adenine

2,4-D-dichlorophenoxyacetic acid

1. INTRODUCTION

With around 70 million hectares of tropical forest covering nearly 60% of its territory, Peru has the fourth largest area of tropical forest in the world (see App.1); after Brazil, Democratic Republic of Congo and Indonesia (FAO and ITTO, 2011). Furthermore, the official deforestation rate in Peru is about 150,000 ha/year (MINAM, 2009). Currently, 80% of the deforested area (5.5 million hectares) is in a state of neglect and 20% in production: low-productivity agricultural activities, intensive farming, coca cultivation and logging residue (Meza *et al*., 2006).

Logging, deforestation and primitive farming have completely eliminated the remarkable forest potential of species as valuable as mahogany (*Swietenia*). Extraction for export grew exponentially over the last decade (1.900 ^{m3} more than 39.000 ^{m3}), but now was found that 40% of the population of this species are below the minimum diameter for extraction, it means that it was being consumed more than the reserve allows, that situation was aggravated by the failure to find sufficient natural regeneration to replace the trees that are taking advantage (Dourojeanni *et al.*, 2009).

Commercially *Swietenia macrophylla* is the most important mahogany; regeneration of the species is stochastic, depending in nature on large-scale disturbance, and also, this specie is vulnerable according to IUCN red list (World Conservation Monitoring Centre, 1998).

On the other hand *Schizolobium parahyba* has attracted interest among farmers and commercial loggers, the main reasons are the commercial value of the timber for rolled products of excellent quality and the rapid growth of the specie, especially in the early years (Reis *et al.*, 2009). However, for a reforestation program to be successful economically and environmentally, it is necessary to produce good quality seedlings that show uniform growth and a lower percentage of mortality in the field and therefore less need for replanting (Rosa *et al.*, 2009).

Plant tissue culture is one of the most important conservation techniques because it offers great potential for rapid cloning from a minimum of plant material; in addition, it plays a key role in the reproduction of plant material required for different purposes such as breeding, genetic and biotechnological research, and the acquisition of industrial raw material (Hasançebi *et al.*, 2011).

The aim of the thesis is to find out suitable methods for in -vitro propagation of two Amazonian tree species *Schizolobium parahyba* (Vellozo) Blake var. *amazonicum* (Huber ex Ducke) Barneby and *Swietenia macrophylla* G. King.

2. LITERATURE REVIEW

2.1. Advantages of vegetative propagation

The concept of vegetative propagation is that an exact copy of the genome of a mother plant is made and continued in new individuals. Whereas sexual reproduction by seeds provides opportunity for variation and evolutionary advancement, vegetative propagation aims at the identical reproduction of plants with desirable features such as high productivity, superior quality, or high tolerance to biotic and/or abiotic stresses, and as such, plays a very important role in continuing a preferred trait from one generation to the next. The most important vegetative propagation techniques (Figure 1) for tree species are the propagation by stem or root cuttings, grafting and budding, and various methods and techniques of layering and micropropagation (Jaenicke and Beniest, 2002).

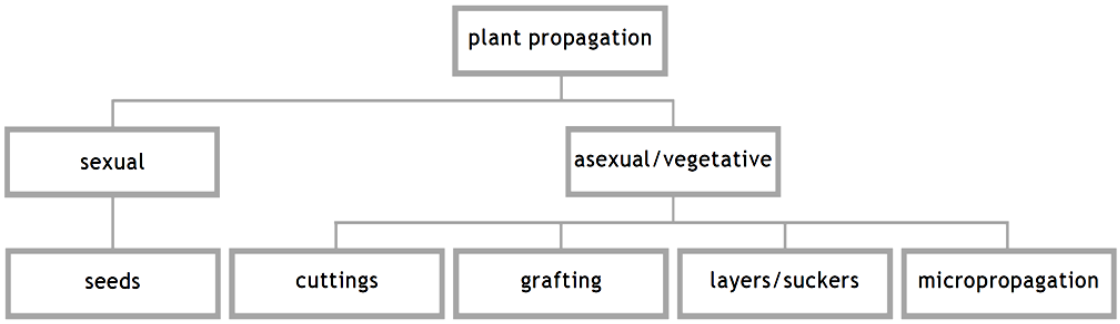

Figure 1. Types of plant propagation

2.1.1. Micropropagation of woody species

Micropropagation is a method of propagating plants by culturing very small parts of it called explants. This technique provides a rapid and reliable system for reproduction of a large number of genetically uniform disease-free plantlets. Micropropagation is one of the important contributions of plant tissue culture to commercial plant propagation and has vast significance, also there are many methods of propagation (Figure 2) like meristem culture, shoot tip culture, single node culture, seed culture, adventive (direct) organogenesis, indirect organogenesis, somatic embryogenesis and shoots from floral meristems (Baran and Ghosh, 2005).

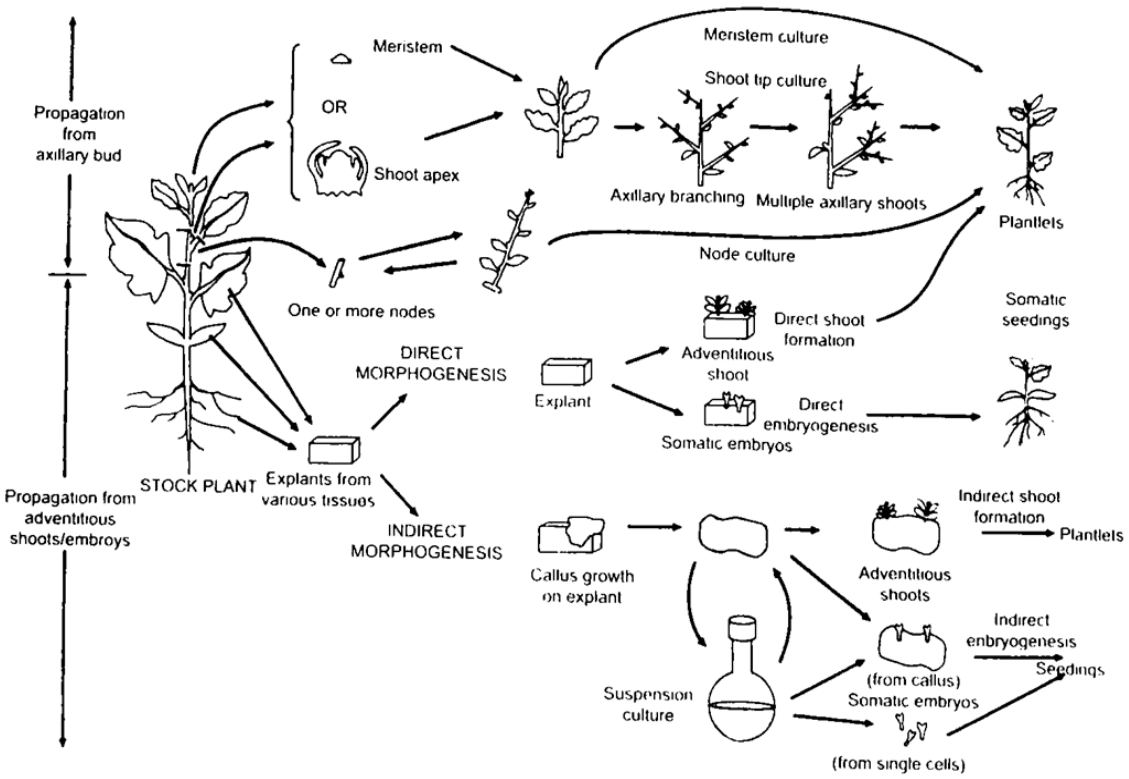

Figure 2. Principal methods of micropropagation (Baran and Ghosh, 2005)

In a number of woody species process of micropropagation had been optimized. Schottz *et al*. (2007) developed a multiplication protocol for *Swietenia macrophylla* using juvenile material. In this study, different media containing cytokinins 6-benzylaminopurine (BAP) and isopentenyl adenine (2-iP) were tested. The highest multiplication rate (5.7) was obtained when the MS culture medium was supplemented with 4 mgl^{-1} BAP and 0.5 mgl^{-1} 2-iP. Tacoronte *et al*. (2004) developed a study of *in vitro* propagation *Swietenia macrophylla* from nodal segments. Explants were cultivated on a half- strength MS medium supplemented with different combinations of naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), within a range of 0-3 mgl^{-1} for both hormones. The highest axillary bud elongation was achieved on medium containing 1.94 mgl^{-1} BAP and 0.38 mgl^{-1} NAA.

In woody species *Khaya senegalensis*, also belonging to Meliaceae family, process of micropropagation had been optimized (Hung and Trueman, 2011). For *in vitro* shoot proliferation and plantlet formation, plant growth regulators 6-benzylaminopurine (BAP), kinetin (KIN), naphthalene acetic acid (NAA) and gibberellic acid (GA_3) were tested. Shoot proliferation was more effective in media containing 6-benzylaminopurine than in media containing other growth regulators, and

optimal proliferation from seed of three different sources was consistently obtained in medium containing 1mgl^{-1} 6-benzylaminopurine (BAP). Shoots from this medium were converted to plantlets at high frequencies (76–90%) after treatment with 4 mgl^{-1} indole-3-butyric acid (IBA), and almost all plantlets were successfully acclimatized to nursery conditions.

Rabelo de Souza *et al.* (2009) evaluated the influence of full-strength MS culture medium and at half-strength of its salts (½ MS), the presence of gibberellic acid (GA_3) and citric acid on the *in vitro* culture of *Schizolobium parahyba* var. *amazonicum* embryonic axes. The embryonic axes were extracted from seeds and inoculated on basic MS and ½ MS culture media, supplemented or not with citric acid (1 g/l^{-1}) and GA_3 (3 mgl^{-1}). Successful development of *S. parahyba* seedling was achieved on MS medium at half strength of its salts, the addition of citric acid not being necessary, and the presence of 3 mgl^{-1} GA_3.

In *S. parahyba* var *amazonicum*, pre-germination of seeds is also important for *in vitro* germination. Rabelo de Souza *et al*. (2009) proposed a protocol for mechanic scarification of seeds with sandpaper. Thereafter, the seeds were placed in 100 ml beaker with water and subjected to several washes in water and detergent. In laminar flow, the seeds were sterilized in 70% commercial alcohol for three minutes and immersed in a solution of sodium hypochlorite (NaOCl) to 3%, for twenty minutes. Subsequently they were washed four times in distilled sterile water and immersed in distilled water with temperature around 70 ° C, where they remained for 24 hours.

2.2. *Swietenia macrophylla* G. King.

2.2.1. Botanic description

Swietenia macrophylla is a large deciduous tree with an umbrella-shaped crown, frequently reaching heights of over 30 m and diameter at breast height (DBH) of more than 1.5 m (Figure 3). The trunk is straight and cylindrical, slightly grooved, with well-developed spurs. The crown of young trees is narrow, but old trees have a broad, dense and highly branched crown. The outer bark of older trees (Figure 4) is scaly, shaggy, deeply longitudinally furrowed and brownish-grey to reddish-brown, and the inner bark is red-brown or pinkish-red. The leaves are usually paripinnate, sometimes imparipinnate (Figure 5), 12–45 cm long, and are made up of 3–6 pairs of lanceolate or ovate leaflets (Krisnawati *et al.*, 2011; Orwa *et al.*, 2009; Reynel *et al.*, 2003).

