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Research Article

In Vitro Propagation of Pink Lapacho: Response Surface Methodology and Factorial Analysis for Optimisation of Medium Components

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Handroanthus impetiginosus, pink lapacho, is a timber, ornamental, and medicinal tree. Experiments on the *in vitro* propagation of *H. impetiginosus* were conducted using nodal segments cultivated in both Murashige and Skoog salts with Gamborg vitamins (MSG) and Woody Plant Medium (WPM) with different concentrations of 6-benzylaminopurine (BA) and indole butyric acid (IBA). Morphogenic responses were differentially affected by salt compositions and their interactions with plant growth regulators in each micropropagation stage. According to response surface analysis, the optimum multiplication rate with 1 μM IBA ranged from 16.7 to 21.3 μM BA in WPM, and the inhibitors of endogenous auxins could increase multiplication rates. A pulse with 50 μM IBA in 1/2 MSG produced 83% rooting with 3.2 roots per shoots and higher fresh and dry weights of shoots and roots. In the acclimatisation stage, 50% of plants survived after 1 year. This methodology optimised the culture media for the *in vitro* propagation of the *H. impetiginosus* clonal pool and could be applied to related species, several of which are categorised as vulnerable on the International Union for the Conservation of Nature Red List.

1. Introduction

Molecular phylogenetic studies resulted in the segregation of the *Handroanthus* genus in the Bignoniaceae family from the *Tabebuia* Gomes ex DC genus [1]. *Handroanthus impetiginosus* (Mart. ex DC) Mattos (*Tabebuia impetiginosa*), pink lapacho, is a tree native to northwest Argentina, southeastern Bolivia, Paraguay, and the Atlantic forest in Brazil. It is used as a timber resource, and its stem bark has anti-inflammatory, antimicrobial, diuretic, and anticarcinogenic medicinal properties [2–4]. The most interesting pharmacological compounds are bioactive naphthoquinones, lapachol, and α- and β-lapachone, which exhibit activity against the agents of schistosomiasis, Chagas' disease, and malaria and induce apoptosis in various human cancer cells [5, 6]. *H. impetiginosus* is also cultivated as an ornamental plant for its pink flowers in spring and is commonly used in urban forestry programs and landscaping [3].

Increasing human activity in the biomes where wild *H. impetiginosus* grows (agricultural practices, grazing) and climatic changes have led to its inclusion on the list of species under threat [2]. Moreover, seed production can vary greatly from one year to the next, and insect attack may destroy up to 95% of the seeds produced [3]. Therefore, conservation programs aimed at diminishing the risks of extinction are required because of the ecological and economic importance of this species [2].

In vitro culture is a biotechnological tool that exploits the natural totipotency of plant cells and produces many disease-free plants, which helps preserve the genetic resources of trees and crops [7, 8]. Medium formulation is an important factor in *in vitro* culture, and several nutrient combinations have proven successful for many crops. Although the Murashige and Skoog (MS) medium [9] has been successful in propagating many species, it has not been satisfactory for some woody species, and Woody Plant Medium (WPM) [10]

has been used as an alternative. The varied results can be explained by the different chemical and osmotic compositions of the media and individual species requirements. For example, WPM does not contain either cobalt or iodine micronutrients and has 4 times less nitrogen and a higher level of sulphate than MS medium [11, 12].

As far as we know, the bibliography on *in vitro* propagation of the *Handroanthus* and *Tabebuia* genera is limited to specific studies of certain species. The *in vitro* and *ex vitro* seed germination of *T. serratifolia* (Vahl) Nich and an adventitious shoot induction protocol for *T. donnell-smithii* rose adult tissues have been published [11, 13, 14]. In addition, *T. rosea* Bertol DC micropropagation and *T. roseo-alba* (Ridl.) Sand. *ex vitro* and *in vitro* leaf anatomy have been described [15, 16]. Studies of *H. impetiginosus* have been limited to morphology, temperature requirements, and the effect of gibberellins on seed germination [2] as well as the anatomy of normal and hyperhydric shoots cultivated *in vitro* [17]. Consequently, any improvement in the *in vitro* culture protocol for *H. impetiginosus* will provide information for conservation programs and the mass propagation of species of the *Handroanthus* genus. In the present study, we examined the effect of 2 basic culture media supplemented with different phytohormone combinations at all stages of *H. impetiginosus* micropropagation. Moreover, response surface analysis was used to determine the hormonal range for optimum proliferation.

2. Materials and Methods

2.1. Culture Media and Conditions. Two basic media formulations were tested at all *in vitro* stages: Murashige and Skoog salts [9] with Gamborg vitamins [18] (MSG) and WPM [10]. Both media were supplemented with 100 $mg \cdot L^{-1}$ myoinositol, 20 $g \cdot L^{-1}$ sucrose, and agar at different concentrations according to the medium and propagation stage. MSG was supplemented with 7 $g \cdot L^{-1}$ agar at the germination and multiplication stages, and agar concentration was decreased to 6 $g \cdot L^{-1}$ at the rooting stage. WPM was supplemented with 6 $g \cdot L^{-1}$ agar for all assays. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 20 min. The cultures were incubated in a growth chamber at 25 ± 2°C with 55–60% relative humidity under Phillips fluorescent daylight tubes (55 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 16-h photoperiod.

2.2. Initiation of Tissue Culture. *H. impetiginosus* seeds were obtained from a population of adult trees in Salta, in northwestern Argentina. The seed coat was removed manually, and the seeds were washed in running water for 1 h, disinfected with sodium hypochlorite solution (1.1% active chlorine) for 30 min, and rinsed 3 times (1 min each) with sterile distilled water. Each axenic seed was grown in a flat-bottom glass tube (55 mL) with 15 mL of basic medium (MSG or WPM) supplemented with activated charcoal (5 $g \cdot L^{-1}$) (Figure 1(a)). After 30 d, germination (radicle elongation > 5 mm) was recorded daily. At the end of the experiment, germination percentage, root surface area as milligrams of $Ca(NO_3)_2$ adhered to the root [19], shoot and root length, fresh and dry weights of shoots and roots, and the presence of hyperhydric shoots were evaluated. The germination rate was evaluated using successive monthly cultures.

2.3. Multiplication of Shoots. After 30 d, 4 cm high epicotyls from successful *in vitro* germinations were selected for the multiplication stage. The clonal pool epicotyls were cut (1–1.5 cm each) and placed in MSG or WPM supplemented with various 6-benzylaminopurine (BA) concentrations (0, 5, and 10 μM). Seven shoots per glass flask (350 mL) were cultured in 60 mL of medium (Figures 1(b)-1(c)). In all treatments, the average number of shoots per initial shoot (multiplication rate, MR), length of shoots and internodes, shoot number (initial plus adventitious shoots) per explant, survival and hyperhydricity percentage, and callus formation were recorded after 30 d.

In addition, the effects of BA and indole butyric acid (IBA) on shoot multiplication were investigated by growing shoots in WPM supplemented with BA (0, 5, 10, 20, 30, or 40 μM) and IBA (0, 1, 2, or 4 μM). The parameters evaluated were the same as those described for the previous multiplication assays.

2.4. Rooting of Shoots. To reduce variability in the initial physiological status, only shoots from the optimal multiplication medium were used. Shoots 15–20 mm in length with 2-3 nodes obtained by multiplication in WPM supplemented with 20 μM BA and 1 μM IBA were selected to initiate the rooting stage. The salt concentration in the rooting medium (RM) was reduced by half (1/2 MSG or 1/2 WPM). Glass tubes (55 mL), each containing 15 mL of medium, were used. One shoot per tube was cultured in RM supplemented with IBA (0, 10, 20, 30, 40, or 50 μM) for root induction for 3 d and was subsequently transferred to auxin-free RM for root development until the end of the experiment (40 d) (Figures 1(d)-1(e)). The shoot and root length, numbers of leaves and roots, fresh and dry weights of shoots and roots, root surface area, and rooting percentage were recorded. The presence/absence of basal callus and hyperhydricity were also evaluated.