Flowers are unisexual and the tree monoecious (Schmidt and Jøker, 2000; Krisnawati *et al.*, 2011), the flowers are pollinated by insects. It is believed that the family Meliaceae is pollinated primarily by bees and moths (Bauer and Francis, 1998); hybridisation is frequent, especially with *S. mahagoni* where the species grow together; usually only one flower of the inflorescence develops into a fruit, the others being aborted. Development from flower to mature fruit takes 9-12 months. Flowering and fruiting are regular annual from 10 to 15 years of age but fruit set can be low due to lack of pollinators (Schmidt and Jøker, 2000).

S. macrophylla is a species with male flowers that open after female ones, to promote cross-pollination, the mating system of the species is predominantly exogamous (Pennington 2002, Loveless and Gullison 2003 cited by Marmillod, 2007).
The fruits (Figure 5) are capsular, oblong or ovoid, 11.6–38.7 cm in length, 6.7–12.0 cm in diameter and light grey to brown with 4–5 valves. Each fruit contains 22–71 developed seeds (Figure 5) (Krisnawati *et al.*, 2011).

Seeds are hanging from the columella by their wing, leaving conspicuous scars after their release. Usually in each fruit 35-45 seeds can be found (Schmidt and Jøker, 2000). '*Swietenia*' commemorates Gerard von Swieten (1700-1772), botanist and physician to Maria Theresa of Austria. The specific name, '*macrophylla*', means large leaved and comes from Greek words 'makros' (large) and 'phyllon' (leaf), (Orwa *et al.*, 2009).

Figure 3. (Left) Five-year-old *S. macrophylla* trees of characteristic shape, planted by a smallholder in South Kalimantan Figure 4. (right) Bark of 15-year-old *S. macrophylla* tree (Krisnawati *et al.*, 2011).

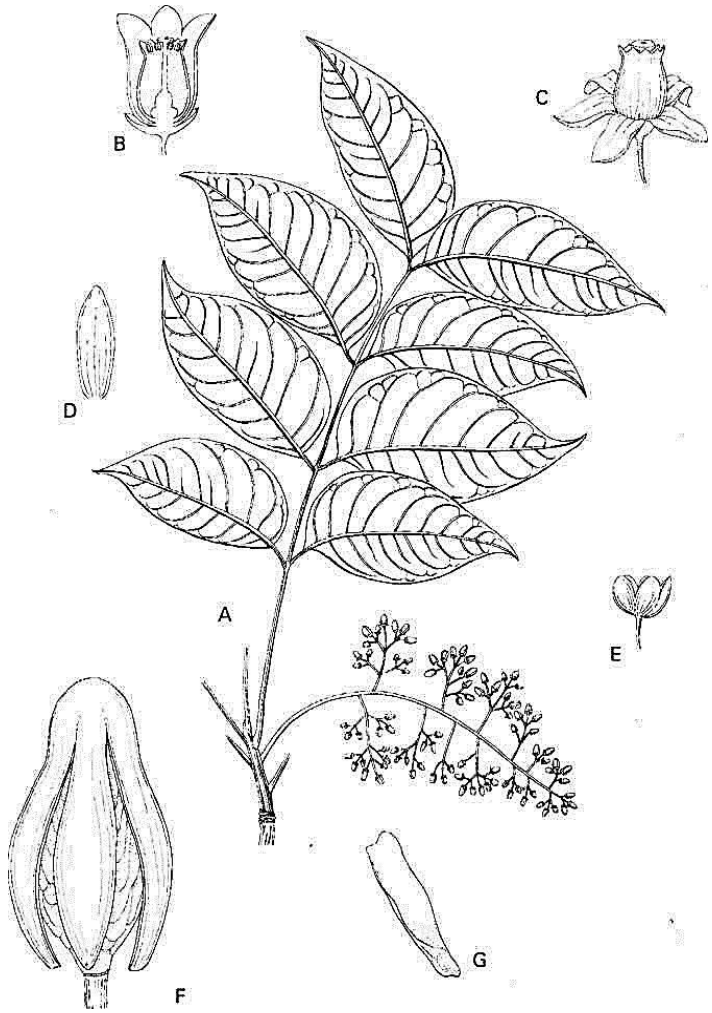

Figure 5. *Swietenia macrophylla* ("Caoba") A. Twig with leaves and inflorescence B. Flower, longitudinal section C. Flower, side view D. Petal E. Calyx F. Fruit G. Seed (Reynel *et al.*, 2003).

2.2.2. Origin and distribution

Swietenia macrophylla grows naturally in Belize, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Perú and Venezuela (Figure 6). However, it is nearly extinct in Ecuador, Colombia, Panama and Costa Rica; close to commercial extinction in Bolivia; declining in Mexico, Belize and Brazil; and in severe decline in Guatemala, Peru, Nicaragua and Honduras; the species has been extensively planted mainly in Southern Asia and the Pacific including India, Indonesia, Philippines and Sri Lanka, it has also been introduced into West Africa (Krisnawati *et al.*, 2011).

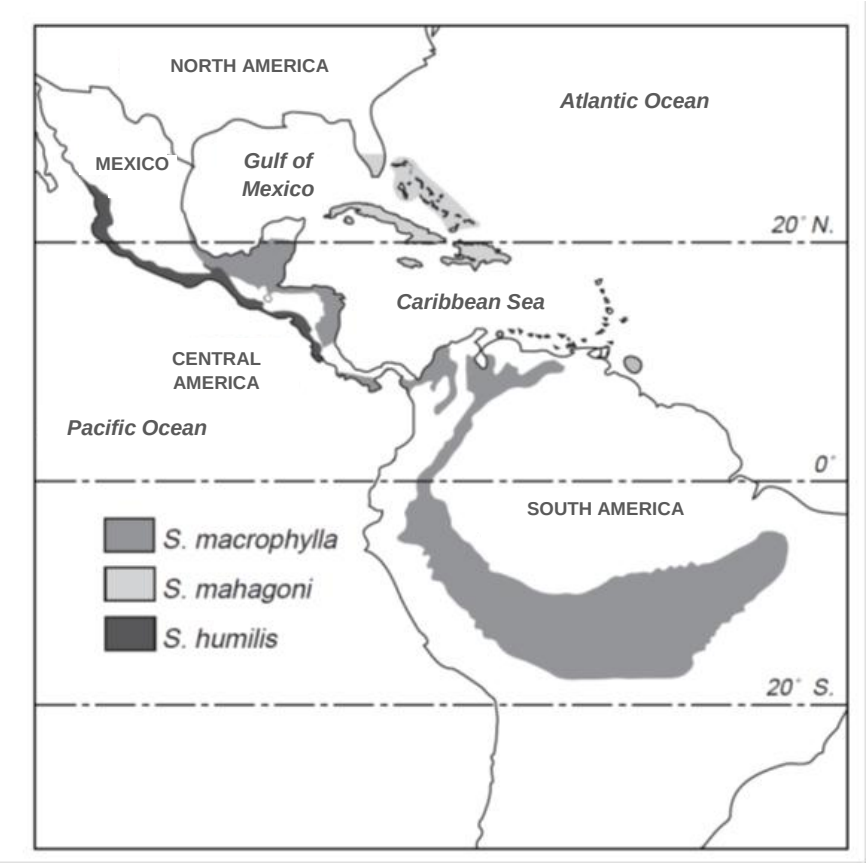

Figure 6. Natural distributions of Honduras mahogany, *Swietenia macrophylla*, and two other species of *Swietenia* in the Neotropics (Bauer and Francis, 1998).

2.2.3. Silviculture management

The *Swietenia macrophylla* King, according to Lamb, 1966 and Barros *et al*, 1992 cited by Contente *et al.*, 2011, given the large area of occurrence of mahogany, and only in the Brazilian Amazon (Figure 6) covers about 1.5 million km², it occurs in various soil conditions, from deep soil, poorly drained, acidic clay soils to well-drained alkaline soils that come from limestone plateau. However, the species has been shown to tolerate a very wide range of soil types (Mayhew and Newton, 1998. cited by Brown *et al.*, 2003).

Mahogany does not grow well in shaded forests and competition at the root system level; thus, the species behaves as heliophile growing in an open forest, in the seedling phase, the attack of yellow caterpillars (*Steniscadia poliophaea*) that destroy the leaflets is common (Figure 7), although the seeds, fruits and seedlings present different predation forms, the microlepidopter *Hypsipyla grandella* Zeller attacking the apical branches is considered the major pest of *Swietenia macrophylla* King. The *H.*

grandella Zeller, attacks the species in natural forest in the form of enrichment of these forests, but also in environments of open forests (Contente *et al.*, 2011).

Figure 7. Yellow caterpillar attacks in mahogany seedlings sprouts in natural forest (Contente *et al.*, 2011).

The planting of mahogany is severely limited by the attack of the shoot-borer *Hypsipyla grandella,* pruning is sometimes advocated as one means of controlling and/or mitigating shoot-borer attack (Cornelius, 2001). Older trees are not susceptible to attack (Krisnawati *et al.*, 2011).

The ecological amplitude of *S. macrophylla* is because the species behave as a pioneer or successional species, which can occur in disturbed areas (after catastrophic disturbance) or zones of ecological tension in greater quantity than in conditions where vegetation can reach balance or climax, presenting a great potential to regenerate artificially (Brown *et al.*, 2003; Contente *et al.*, 2011).

2.2.4. Natural reproduction

Records of flowering during the dry season, from August to October and fruiting mostly towards the end of the year. In other South American countries like Venezuela, the species is deciduous part of the year. This tree begins to bloom between 12-15 years (Reynel *et al.*, 2003).

2.2.5. Uses

Swietenia macrophylla is one of the most valuable furniture timbers in the world due to the decorative and attractive timber (Figure 8) with good technical characteristics; it is widely planted in the tropics in reforestation and afforestation programmes. In agroforestry systems it is used for shade and fuelwood (Schmidt and Jøker, 2000).

Figure 8. Items made of *S. macrophylla* timer: (a) door pane, (b) cupboard, (c) flooring and (d) urn (Krisnawati *et al.*, 2011).

In Indonesia, it is also used in agroforestry systems, for example in Java with maize, upland rice and cassava, and in our study village in South Kalimantan with cassava, corn, peanuts and pumpkin (Krisnawati *et al*, 2011).

2.3. *Schizolobium parahyba* (Vellozo) Blake var. *amazonicum* (Huber ex Ducke) Barneby var. *amazonicum*

2.3.1. Botanical description

Schizolobium parahybum var. *amazonicum* is an amazing fast growing tree, about 40m (130 feet) tall, growing more than 5m in 18 months, with a trunk 20cm wide; the leaves are remarkably long and fern like reaching lengths up to 2m (6 feet) long, they are supposedly the largest compound leaves in the world. The leaves are subdivided having 15 paired pinnate and each has 10-30 pairs of oblong leaflets (Figure 9). The tree puts on an abundant display of bright yellow flowers carried in large clusters, 30cm (12 inches) long, with a profusion of yellow, pea-shaped flowers. The fruit is a flat pod, spoon or teardrop shaped, firm and leathery. The large flat seeds are oval shaped and have a hard coat (Allen and Allen, 1981).

During the flowering of *S. parahyba*, the most common insects around flowers are bees from the family Apidae and butterflies from the family Pieridae, Nymphalidae and probably Heliconidae (Justiniano *et al.*, 2001).

2.3.2. Origin and distribution

The native range is located in the countries of Brazil, Colombia, Mexico; and exotic range is located in Costa Rica, Fiji, Indonesia, Kenya, Sri Lanka, US (Allen and Allen, 1981; Orwa *et al.*, 2009).