2.5. Acclimatisation. The plantlets with a well-developed root system from all the rooting treatments in 1/2 MSG were removed from the flasks, and their roots were washed with running water to remove the agar. They were transplanted into a potting mixture (peat, vermiculite, and perlite: 1 : 1 : 1) in a 3-stage process. Initially, the plantlets from the rooting stage were placed in a plug tray (3 cm diameter multialveoli) inside a transparent, sealed polyvinyl chloride container (Figures 1(f)-1(g)) and maintained in growth chambers at 25 ± 2°C with a 16 h photoperiod at 115 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 4 weeks, the plantlets were exposed to a gradually reduced humidity regime by partially opening the container for 3 weeks and subsequently fully opening the container for an additional 3 weeks. In the second stage, the plants were transferred to 10 cm diameter plastic pots and incubated in a growth chamber for 4 weeks and then in a greenhouse for 8 weeks (Figure 1(h)). Finally, plants were placed in 16 cm

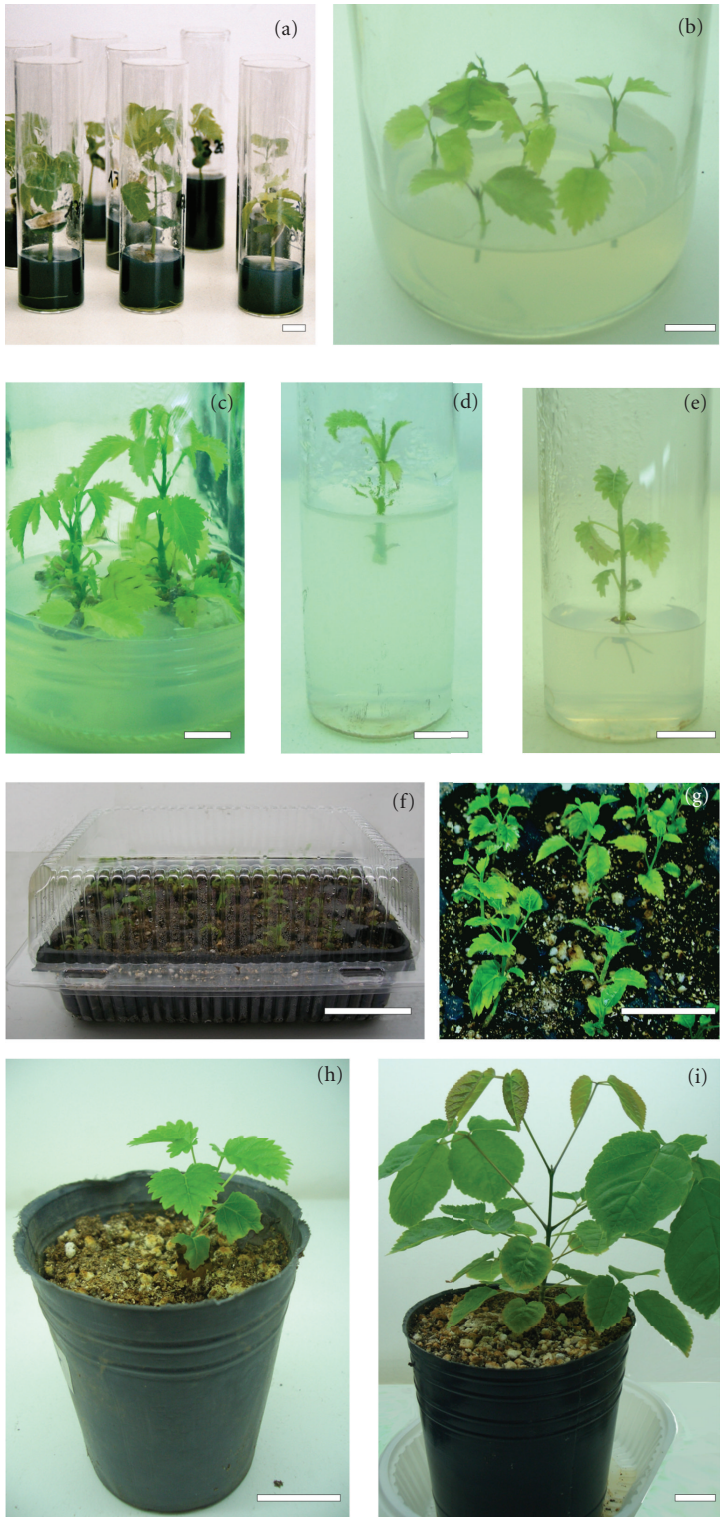

Figure 1: *In vitro* propagation of *Handroanthus impetiginosus*. Initiation stage: (a) germinated seedlings. Multiplication stage: (b) initial explants, (c) shoot after 30 days culture. Rooting stage: (d) initial shoot, and (e) rooted shoot. Acclimatization stage: (f) plantlets in plastic tray for initial stage of acclimatization, (g) detail of plantlets inside plastic tray, (h) plant acclimatization in plastic pot after 90 days culture, and (i) acclimatized plant after one-year culture. Bars (a)–(e): 1 cm, (f)–(i): 3 cm.

diameter plastic pots (Figure 1(i)). The plant survival rate was recorded periodically.

2.6. Experimental Design and Statistical Analysis. The experiments were performed with a completely randomised design with at least 2 replications per experiment. In the initiation and rooting stages, 20–25 repetitions per treatment were realized, and, during acclimatisation, 5 plantlets per treatment were studied. The multiplication assays comprised 8 flasks with 7 shoots each (total of 56 shoots) per treatment level. The data analysis was performed using factorial analysis of variance (ANOVA) and multiple comparisons of means (Tukey's test) using SPSS v12.0. Optimal MR was determined using response surface methodology (RSM) based on the method used by Lenth [20]. The data were evaluated to find the best fitting polynomial model for predicting BA and IBA concentrations and were obtained with the following quadratic model: $\mathrm{MR} = \beta_0 + \beta_1 X + \beta_2 Y + \beta_3 XY + \beta_4 X^2 + \beta_5 Y^2$, where X and Y are the codec values of BA and IBA concentrations, respectively.

3. Results

3.1. Initiation of Tissue Culture. The germination percentage without contamination was 80–85%. No differences were observed in the germination percentage after 30 d of *in vitro* culture of *H. impetiginosus* in either tested media (MSG or WPM). However, the seeds grown in WPM showed significantly earlier germination during the first week of culture. The seedlings showed no malformations, and microcuttings suitable for *in vitro* multiplication were produced (Figure 1(a)). The germination rate was maintained in successive cultures for 6 months.

The analysis of growth parameters showed a significant increase in root length in seedlings grown in MSG compared to seedlings grown in WPM. However, this enhancement did not translate into changes in shoot length, fresh and dry weights of shoots and roots, or root surface area (Table 1).

3.2. Multiplication of Shoots. The shoots developed in all the tested media exhibited expanded green leaves (Figure 1(c)). Shoot elongation was detected in all the media assayed except for MSG supplemented with 10 μM BA, which produced the shortest shoots. The factorial ANOVA showed that shoot number was significantly affected by BA concentration ($F = 4.494$, $P = 0.012$) and its interaction with media type ($F = 5.182$, $P = 0.006$). Internode length was also significantly affected by the basal salt medium formulation ($F = 20.064$, $P = 0.000$) and its interaction with BA concentration ($F = 3.003$, $P = 0.050$). Shoot length decreased in a BA concentration-dependent manner in both culture media and the highest elongation was obtained in WPM. The presence of BA produced some hyperhydric shoots with morphological abnormalities such as translucent, thick, fragile shoots with curled leaves, mainly in MSG (Table 2). MR was maintained for at least 1 year in shoots cultured in WPM but decreased gradually in shoots cultured in MSG because of internode shortening and shoot death. Therefore, WPM was selected as the medium for additional experiments using different auxin-cytokinin combinations.

Table 1: Effect of different formulations of basic culture media on seedling growth parameters on *in vitro* germination of *Handroanthus impetiginosus* after 30-day culture.