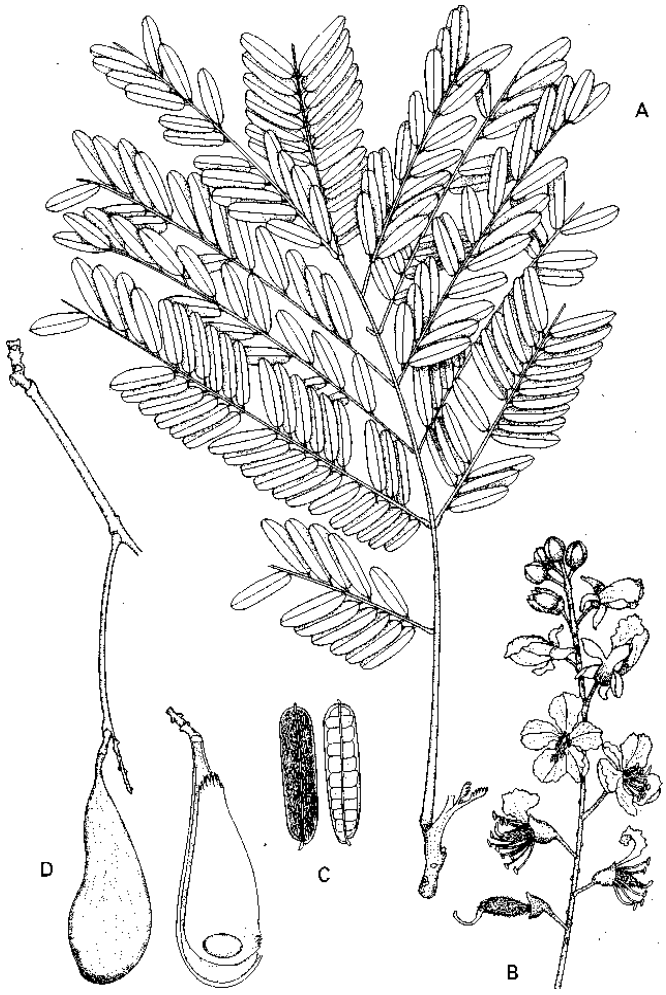

Figure 9. *Schizolobium parahyba* var. *amazonicum* ("Pino chuncho) A. Twig with leaf B. Inflorescence C. Leaf blades D. Fruit and seed (Reynel *et al.*, 2003).

2.3.3. Silviculture management

In Brazil, reports of serious attacks of various insects, the main is “longhorn beetle” (*Acanthoderes jaspidea*), which attacks fast growing trees for the first four years (Alvarez, 2008).

2.3.4. Natural reproduction

Records of flowering in the late dry season, from October to November, and fruiting in the early rainy season from November to December. The tree is defoliated before flowering (Reynel *et al.*, 2003).

2.3.5. Uses

The Brazilian Fern Tree (*Schizolobium parahyba*) is a promising agroforestry species, intercropping being possible because of its light shade. Also, the tree protects

surrounding soil from soil erosion and the enormous amount of biomass shed by the tree improves soil fertility (Allen and Allen, 1981).

The wood of this species is a potential source of paper pulp, plywood and fuel-wood. Also, the wood is used in manufacture of wood-plastic composites in combination with methyl methacrylate (a plastic monomer). The timber is rarely utilized because of its repulsive smell when fresh (Allen and Allen, 1981; Reynel *et al.*, 2003; Orwa *et al.*, 2009).

This spectacular tree is cultivated as an ornamental (Figure 10), especially beautiful in flower with masses of yellow gold blossoms. Ideal to add tropical drama to a sunny and sheltered position (Allen and Allen, 1981).

Figure 10. Flowers of *Schizolobium parahyba* (Vell.) S. F. Blake (Tropicos.org, 2012)

3. OBJECTIVES OF THE STUDY

The objective of the thesis was to find out suitable methods for *in vitro* propagation of two Amazonian tree species *Schizolobium parahyba* (Vellozo) Blake var. *amazonicum* (Huber ex Ducke) Barneby and *Swietenia macrophylla* G. King. The main aim was to verify the influences of two different growing media and different concentrations of cytokinins and auxins on shoot and root induction.

Specific objectives are following:

- Establishment of *in vitro* culture.
- Multiplication of plant material for establishment of experiment.
- Optimization of multiplication process in *Schizolobium parahyba* and *Swietenia macrophylla*.

The addition of growth regulators to the culture medium may facilitate the development of shoots in *Schizolobium parahyba* and *Swietenia macrohylla* via direct organogenesis.

4. MATERIALS AND METHODS

4.1. Plant material

The initial plant material used for this experiment was 84 seeds of *Swietenia macrophylla* and 92 seeds of *Schizolobium parahyba*; which were obtained near the city Pucallpa, which is situated in Peruvian Amazon basin in Ucayali Region, Peru (Figure 11). The seeds were collected from randomly selected trees, healthy and free from visual disease symptoms.

The city of Pucallpa is located at 8° 23' south latitude, 74° 50' longitude west and rises 154 meters above sea level (Pashanasi, 2001). According to Koppen's classification, the central Ucayali has a humid and dry; Ucayali ecosystem belongs to tropical rainforest, (Pidwirny and Saundry, 2011); with an average temperature of 27.6°C, with a maximum of 33.6°C and minimum 21.6°C, the average annual rainfall of 1675.5 mm and a relative humidity of 86.3% (Díaz *et al.*, 2007).

Also, another Amazonian species like *Amburana cearensis* (Allemão) A.C. Smith, *Cedrela odorata* L. *Dipteryx micrantha* Harms, *Guazuma crinita* Martius, *Myroxylon balsamum* (L.) Harms were tested for responsibility *in vitro*, but only *Swietenia macrophylla* and *Schizolobium parahyba* provided good results and were used for further research.

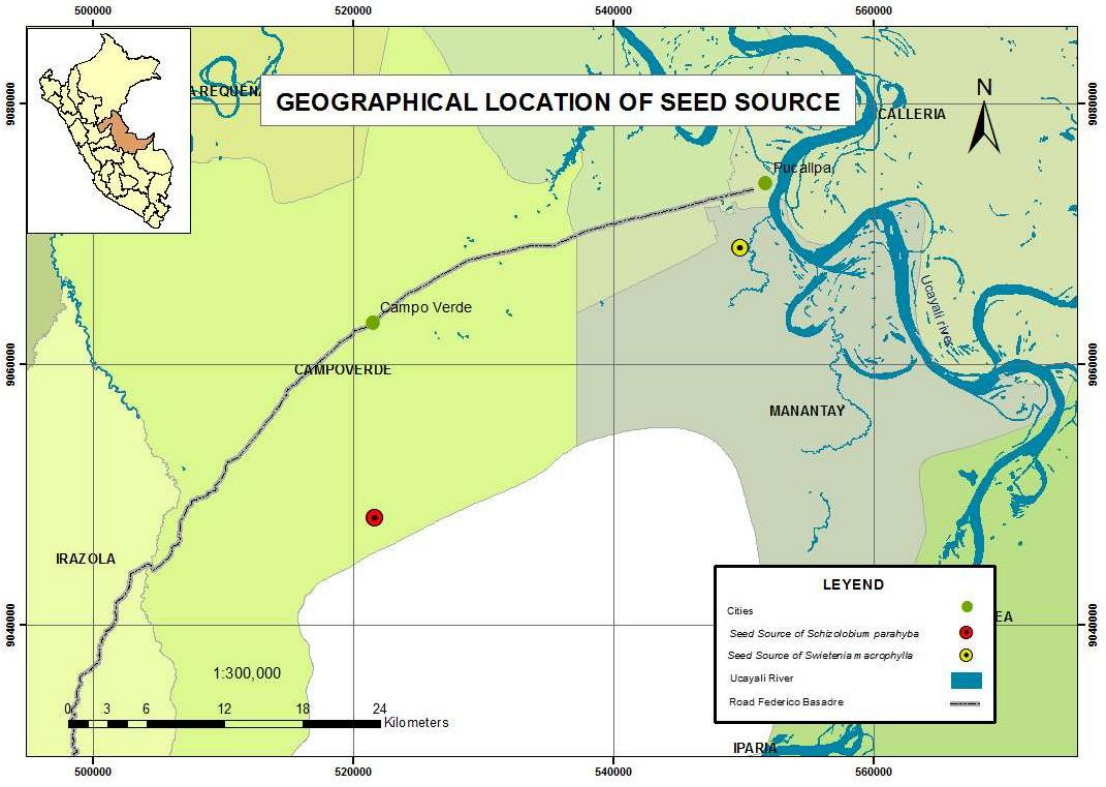

Figure 11. Geographical location of seed source for both species.

4.2. Establishment of *in vitro* culture

In *Swietenia macrophylla* G. King, the seed coat had been manually removed from 84 seeds (Figure 12).

Figure 12. Manual scarification of *Swietenia macrophylla*.

Thereafter, seeds were submerged in a sterilized flask with 70% ethanol for one minute (or this step was omitted), and then sterilized for 15-20 minutes in 1, 1.5 or 2.5% of sodium hypochlorite (NaClO). Eventually, the material has been in laminar flow cabinet rinsed in distilled sterile water (3 times for 1 minute). After surface sterilization, seeds have been sown on solidified MS medium (Murashige and Skoog, 1962) containing 30 g l^{-1} sucrose, 6 g l^{-1} agar, 100 mg l^{-1} *myo*-inositol, 200 mg l^{-1} casein, 200 mg l^{-1} glutamin (pH 5.7) (Figure 13). Cultures were maintained in the cultivation room under following conditions: temperature 25±2º C, photoperiod 18/6 hours under light/dark conditions, light intensity 2000 lx (fluorescent lamps NARVA LT 36 W/010). The plants had been cultivated until the seedlings reached mean height of 5-7 cm. Thereafter, they were used for establishing of propagation experiments.

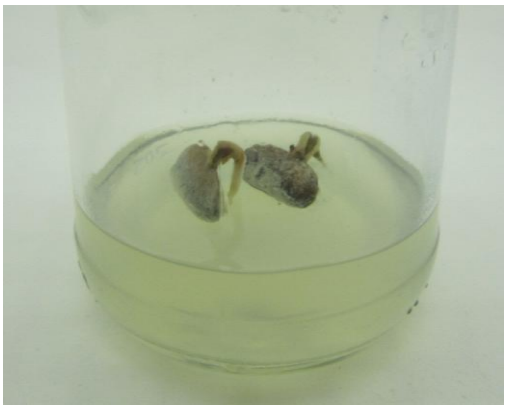

Figure 13. *In vitro* germination of *Swietenia macrophylla* seeds.

Also for *Schizolobium parahyba* var. *amazonicum*, the seed coat of the 92 seeds has been removed manually, but at first the seeds must had been submerged in water for 24 hours or more (Figure 14). After the removal of seed coats, seed were transferred into a sterilized flask with 70% ethanol for one minute (or this step was omitted), and then they were sterilized for 15-20 minutes in 1.5% or 1% of sodium hypochlorite (NaClO). Eventually, the material has been in laminar flow cabinet rinsed in distilled sterile water (3 times for 1 minute). After sterilization, seeds have been sown on solidified MS medium (Murashige and Skoog, 1962) containing 30 g l^{-1} sucrose, 6 g l^{-1} agar, 100 mg l^{-1} *myo*-inositol, 200 mg l^{-1} casein, 200 mg l^{-1} glutamin (pH 5.7) (Figure 15). The cultures were maintained under the conditions described above. When plantlets reached height of 5-7 cm, they were used for multiplication experiment.

Figure 14. Seeds of *Schizolobium parahyba* var. *amazonicum* sumerged in water for 24 hours before surface sterilization.

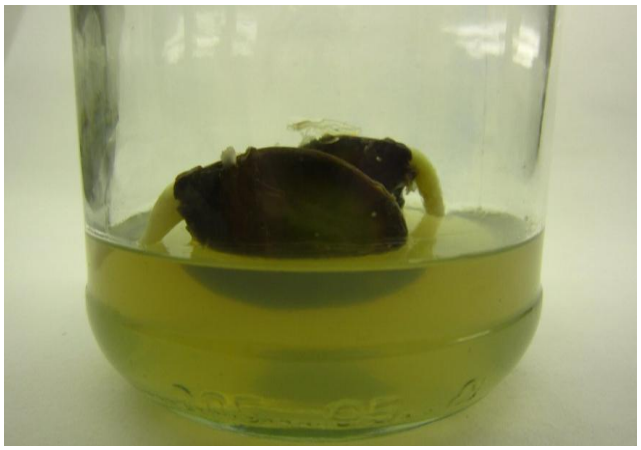

Figure 15. Seeds of *Schizolobium parahyba* in MS medium.

4.3. Multiplication experiment

For multiplication experiment purposes, 86 seedlings of *Schizolobium parahyba* were divided so that two parts each bearing one axilar meristem, and one apical segment, could have been used (Figure 16 and 17).

Nine MS media (Murashige and Skoog 1962) and five WPM media (Lloyd and McCown, 1981), containing different concentration of plant growth regulators were tested for multiplication of *Schizolobium parahyba* (Table 1). In both experiments, 10 repetitons were used in each treatment (for MS media and WPM media).

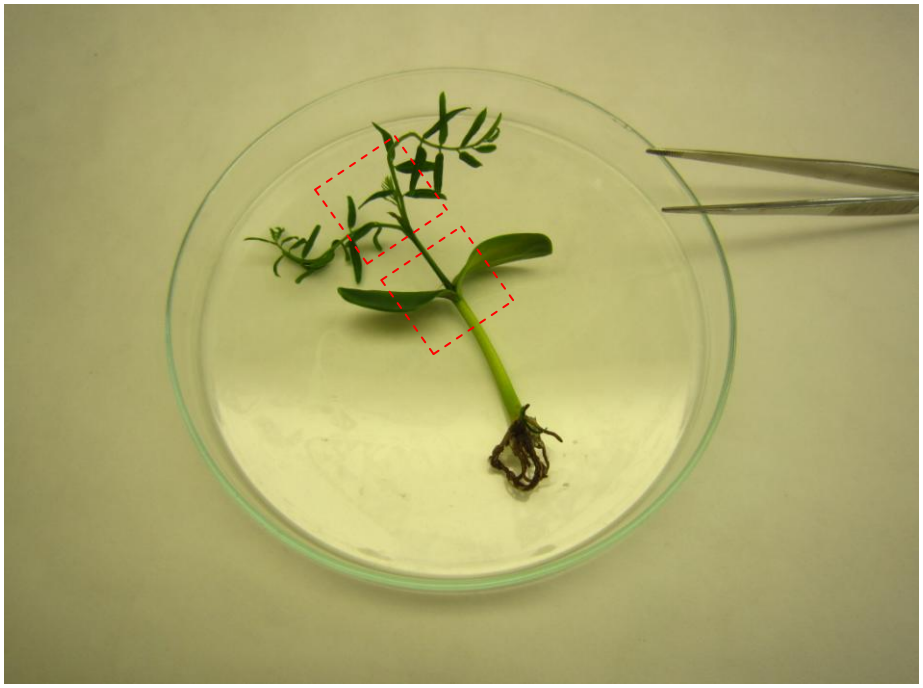

Figure 16. Seedling of *Schizolobium parahyba* divided in two parts.

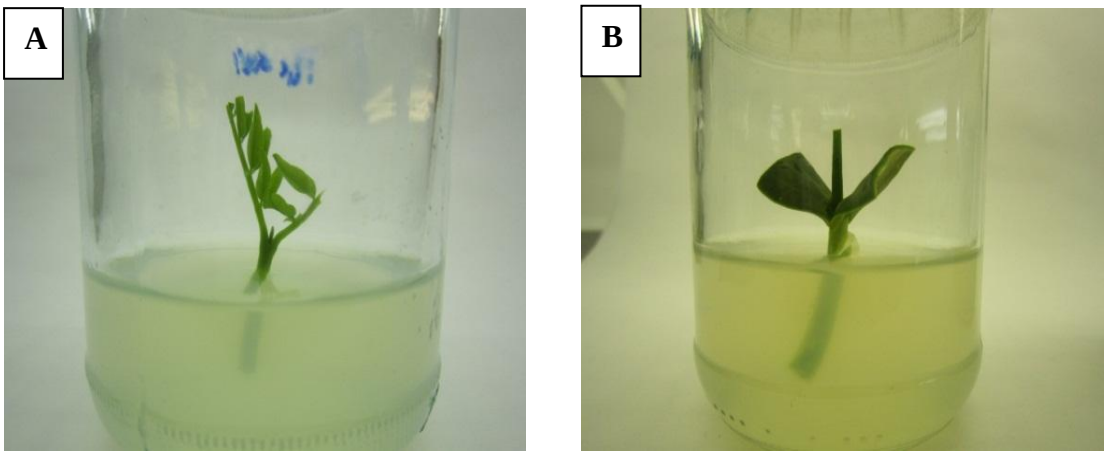

Figure 17. Two parts of *Schizolobium parahyba*. A apical segment B. axilar meristem.

Table 1. Media used for multiplication of *Schizolobium parahyba* var. *amazonicum*

Treatment	N° of repetitions:	N° of repetitions:	Total N° of repetitions per treatment
	Axillary segments	Apical segments	
MS	4	6	10
MS + 0.1 mg l^{-1} BAP	4	6	10
MS + 0.5 mg l^{-1} BAP	4	6	10
MS + 1.5 mg l^{-1} BAP	2	8	10
MS + 3.0 mg l^{-1} BAP	4	6	10
MS + 0.1 mg l^{-1} BAP + 0.1 mg l^{-1} IBA	4	6	10
MS + 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} IBA	4	6	10
MS + 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} IBA	4	6	10
MS + 3.0 mg l^{-1} BAP + 0.1 mg l^{-1} IBA	4	6	10
WPM	5	5	10
WPM + 0.1 mg l^{-1} BAP	6	4	10
WPM + 0.5 mg l^{-1} BAP	4	6	10
WPM + 1.5 mg l^{-1} BAP	2	8	10
WPM + 3.0 mg l^{-1} BAP	3	7	10

For multiplication experiment purposes, 80 seedlings of *Swietenia macrophylla* were used, only apical segments for propagation purposes (Figure 18).

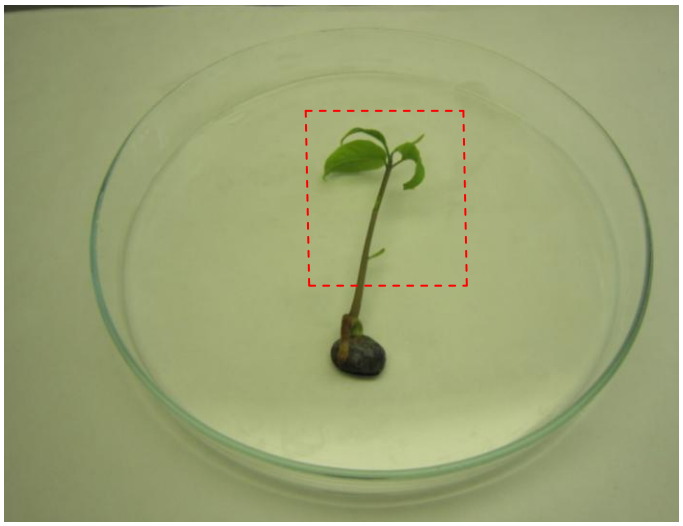

Figure 18. Seedling of *Swietenia macrophylla* used for *in vitro* culture

Also, five MS media (Murashige and Skoog 1962) and five WPM (Lloyd and McCown, 1981) media, containing different concentration of plant growth regulators were tested for multiplication of *Swietenia macrophylla* (Table 2). In each treatment, 7 and 9 repetitions were used, respectively (MS and WPM media).

Table 2. Media use for multiplication of *Swietenia macrophylla* G. King.

Treatment	Total N° of repetitions per treatment
MS	7
MS + 0.1 mg l^{-1} BAP	7
MS + 0.5 mg l^{-1} BAP	7
MS + 1.5 mg l^{-1} BAP	7
MS + 3.0 mg l^{-1} BAP	7
WPM	9
WPM + 0.1 mg l^{-1} BAP	9
WPM + 0.5 mg l^{-1} BAP	9
WPM + 1.5 mg l^{-1} BAP	9
WPM + 3.0 mg l^{-1} BAP	9

4.4. Induction of rooting

After one-month cultivation of plants on multiplication media, one half of all plants was transferred to rooting medium containing for 0.1 mg l^{-1} naphthaleneacetic (NAA) and the other half to MS without any growth regulators.

All laboratory experiments have been carried out in a Laboratory of Plant Tissue Cultures (Faculty of Forestry and Wood Sciences, CULS Prague).

4.5. Experiment evaluation

Multiplication experiment was evaluated once a week during 4-weeks cultivation. Total number of shoots and their mean length (height) was measured. Induction of roots was evaluated 3-weeks after cultivation of plants on rooting media.

Statistical significance was determined by analysis of variance (ANOVA) and the least significant ($P \leq 0.05$) differences among mean values were estimated using Tukey´s SD test [StatSoft STATISTICA 9.0].

5. RESULTS

5.1. Establishment of *in vitro* culture

For establishment of *in vitro* culture of *Schizolobium parahyba*, pre-treatment of seeds with sterilized water for 24 hours was necessary, because the testa is hard and with the water, the testa can be removed easily. The optimal concentration of NaClO was 1.5% without application of ethanol 70%, where the percentage of contamination was 0%. The first germination began after 4 days of cultivation and the final percentage of germination was 63%.

Lower concentration of NaClO was not suitable for sterilization, because the seeds were more contaminated. Surprisingly, also addition of 70% of ethanol caused stronger contamination when compared with not treated seeds with ethanol. Longer pre-treatment of seeds in water decreased percentage of germination (Table 3).

Table 3. Experiments for establishment of *in vitro* culture of *Schizolobium parahyba*

N°	Number of seeds	Pre-treatment of seeds with water	Sterilization		Germination				Contamination	
			Concentration		First germination (days)	% germination after			N° of seeds	%
			NaClO	Ethanol		7 days	15 days	30 days		
1	56	24 hours	1.50%	-	4	48	59	63	0	0
2	22	24 hours	1.50%	70%	5	41	55	55	1	5
3	14	48 hours	1%	-	12	0	15	35	1	10

Also before establishment of *in vitro* culture of *Swietenia macrohylla*, it was necessary to remove the seed coat (Figure 12).

The optimal concentration of NaClO was 2.5% without ethanol, where the percentage of contamination was 0%. The first germination began after 19 days of cultivation and the percentage of germination was 60%. Lower concentration of NaClO (1 and 1.5%) caused stronger contamination of seeds (Table 4).