	MSG[1]	WPM[2]
Shoot length (mm)	59.6 ± 18.0 a	56.0 ± 20.8 a
Shoot fresh weight (mg)	487.3 ± 108.2 a	411.6 ± 91.4 a
Shoot dry weight (mg)	66.9 ± 15.8 a	96.5 ± 22.8 a
Root length (mm)	131.4 ± 26.4 a	109.7 ± 33.4 b
Root fresh weight (mg)	211.8 ± 30.9 a	201.9 ± 17.5 a
Root dry weight (mg)	40.5 ± 13.3 a	34.1 ± 7.0 a
Root surface area (mg)[3]	140.8 ± 75.5 a	142.3 ± 73.3 a
Hyperhydric shoots (%)	0	0

[1]MSG: Murashige and Skoog [9] salts with Gamborg vitamins [18] medium. [2]WPM: Woody Plant Medium [10]. [3]Expressed as mg of saturated solution $Ca(NO_3)_2$ according to [19]. Different letters in the same row indicate significant differences between treatments by Tukey's multiple range test ($P \leq 0.05$). Data are the averages of 3 replications of 25 seeds.

The relationship obtained through response surface analysis between MR with codec BA (X) and IBA (Y) concentrations was $\mathrm{MR} = 3.4179 + 0.3820XY - 0.7349X^2$. The model used had a high level of significance ($P = 0.004$). Using partial derivation and scale conversion, the relationship to maximise MR was determined to be linear and not focused at the origin. The response surface plot and level curves indicated that maximum MR was around 20 μM BA with all the IBA concentrations assayed (Figure 2). By applying the restriction that hormone levels cannot be negative, at least 12.0 μM BA was required to induce optimum MR. In addition, 1 μM IBA required a BA concentration ranging from 16.7 to 21.3 μM.

In WPM, reduced shoot proliferation was obtained in the highest BA concentration tested (30 and 40 μM) without IBA (Table 3). The factorial ANOVA showed that both individual factors (BA and IBA) and their interactions were significant ($P \leq 0.01$) in all the parameters evaluated. Callus proliferation was observed in the medium with high BA concentration (20–40 μM) without auxin. Hyperhydricity in the shoots was low (≤5%) in all treatments.

The medium supplemented with 20 μM BA and 1 μM IBA produced the highest MR (4.37), induced greater elongation, and reduced callus formation; thus, shoots derived from this treatment were selected for rooting assays.

3.3. Rooting of Shoots. Higher rooting percentages were observed in shoots induced with 30 and 50 μM IBA cultured in 1/2 MSG (88% and 83%, resp.) (Table 4). The shoots induced with 50 μM IBA and cultured in 1/2 MSG-auxin-free medium showed significant increases ($P \leq 0.05$) in root number (49%), root length (84%), root fresh and dry weights (269% and 248%, resp.), root surface area (202%), leaf number (50%), and shoot fresh and dry weights (120% and 134%, resp.) at the end of the experiment (40 d) compared to those developed in 1/2 WPM with identical IBA induction (Tables 4 and 5). In the auxin-free pulse, only 25–43% of shoots exhibited roots in both basic media (Table 4).

Table 2: Effect of different concentrations of benzylaminopurine (BA) on *in vitro* shoot multiplication of *Handroanthus impetiginosus* on MSG and WPM basic media after 30-day culture.

Media	BA (μM)	MR[1]	Shoots length (mm)	Internodes length (mm)	Shoots number[2]	Callus formation[3]	Hh[4] (%)	Survival (%)
MSG	0	1.68 ± 0.61 a	19.06 ± 5.02 a	5.77 ± 2.29 bc	1.26 ± 0.22 ab	−	0	98
WPM	0	1.66 ± 0.40 a	21.25 ± 6.58 a	6.78 ± 1.96 ab	1.00 ± 0.00 b	−	0	100
MSG	5	2.51 ± 0.80 a	17.06 ± 4.66 ab	5.08 ± 1.98 c	1.54 ± 0.26 a	+	3	86.5
WPM	5	1.58 ± 0.48 a	18.92 ± 4.67 a	7.72 ± 2.37 a	1.23 ± 0.26 ab	−	0	100
MSG	10	1.52 ± 0.53 a	13.69 ± 6.66 b	4.95 ± 1.74 c	1.20 ± 0.19 ab	++	5	83.8
WPM	10	2.01 ± 0.50 a	17.02 ± 6.47 ab	5.95 ± 1.36 bc	1.47 ± 0.38 a	+	2	98

MSG: Murashige and Skoog [9] salts with Gamborg's vitamins [18] medium. WPM: Woody Plant Medium [10]. [1]Multiplication rate: number of usable shoots obtained per initial shoot. [2]Initial and adventitious shoots per explant. [3]Callus absence (−) or presence (+). [4]Hh: percentage of hyperhydric shoots in scale 1 to 5. Different letters in the same column indicate significant differences between treatments by Tukey's multiple range test ($P \leq 0.05$). Data are the averages of 2 replications of at least 56 shoots each.