Table 4. Experiments for establishment of *in vitro* culture of *Swietenia macrophylla*

N°	Sterilization			Germination				Contamination	
	Number of seeds	Concentration NaClO	Concentration Ethanol	First germination (days)	% germination after			N° of seeds	%
					7 days	15 days	30 days		
1	12	1%	-	11	0	17	25	0	0
2	40	1.50%	-	12	0	33	63	8	20
3	22	1.50%	70%	12	0	23	64	2	9
4	10	2.50%	-	19	0	0	60	0	0

5.2. The effect of plant growth regulators on parameters of *Schizolobium parahyba*

5.2.1. Evaluation of shoot multiplication on MS-derived media

Since the type of growth of apical and axillary segment was different during the experiment, the results were differentiated according to the initial character of explants. Overall, the apical segments provided shorter shoots compared to axillary ones, and apical segment produced more shoots than axillary segments, although the differences were not mostly statistically significant for both characteristics (Table 5).

The highest plants were obtained from axillary segment on media with lower concentration of BAP (0.1 or 0.5 mg l^{-1}), with or without auxin addition (increase of plant height 3.63-3.86 cm (Figure 19). In all treatments with IBA it had been proved that addition of auxin decreases growth of plants thought the differences were not statistically different (Table 5).

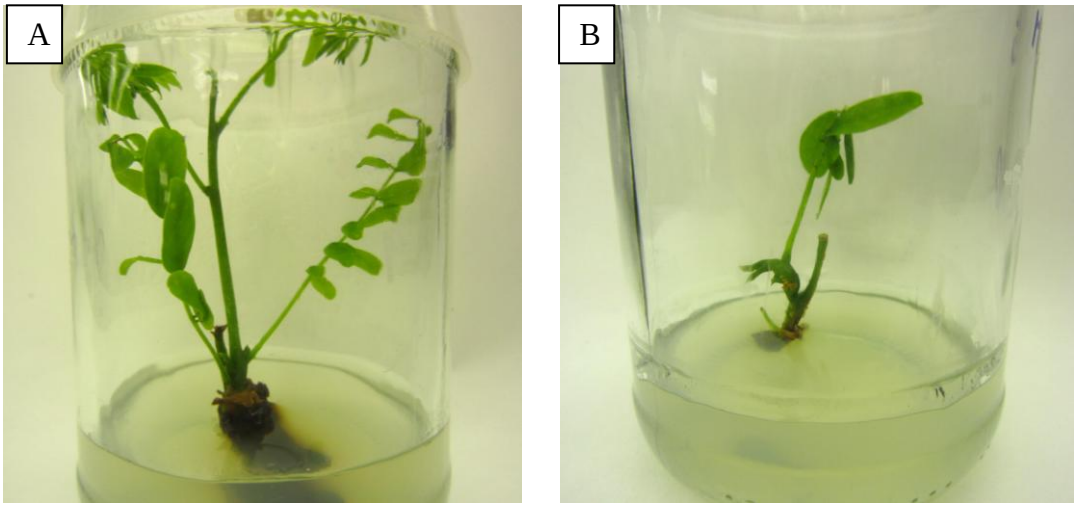

Figure 19. Explants from axillary buds of *S. parahyba* A. Explant with the cytokinin BAP (0.1mg l^{-1}); B. Explant on MS media.

For growth of *in vitro* plants, higher concentrations of cytokinins (BAP 1.5 or 3 mg l^{-1}) seems to be improper, as they suppress the growth of plants, regardless of auxin presence in the medium (increase of plant height 0.60-1.88 cm) (Figure 20).

Table 5. Characteristics of *in vitro* cultivated plants measured after 4 weeks on MS-derived media.

Treatment		Characteristics	
Nutrient concentration	Type of explant	Plant height (cm)	N° of shoots/explant
MS	Apical segment	0.60 ± 0.43 ^{a}	1.00 ± 0.50 ^{ab}
MS	Axilar segment	1.08 ± 0.35 ^{a}	1.00 ± 0.40 ^{a}
MS + 0.1 mgl^{-1} BAP	Axilar segment	3.63 ± 0.35 ^{d}	2.50 ± 0.40 ^{abcd}
MS + 0.1 mgl^{-1} BAP	Apical segment	1.07 ± 0.43 ^{a}	4.25 ± 0.50 ^{cd}
MS + 0.1 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Axilar segment	3.28 ± 0.35 ^{cd}	2.33 ± 0.40 ^{abc}
MS + 0.1 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Apical segment	1.42 ± 0.43 ^{abc}	3.50 ± 0.50 ^{bcd}
MS + 0.5 mgl^{-1} BAP	Axilar segment	3.86 ± 0.35 ^{d}	4.16 ± 0.40 ^{cd}
MS + 0.5 mgl^{-1} BAP	Apical segment	1.47 ± 0.43 ^{abc}	4.50 ± 0.50 ^{cd}
MS + 0.5 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Axilar segment	3.13 ± 0.35 ^{bcd}	3.33 ± 0.40 ^{bcd}
MS + 0.5 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Apical segment	1.20 ± 0.43 ^{ab}	4.75 ± 0.50 ^{d}
MS + 1.5 mgl^{-1} BAP	Axilar segment	1.88 ± 0.30 ^{abc}	3.25 ± 0.35 ^{cd}
MS + 1.5 mgl^{-1} BAP	Apical segment	0.99 ± 0.60 ^{abc}	3.00 ± 0.70 ^{abcd}
MS + 1.5 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Axilar segment	1.75 ± 0.35 ^{abc}	3.66 ± 0.40 ^{cd}
MS + 1.5 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Apical segment	1.12 ± 0.43 ^{ab}	4.00 ± 0.50 ^{cd}
MS + 3.0 mgl^{-1} BAP	Axilar segment	1.48 ± 0.35 ^{abc}	3.16 ± 0.40 ^{bcd}
MS + 3.0 mgl^{-1} BAP	Apical segment	0.60 ± 0.43 ^{a}	3.50 ± 0.50 ^{bcd}
MS + 3.0 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Axilar segment	1.05 ± 0.35 ^{a}	3.00 ± 0.40 ^{abcd}
MS + 3.0 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Apical segment	1.12 ± 0.43 ^{ab}	3.50 ± 0.50 ^{bcd}

Each value represents the mean ± SD. Values within the same column followed by different lower-case letters are significantly different at $P \leq 0.05$ according the Tukey's test.

The number of total shoots was not significantly affected by concentration of plant growth regulators in the cultivation media (Table 5). Optimal concentrations of BAP for maximal production of shoots ranged between 0.5 and 1.5 mg l^{-1} BAP, regardless of auxin addition in the media. These results are not, however, significantly different from most other treatments.

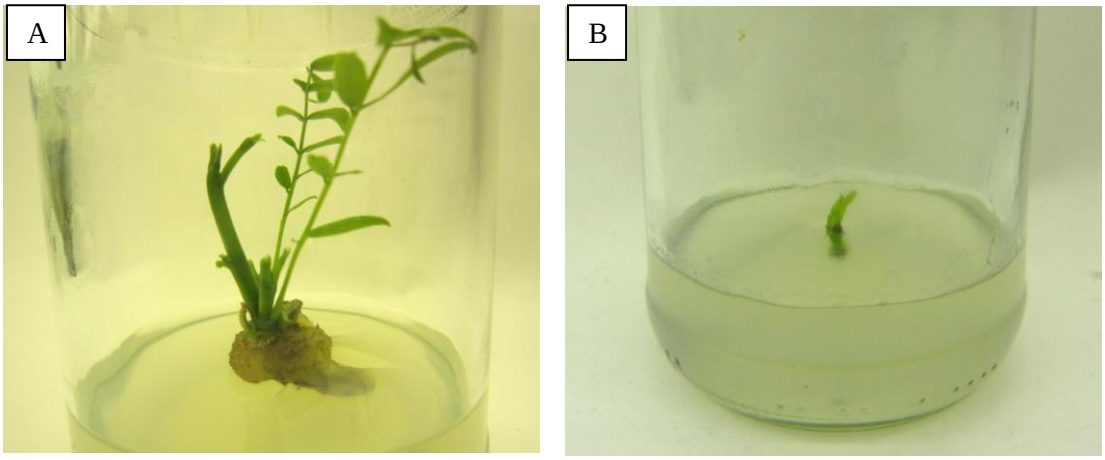

Figure 20. Explants from axillary buds of *Schizolobium parahyba*; A. Explant with the cytokinin BAP (0.5mg l^{-1}); B. Explant with BAP (1.5mg l^{-1}).

For effective multiplication of *S. parahyba*, optimal combination of growing and shoot-inducing conditions must be chosen. Thus, according to obtain results it can be concluded, that MS medium supplemented with 0.5 mg l^{-1} BAP can provide satisfactory results either for axillary or apical meristems, (Figure 20).

For growth of *S. parahyba* MS medium without plant growth regulators seems to be unsuitable (Figure 21), as the growth of plants and number of produced shoots is very low. Also media supplemented with higher concentrations of BAP (1.5 and 3.0 mg l^{-1}) are not optimal for multiplication.

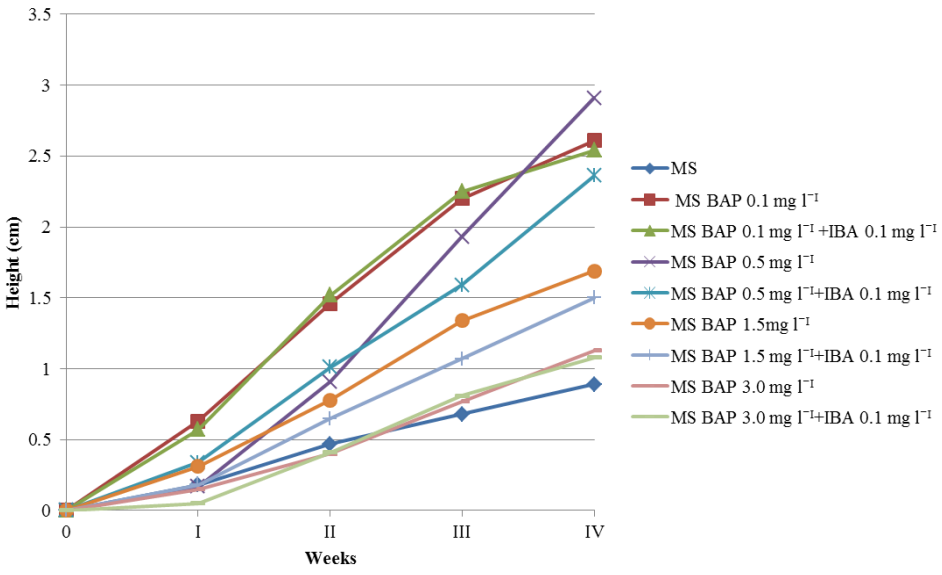

Figure 21. Increase of plant height of *S. parahyba* in MS media.

In our experiment, the formation of callus started the 7th day of *in vitro* culture (for all treatments, except for MS-control treatment), and the percentage of callus formation was approximately 80%. Of this number, 64% were observed in axillary segments and 36% in apical segments. On media supplemented with BAP (0.5 mg l^{-1}) with or without the addition of auxin IBA, in all the explants callus was produced.

All the result and statistical differences for the final plant height and number of produced shoots can be found in table 5.

5.2.2. Evaluation of shoot multiplication on WPM-derived media

In this experiment the axillary segments were rather higher compared to apical ones. However, the differences were not mostly statistically significant (Table 6). The number of shoots was comparable in all treatment containing plant growth regulators.

The highest plants were obtained from axillary segments on media with lower concentration of BAP (3.12 and 3.81 cm for 0.1 and 0.5 mg l^{-1} BAP, respectively) (Table 6).

Table 6. Characteristics of *in vitro* cultivated plants measured after 4 weeks on WPM-derived media

Treatment	Characteristics		
Nutrient concentration	Type of explant	Plant height (cm)	N° of shoots/explant
WPM	Apical segment	0.80 ± 0.36 ^{a}	1.00 ± 0.46 ^{b}
WPM	Axilar segment	0.72 ± 0.36 ^{a}	1.00 ± 0.46 ^{b}
WPM + 0.1 mgl^{-1} BAP	Apical segment	1.73 ± 0.33 ^{ab}	4.50 ± 0.42 ^{c}
WPM + 0.1 mgl^{-1} BAP	Axilar segment	3.12 ± 0.40 ^{bc}	2.25 ± 0.51 ^{ab}
WPM + 0.5 mgl^{-1} BAP	Apical segment	1.62 ± 0.40 ^{ab}	2.75 ± 0.51 ^{abc}
WPM + 0.5 mgl^{-1} BAP	Axilar segment	3.81 ± 0.33 ^{c}	3.83 ± 0.42 ^{ac}
WPM + 1.5 mgl^{-1} BAP	Axilar segment	2.02 ± 0.28 ^{ab}	3.87 ± 0.36 ^{ac}
WPM + 1.5 mgl^{-1} BAP	Apical segment	1.40 ± 0.57 ^{ab}	4.50 ± 0.73 ^{ac}
WPM + 3.0 mgl^{-1} BAP	Axilar segment	1.65 ± 0.30 ^{ab}	2.71 ± 0.39 ^{abc}
WPM + 3.0 mgl^{-1} BAP	Apical segment	1.10 ± 0.46 ^{ab}	2.00 ± 0.59 ^{ab}

Each value represents the mean ± SD. Values within the same column followed by different lower-case letters are significantly different at $P \leq 0.05$ according the Tukey's test.

For growth of *in vitro* plants higher concentrations of cytokinins (BAP 1.5 or 3 mg l^{-1}) seems to be improper, as they suppress the growth of plants (1.10-2.02 cm). Moreover, in these treatments black callus on the basis of explants had been observed.

Development of shoots in *S. parahyba* was maximal from the apical segments on media with 0.1 and 1.5 mg l^{-1} of BAP (4.50 cm for both treatments). These results were significantly higher compared to other treatments (Table 6).

WPM medium without plant growth regulators seems to be unsuitable for multiplication (Figure 22). All the results and statistical differences for the final plant height can be found in table 6.

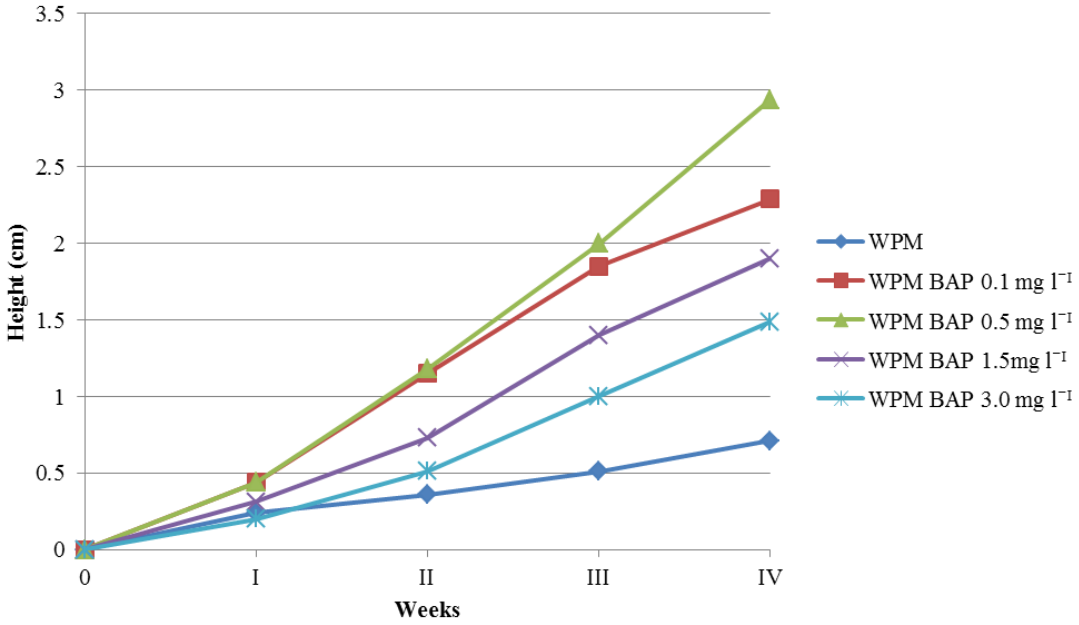

Figure 22. Increase of plant height of *S. parahyba* in WPM

5.2.3. Comparison of shoot multiplication on MS-derived and WPM-derived media

On both MS-derived and WPM-derived media the development of plantlets was similar. Media without any plant growth regulators did not produce any new shoots and the original ones were after the experiment very short. Therefore, these media are not proper for plant multiplication.

Plantlets on media with low concentrations of BAP (0.1 mg l^{-1} and 0.5 mg l^{-1}) produced relatively high number of long shoots, while on media with higher concentration of BAP (1.5 mg l^{-1} and 3.0 mg l^{-1}) produced comparable number of shoots, which were however very short.

In MS medium rather better results were obtained for medium with 0.5 mg l^{-1} BAP, while in WPM medium 0.1 mg l^{-1} BAP. All the results with standard deviation and statistical significances are presented in Table 1A In Appendix.

5.3. The effect of plant growth regulators on parameters of *Swietenia macrophylla*

5.3.1. Evaluation of shoot multiplication on MS-derived media

After four weeks of cultivation on 5 different media the increase of plant height was comparable in all treatments (Table 7). Although the highest increase was achieved in shoots cultivated on MS medium without any plant growth regulators (0.97 cm) (Figure 23 and 24), there was no statistical difference when compared with the other treatments. No production of new shoots occurred during the cultivation.

Table 7. Characteristics of *in vitro* cultivated plants measured after 4 weeks on MS-derived media

Nutrient concentration	Plant Height (cm)
MS	0.97 ± 0.25 [a]
MS + 0.1 mgl^{-1} BAP	0.50 ± 0.15 [a]
MS + 0.5 mgl^{-1} BAP	0.52 ± 0.38 [a]
MS + 1.5 mgl^{-1} BAP	0.62 ± 0.24 [a]
MS + 3.0 mgl^{-1} BAP	0.61 ± 0.37 [a]

Each value represents the mean ± SD. Values within the same column followed by different lower-case letters are significantly different at $P \leq 0.05$ according the Tukey's test.

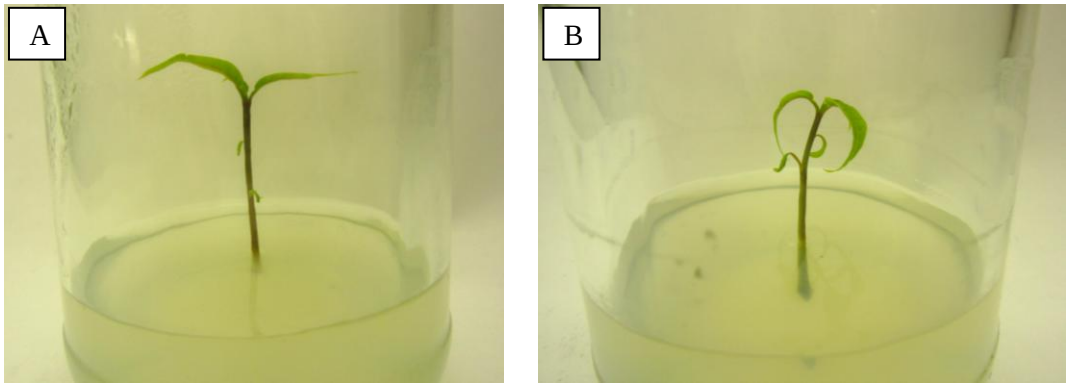

Figure 23. Explants of *Swietenia macrophylla*; A. Explant on MS media (without PGRs); B. Explant with the cytokinin BAP (3.0 mg l^{-1}).

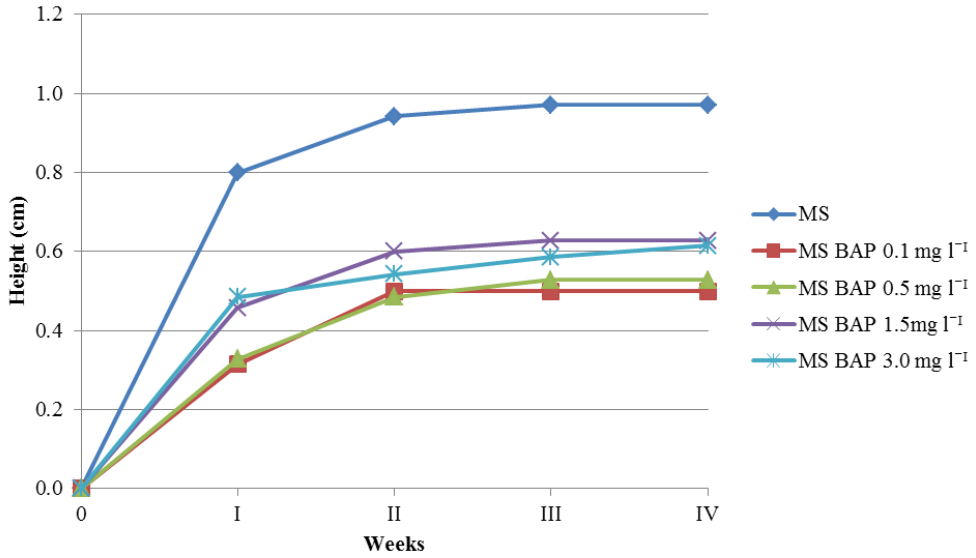

Figure 24. Increase of plant height of *S. macrphylla* in MS

5.3.2. Evaluation of shoot multiplication on WPM-derived media

On WPM medium significantly higher increase of plant height was achieved on medium without any plant growth regulators (0.45 cm). In other treatments, no statistically different values were obtained (Table 8).

The evaluation of the increase of plant height shown that the control treatment (WPM) demonstrated better growth from the first week of evaluation in contrast with the other treatments, that have a similar growth between them, also demonstrated a decrease of growth in the last week of evaluation. On the other hand, the treatment with the cytokinin BAP (0.1 mg l^{-1}) demonstrated a constant growth (Figure 25 and 26).

Table 8. Characteristics of *in vitro* cultivated plants measured after 4 weeks on WPM

Nutrient concentration	Plant Height (cm)
WPM	0.45 ± 0.16 ^{b}
WPM + 0.1 mgl^{-1} BAP	0.20 ± 0.09 ^{a}
WPM + 0.5 mgl^{-1} BAP	0.23 ± 0.08 ^{a}
WPM + 1.5 mgl^{-1} BAP	0.22 ± 0.10 ^{a}
WPM + 3.0 mgl^{-1} BAP	0.23 ± 0.08 ^{a}

Each value represents the mean ± SD. Values within the same column followed by different lower-case letters are significantly different at $P \leq 0.05$ according the Tukey's test.