Table 3: Effect of benzylaminopurine (BA) and indole butyric acid (IBA) concentrations on *in vitro* shoot multiplication of *Handroanthus impetiginosus* on WPM basic medium after 30-day culture.

BA (μM)	IBA (μM)	MR[1]	Shoot length (mm)[2]	Internode length (mm)[3]	Shoot number[4]
0	0	1.75 ± 0.40	18.92 ± 7.37 ab	6.17 ± 2.07 abc	1.00 ± 0.00 d
5	0	1.72 ± 0.48	16.05 ± 3.78 bc	7.13 ± 2.00 a	1.28 ± 0.32 cd
5	1	2.44 ± 0.68	16.72 ± 6.03 bc	6.40 ± 2.20 ab	1.61 ± 0.42 bcd
5	2	2.54 ± 0.77	15.01 ± 7.37 bc	6.65 ± 2.01 ab	1.72 ± 0.49 bcd
5	4	2.24 ± 0.46	16.32 ± 5.04 bc	6.93 ± 1.63 ab	1.35 ± 0.33 cd
10	0	2.13 ± 0.50	15.22 ± 4.90 bc	5.66 ± 1.33 bcd	1.56 ± 0.45 bcd
10	1	3.58 ± 0.91	22.60 ± 7.26 a	7.22 ± 2.28 a	1.78 ± 0.48 bcd
10	2	1.94 ± 0.50	15.65 ± 4.64 bc	6.53 ± 1.52 ab	1.44 ± 0.38 bcd
10	4	1.51 ± 0.38	13.76 ± 3.85 cd	5.30 ± 1.47 cd	1.19 ± 0.25 cd
20	0	3.72 ± 1.15	16.19 ± 6.12 bc	6.38 ± 1.21 ab	3.00 ± 0.79 a
20	1	4.37 ± 0.98	18.86 ± 8.71 ab	6.43 ± 2.67 ab	1.94 ± 0.52 bc
20	2	3.30 ± 0.82	18.44 ± 6.79 ab	6.41 ± 1.31 ab	1.94 ± 0.54 bc
20	4	3.92 ± 1.28	16.72 ± 7.17 bc	6.90 ± 1.68 ab	2.17 ± 0.35 b
30	0	1.00 ± 0.00	10.31 ± 1.04 e	5.00 ± 0.00 de	1.00 ± 0.00 d
30	1	2.62 ± 0.65	19.76 ± 4.41 ab	6.30 ± 1.26 ab	1.44 ± 0.35 bcd
30	2	3.06 ± 1.02	15.26 ± 4.53 bc	5.15 ± 1.16 de	1.94 ± 0.63 bc
30	4	3.78 ± 1.22	11.90 ± 3.43 de	4.90 ± 0.96 de	3.40 ± 0.98 a
40	0	1.38 ± 0.38	10.40 ± 1.11 e	4.85 ± 0.47 de	1.10 ± 0.22 d
40	1	2.67 ± 0.74	16.53 ± 7.99 bc	6.38 ± 1.97 ab	1.39 ± 0.39 cd
40	2	1.94 ± 0.57	15.27 ± 4.15 bc	5.25 ± 0.53 cd	1.10 ± 0.15 d
40	4	2.81 ± 0.99	10.56 ± 2.00 e	4.48 ± 1.12 e	3.15 ± 1.03 a

WPM: Woody Plant Medium [10]. [1]Multiplication rate: number of useful shoots obtained per initial shoot. [2]Average of shoot length for all shoots obtained. [3]Average of internode length for all shoot obtained. [4]Initial and adventitious shoots. Different letters indicate significant differences between treatments by Tukey's multiple range test ($P \leq 0.05$). Data are the averages of 2 replications of at least 56 shoots each.

When shoots cultured in 1/2 MSG and induced with 50 μM IBA were compared to noninduced shoots, significant differences were observed in all rooting parameters except root surface area and in all shoot development parameters. Using the same medium, similar stimulatory responses were obtained with the 30 μM IBA pulse, but root number decreased significantly (Tables 4 and 5).

Bifactorial ANOVA showed that the IBA concentration pulse significantly affected all parameters ($P \leq 0.01$) except shoot length. Basal callus proliferation or hyperhydricity was not observed in shoots cultured in either rooting media without growth regulator induction. In treatments with IBA induction, basal callus was observed only sporadically (≤5%).

3.4. Acclimatisation. Plant survival averaged 50% after 5 months of acclimatisation, and no significant differences ($P \leq 0.05$) were observed in the plant survival derived from all rooting treatments. After 7 weeks with a gradual decrease in humidity (from 100% to 50–60%) and 3 weeks in a growth

Table 4: Effect of indole butyric acid (IBA) concentration and different formulations of basic culture media (1/2 WPM, 1/2 MSG) on root development parameters of *in vitro* rooting of *Handroanthus impetiginosus* with three days of auxin pulse induction, after 40-day culture.

Medium	IBA (μM)	Rooting percentage	Root number	Root length (mm)	Fresh root weight (mg)	Dry root weight (mg)	Root surface area[1]
1/2 WPM	0	42.65 ± 9.67 cd	2.15 ± 0.68 bc	16.14 ± 2.23 cdef	21.54 ± 0.90 cd	2.47 ± 0.86 cd	6.77 ± 0.59 ef
	10	60.75 ± 12.92 bc	1.81 ± 0.51 bc	10.40 ± 2.71 f	17.30 ± 2.21 de	2.05 ± 1.68 d	7.69 ± 2.06 def
	20	73.63 ± 21.16 abc	1.74 ± 0.47 bc	11.65 ± 3.41 ef	12.27 ± 2.75 e	2.30 ± 1.35 d	5.70 ± 2.51 f
	30	57.77 ± 9.87 bc	2.39 ± 0.53 ab	19.87 ± 5.33 cde	22.86 ± 4.89 cd	3.36 ± 0.83 cd	11.14 ± 3.83 cde
	40	60.99 ± 18.81 bc	2.07 ± 0.65 bc	11.66 ± 2.04 ef	21.04 ± 3.33 cd	2.67 ± 0.68 cd	13.11 ± 2.25 bcd
	50	72.33 ± 12.71 abc	2.15 ± 0.50 bc	15.29 ± 3.19 def	17.92 ± 4.73 de	2.44 ± 2.01 cd	9.68 ± 2.29 def
1/2 MSG	0	25.13 ± 2.66 d	1.63 ± 0.47 bc	15.25 ± 2.28 def	24.45 ± 2.54 cd	2.62 ± 0.53 cd	19.39 ± 2.63 abc
	10	58.68 ± 9.09 bc	1.80 ± 0.34 bc	51.56 ± 4.59 a	33.24 ± 6.81 bc	4.36 ± 0.82 bc	23.83 ± 4.13 a
	20	60.64 ± 11.29 bc	1.79 ± 0.40 bc	37.35 ± 4.44 ab	30.13 ± 10.38 bcd	3.11 ± 1.13 cd	20.24 ± 6.63 ab
	30	87.94 ± 3.69 a	1.37 ± 0.49 c	27.95 ± 7.64 bc	46.50 ± 7.94 ab	5.90 ± 2.26 ab	25.27 ± 6.75 a
	40	47.50 ± 17.15 cd	2.04 ± 0.54 bc	22.17 ± 6.29 bcd	57.06 ± 9.89 a	2.83 ± 1.03 cd	24.72 ± 7.86 a
	50	83.30 ± 21.99 ab	3.20 ± 0.59 a	28.07 ± 7.90 bc	66.21 ± 10.05 a	8.48 ± 3.33 a	29.27 ± 6.47 a

1/2 WPM: Woody Plant Medium [10] half concentration. 1/2 MSG: Murashige and Skoog [9] salts at half concentration with Gamborg vitamins [18]. [1]Expressed as mg of saturated solution $Ca(NO_3)_2$ according to [19]. Different letters in the same column indicate significant differences between treatments by Tukey's multiple range test ($P \leq 0.05$). Data are the averages of 3 replications of 20 shoots.