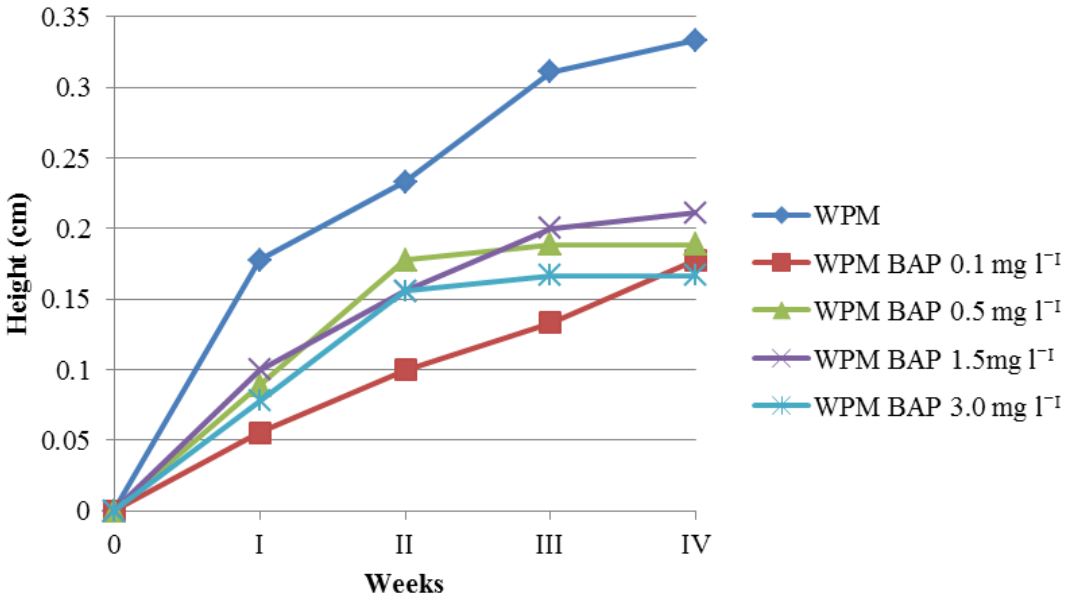

Figure 25. Increase of plant height of *S. macrophylla* in WPM

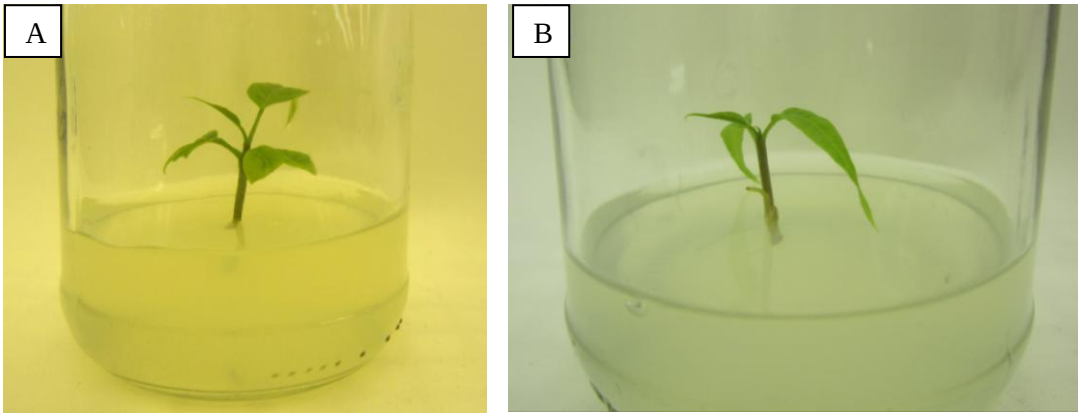

Figure 26. Explants of *Swietenia macrophylla*; A. Explant on WPM (without PGRs); B. Explant with the cytokinin BAP (3.0 mg l^{-1}).

5.3.3. Comparison of shoot multiplication on MS-derived and WPM-derived media

Appendix Table 2A summarizes the results obtained from MS-derived and WPM-derived media. It is evident, that on MS medium plants grew more rapidly in all treatments compared to WPM-derived media. Absolutely highest increase of plant height was achieved on MS medium without plant growth regulators (0.97 cm) and the lowest on WPM medium with 0.1 mg l^{-1} BAP (0.20 cm).

5.4. Rooting of *Schizolobium parahyba* and *Swietenia macrophylla*

In *Schizolobium parahyba* no root formation was observed after four weeks of cultivation on rooting media. On the basis of some explants callus was produced (Figure

28). In *Swietenia macrophylla* only 4 explants produced roots after 4 weeks of cultivation (Figure 27); 3 explants on MS media with auxin NAA 0.1 mg l^{-1} (0.1, 0.2, 1.3 cm) and 1 explant from the control treatment (MS without plant growth regulators).

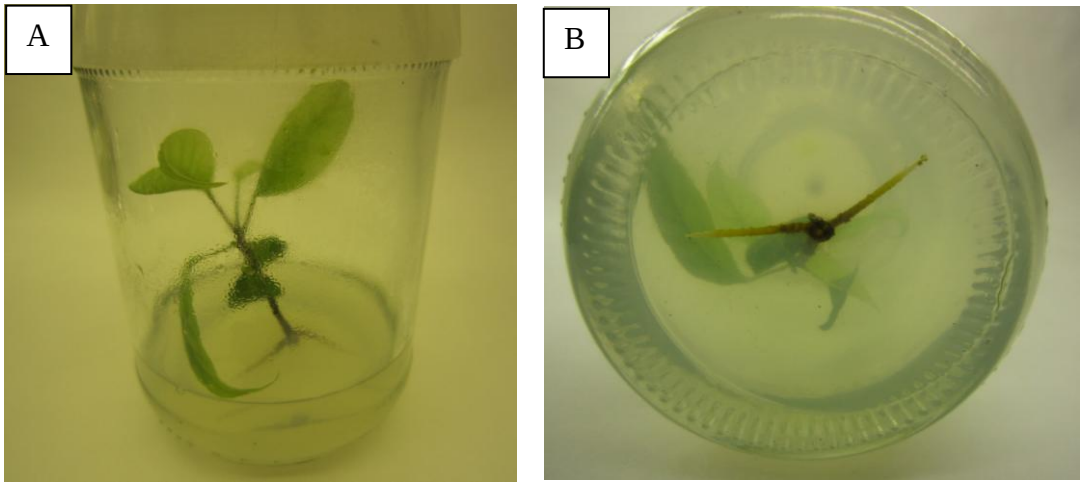

Figure 27. Explant of *Swietenia macrophylla* with small roots in MS media with NAA (0.1 mg l^{-1}). A. Explant at four week of evaluation; B. Roots (1.3 cm).

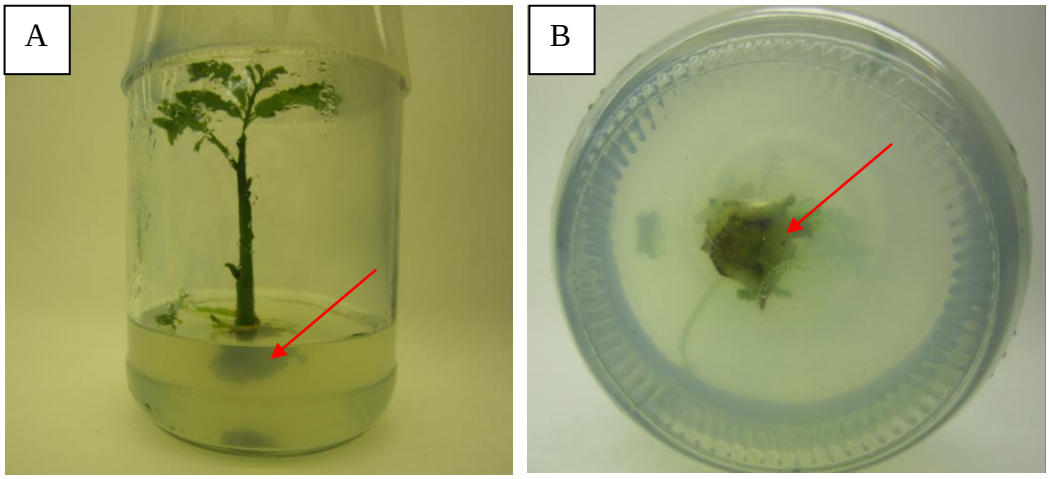

Figure 28. Explant of *Schizolobium parahyba* after four weeks of cultivation on rooting media with NAA (0.1 mg l^{-1}). A. Explant of *S. parahyba*; B. In the basis of the explant there is callus formation.

6. DISCUSSION

6.1. Establishment of *in vitro* culture

6.1.1. *Schizolobium parahyba*

In our experiment, it was necessary to remove the hard testa of seeds to increase the germination. It was done after the 24 hour treatment of seeds in water. In accordance with our results, Cordero and Boshier (2003) reports that the seeds coat (testa) in *Schizolobium parahyba* is impermeable and for better germination it is necessary to make a lateral cut with scissors, and leave the seed for 12 hours in water, which accelerates and homogenizes the germination.

Rabelo de Souza *et al.* (2009) for the disinfection of the *Schizolobium* seeds used commercial alcohol (70%) for 3 minutes and then the seeds were immersed in sodium hypoclorite NaClO (3%) for 20 minutes. Similar sterilization of seeds was used by Hasançebi *et al.* (2011) where the seeds were surface-sterilized in 70% EtOH for 1 min, then in 5% commercial bleach for 15 minutes. In both studies, it was reported that the presence of ethanol is proper for sterilization. These results, however, were not confirmed in this thesis. On the contrary, optimal concentration of sodium hypochlorite was in our experiment lower compared to study of Rabelo de Souza *et al.* (2009) and Hasançebi *et al.* (2011).

6.1.2. *Swietenia macrophylla*

In *Swietenia macrophylla* 2.5% sodium hypochlorite was optimal for seed sterilization. Schottz *et al.* (2007) reported that for sterilization of seeds 2% NaClO solution is sufficient. In his study was confirmed that the use of Benomyl at 0.1% is not necessary to apply, since the effect of sterilization was comparible to that without this agent.

Hung and Trueman (2011) used for sterilization of seeds of another species, belonging to the same family (Meliaceae), *Khaya senegalensis*, 70% ethanol and 1, 5 or 10% NaClO. It was conluded that sodium hypochlorite concentration had no significant effect on seed contamination or the percentage of seeds producing suitable shoots for transfer to shoot induction medium.

6.2. Multiplication phase of micropropagation

6.2.1. Multiplication of *Schizolobium parahyba*

For multiplication of *Schizolobium parahyba* in our experiment, MS and WPM media containing 0.1 or 0.5 mg l^{-1} BAP were successfully used. Also Balaraju (2011), working with *Pterocarpus santalinus*, another species from Fabaceae family, recommend lower concentration of BAP for multiplication of the species, indicating that lower concentrations of cytokinins are suitable for propagation of species of Fabaceae family.

Consequently, Pérez *et al.* (2002) described that the best concentrations for multiplication of *Cedrela odorata* was 0.5 and 1.5 mg l^{-1}BAP, while high concentration of BAP (5.0 mg l^{-1}), or kinetin (1.5 mg l^{-1}), or 2-ip (3.0 and 4.0 mg l^{-1}) had a minimum effect.

On the contrary, MS medium with half of concentration of salts recommended by Rabelo de Souza *et al.* (2009) for *Schizolobium parahyba* embryonic axes culture was as full concentrated in our experiment ineffective. In the study of Rabelo de Souza *et al.,* (2009) addition of plant growth regulator (GA_3) led to negative influence of culture.

In our experiment, callus formation was observed in all treatments, except for control medium without PGRs. Contrarily to these results, Rabelo de Souza *et al.,* (2007) reported that callus formation was more intense on media containing plant growth regular 2,4-D when compared to MS medium free of regulators.

Higher concentrations of BAP (1.5 mg l^{-1} and 3.0 mg l^{-1}) produced shoots, which were however rather short. Puddephat *et al.* (1997) found that increasing the concentration of BAP in culture of *Quercus robur* promoted callus growth, but nodal explants did produce axillary shoots. On the contrary, Khan *et al.* (2011) reported that cytokinins play a key role in DNA synthesis and cell division, which might be the reason for induction of multiple shoots.

6.2.2. Multiplication of *Swietenia macrohylla*

For multiplication phase of micropropagation in *Swietenia macrophylla* MS medium without any plant growth regulators was optimal. Schottz (2007) for multiplication of axillary shoots of the same species recommended MS medium supplemented with combination of BAP and 2-iP. Cytokinins, however, did not accelerate growth of plants in our experiment. George and Sherington (1984) described that decapitation of seedling of *Azadirachta indica* (Meliaceae) can lead to production

of adventitious shoots. This fenomenon was also observed in *S. macrophylla*, where decapitated seedlings produced 1 or 2 shoots per seedling. These shoots were not used for experiment establishment, because of standardization of experiment conditions. However, they could be used as a good source for plant multiplication.

As mentioned in results, it is evident, that on MS medium plants of *S. macrophylla* grew more rapidly in all treatments compared to WPM media. Similar results were reported by Venketeswaran *et al.* (1988), who described that the species of mahogany *S. mahogany* and *S. macrohylla* essentially showed similar response to the culture conditions, compared to our results. The plant material did not show any specific difference in response to the two culture medium, i.e. MS or WPM, therefore most of the experiments were carried out with MS medium only.

6.3. Root induction in *Schizolobium parahyba* and *Swietenia macrophylla*

In our experiment no root formation was achived in *Schizolobium parahyba.* On the basis of some explants callus was produced. Pérez *et al.* (2002) described an *in vitro* culture of *Cedrela odorata* and found that high concentration of BAP (5.0 mg l^{-1}), kinetin (1.5 mg l^{-1}), or 2-ip (3.0 and 4.0 mg l^{-1}) develop callus production on the basis of the explants, which hinder rooting. Similar results were found in our experiment.

Also in *Swietenia macrophylla*, only 4 plants produced roots after 4 weeks of cultivation on MS media with auxin NAA 0.1 mg l^{-1}, and 1 plant cultivated on control treatment (MS without plant growth regulators). Previous studies with *S. macrophylla* (Maruyama, 2006) showed that after 2 months of culture, more than 50% of explants formed roots in all treatments (IBA and NAA; 0.0-0.0, 0.5-0.0, 0.5-0.05 mg l^{-1}), but statistical differences between these treatments were not observed. However, the root system was better in media containing IBA in combination with NAA, increasing the mean number of secondary roots per explant. The absence of IBA in our experiment may had caused, that root formation was not achieved in *Swietenia* culture.

7. CONCLUSION

On the basis of the present results from can be concluded that, for establishment of *in vitro* culture of *Schizolobium parahyba*, pre-treatment of seeds with water for 24 hours and sterilization by NaClO was 1.5% was optimal, and also for establishment of *in vitro* culture of *Swietenia macrohylla* concentration of NaClO was 2.5% was adequate.

In *Schizolobium parahyba,* MS medium supplemented with 0.5 mg l^{-1} BAP or WPM medium supplemented with 0.1 mg l^{-1} BAP can be recommended for micropropagation. Media without plant growth regulators and media containing high concentrations of BAP are not suitable for propagation.

The present study has revealed that for effective *in vitro* propagation of *Schizolobium parahyba*, lower concentrations of BAP can provide satisfactory results for both axillary and apical meristems.

In *Swietenia macrophylla*, optimal growing was achieved on MS medium without any plant growth regulators. Higher increases in plant height were observed in MS-rerived media compared to WPM-derived media.

The formation of roots has not been achieved for both species. In further research it can be recommended to optimize this step using higher concentrations of auxins, different types of auxins, and/or activated charcoal addition in the medium.

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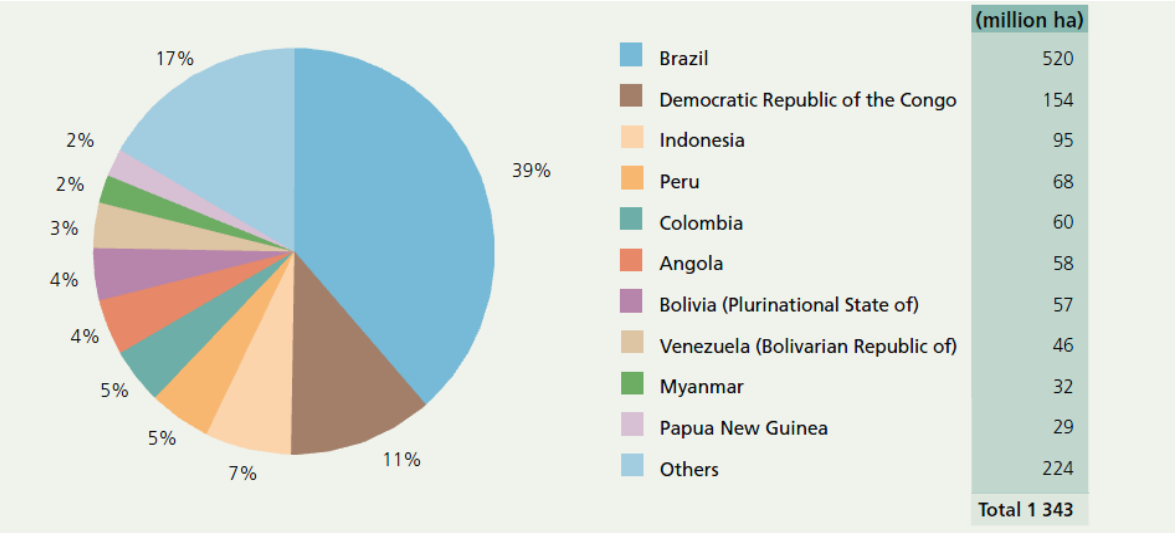
List of Appendixes

Appendix 1. Ten countries with the largest forest area in the three rainforest basin, 2010 (FAO and ITTO, 2011)



	(million ha)
Brazil	520
Democratic Republic of the Congo	154
Indonesia	95
Peru	68
Colombia	60
Angola	58
Bolivia (Plurinational State of)	57
Venezuela (Bolivarian Republic of)	46
Myanmar	32
Papua New Guinea	29
Others	224
Total 1 343	

Table 1A. Characteristics of *Schizolobium parahyba in vitro* cultivated plants measured after 4 weeks in MS and WPM

Treatment	Characteristics		
Nutrient concentration	Type of explant	Plant height (cm)	N° of shoots/explant
WPM	Apical segment	0.80 ± 0.36 [a]	1.00 ± 0.46 [ac]
WPM	Axilar segment	0.72 ± 0.36 [a]	1.00 ± 0.46 [ac]
WPM + 0.1 mgl^{-1} BAP	Apical segment	1.73 ± 0.33 [abcd]	4.50 ± 0.42 [d]
WPM + 0.1 mgl^{-1} BAP	Axilar segment	3.12 ± 0.40 [bcdef]	2.25 ± 0.51 [abcd]
WPM + 0.5 mgl^{-1} BAP	Apical segment	1.62 ± 0.40 [abcde]	2.75 ± 0.51 [abcd]
WPM + 0.5 mgl^{-1} BAP	Axilar segment	3.81 ± 0.33 [f]	3.83 ± 0.42 [d]
WPM + 1.5 mgl^{-1} BAP	Axilar segment	2.02 ± 0.28 [abcde]	3.87 ± 0.36 [d]
WPM + 1.5 mgl^{-1} BAP	Apical segment	1.40 ± 0.57 [abcdef]	4.50 ± 0.73 [d]
WPM + 3.0 mgl^{-1} BAP	Axilar segment	1.65 ± 0.30 [abcd]	2.71 ± 0.39 [abcd]
WPM + 3.0 mgl^{-1} BAP	Apical segment	1.10 ± 0.46 [abcd]	2.00 ± 0.59 [abcd]
MS	Apical segment	0.60 ± 0.43 [a]	1.00 ± 0.50 [abc]
MS	Axilar segment	1.08 ± 0.35 [ab]	1.00 ± 0.40 [a]
MS + 0.1 mgl^{-1} BAP	Axilar segment	3.63 ± 0.35 [ef]	2.50 ± 0.40 [abcd]
MS + 0.1 mgl^{-1} BAP	Apical segment	1.07 ± 0.43 [abc]	4.25 ± 0.50 [d]
MS + 0.1 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Axilar segment	3.28 ± 0.35 [def]	2.33 ± 0.40 [abcd]
MS + 0.1 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Apical segment	1.42 ± 0.43 [abcd]	3.50 ± 0.50 [bcd]
MS + 0.5 mgl^{-1} BAP	Axilar segment	3.86 ± 0.35 [f]	4.16 ± 0.40 [d]
MS + 0.5 mgl^{-1} BAP	Apical segment	1.47 ± 0.43 [abcd]	4.50 ± 0.50 [d]
MS + 0.5 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Axilar segment	3.13 ± 0.35 [cdef]	3.33 ± 0.40 [bcd]
MS + 0.5 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Apical segment	1.20 ± 0.43 [abc]	4.75 ± 0.50 [d]
MS + 1.5 mgl^{-1} BAP	Axilar segment	1.88 ± 0.30 [abcd]	3.25 ± 0.35 [bd]
MS + 1.5 mgl^{-1} BAP	Apical segment	0.99 ± 0.60 [abcd]	3.00 ± 0.70 [abcd]
MS + 1.5 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Axilar segment	1.75 ± 0.35 [abcd]	3.66 ± 0.40 [d]
MS + 1.5 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Apical segment	1.12 ± 0.43 [abc]	4.00 ± 0.50 [d]
MS + 3.0 mgl^{-1} BAP	Axilar segment	1.48 ± 0.35 [abcd]	3.16 ± 0.40 [abcd]
MS + 3.0 mgl^{-1} BAP	Apical segment	0.60 ± 0.43 [a]	3.50 ± 0.50 [bcd]
MS + 3.0 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Axilar segment	1.05 ± 0.35 [ab]	3.00 ± 0.40 [abcd]
MS + 3.0 mgl^{-1} BAP + 0.1 mgl^{-1} IBA	Apical segment	1.12 ± 0.43 [abc]	3.50 ± 0.50 [bcd]

Each value represents the mean ± SD. Values within the same column followed by different lower-case letters are significantly different at $P \leq 0.05$ according the Tukey's test.

Table 2A. Characteristics of *in vitro* cultivated plants measured after 4 weeks in MS and WPM

Nutrient concentration	Plant Height (cm)
WPM	0.45 ± 0.16^{abc}
WPM + 0.1 mgl^{-1} BAP	0.20 ± 0.09^{a}
WPM + 0.5 mgl^{-1} BAP	0.23 ± 0.08^{ab}
WPM + 1.5 mgl^{-1} BAP	0.22 ± 0.10^{a}
WPM + 3.0 mgl^{-1} BAP	0.23 ± 0.08^{ab}
MS	0.97 ± 0.25^{d}
MS + 0.1 mgl^{-1} BAP	0.50 ± 0.15^{abc}
MS + 0.5 mgl^{-1} BAP	0.52 ± 0.38^{abc}
MS + 1.5 mgl^{-1} BAP	0.62 ± 0.24^{cd}
MS + 3.0 mgl^{-1} BAP	0.61 ± 0.37^{bcd}

Each value represents the mean ± SD. Values within the same column followed by different lower-case letters are significantly different at $P \leq 0.05$ according the Tukey's test.