Table 5: Effect of indole butyric acid (IBA) concentration and different formulations of basic culture media (1/2 WPM and 1/2 MSG) on shoot development parameters of *in vitro* rooting of *Handroanthus impetiginosus* with three days of auxin pulse induction, after 40-day culture.

Medium	IBA (μM)	Leaf number	Shoot length	Fresh shoot weight (mg)	Dry shoot weight (mg)
1/2 WPM	0	6.49 ± 1.01 ab	20.28 ± 2.02 b	47.17 ± 3.69 abc	9.98 ± 1.09 abc
	10	4.15 ± 0.88 cd	24.44 ± 2.20 ab	37.64 ± 3.01 bcd	8.35 ± 1.27 abc
	20	4.06 ± 0.91 d	21.92 ± 2.89 ab	31.21 ± 4.05 cd	6.81 ± 1.53 abcd
	30	5.32 ± 0.78 abcd	23.89 ± 2.51 ab	47.09 ± 6.16 abc	10.47 ± 2.03 ab
	40	4.45 ± 0.99 bcd	20.75 ± 1.20 b	29.60 ± 4.12 d	5.92 ± 1.28 cd
	50	4.04 ± 1.11 d	21.56 ± 1.86 ab	28.13 ± 5.76 d	4.75 ± 2.35 d
1/2 MSG	0	5.52 ± 1.06 abcd	21.17 ± 1.75 b	42.65 ± 4.68 abcd	7.39 ± 1.37 abcd
	10	4.55 ± 0.90 bcd	22.14 ± 2.59 ab	36.36 ± 6.39 bcd	6.78 ± 2.98 abcd
	20	6.64 ± 1.28 a	23.50 ± 2.62 ab	54.62 ± 4.66 ab	6.32 ± 0.27 bcd
	30	5.35 ± 1.22 abcd	24.24 ± 2.60 ab	51.36 ± 5.43 ab	6.79 ± 1.63 abcd
	40	6.16 ± 1.85 ab	29.06 ± 4.36 a	58.49 ± 9.48 a	5.92 ± 0.61 cd
	50	6.05 ± 1.46 abc	24.78 ± 2.78 ab	61.94 ± 6.27 a	11.13 ± 2.16 a

1/2 WPM: Woody Plant Medium [10] half concentration. 1/2 MSG: Murashige and Skoog [9] salts at half concentration with Gamborg vitamins [18]. Different letters in the same column indicate significant differences between treatments by Tukey's multiple range test ($P \leq 0.05$). Data are the average of 3 replications of 20 shoots.

chamber, plant survival varied from 61% to 72%. Transplanting to pots reduced survival rate by approximately 10% and transferring to greenhouse conditions, in which day and night temperatures fluctuated between 15°C and 30°C, resulted in an additional 5% loss (Figure 3). Visual screening of plants indicated no morphological variations.

4. Discussion

Although chlorine reportedly causes phytotoxicity and decreases germination percentage in Bignoniaceae species, such as *T. serratifolia* [11], sodium hypochlorite was an effective disinfectant in *H. impetiginosus*. In accordance with that described for *T. serratifolia* [11, 21], different basic media formulations produced no significant difference in germination percentage in *H. impetiginosus* after 30 d of culture. The germination capacity was maintained for 6 months in the *in vitro* cultures; under field conditions, however, germination capacity reportedly does not exceed 3 months [3].

In the exalbuminous seeds of *H. impetiginosus*, reserves are located in the embryo and imbibition may play a vital role in their germinative capacity [2]. The osmotic potential values of the culture medium obtained using van't Hoff's equation, $\Psi_o = -cRT$ (c: solute concentration, R: gas constant, and T: temperature), were −0.375 MPa in MSG and −0.248 MPa in WPM. The higher water potential in WPM could explain the earlier germination of *H. impetiginosus* seeds.

At the multiplication stage, both tested basal media were comparable in terms of shoot MR, although higher shoot and

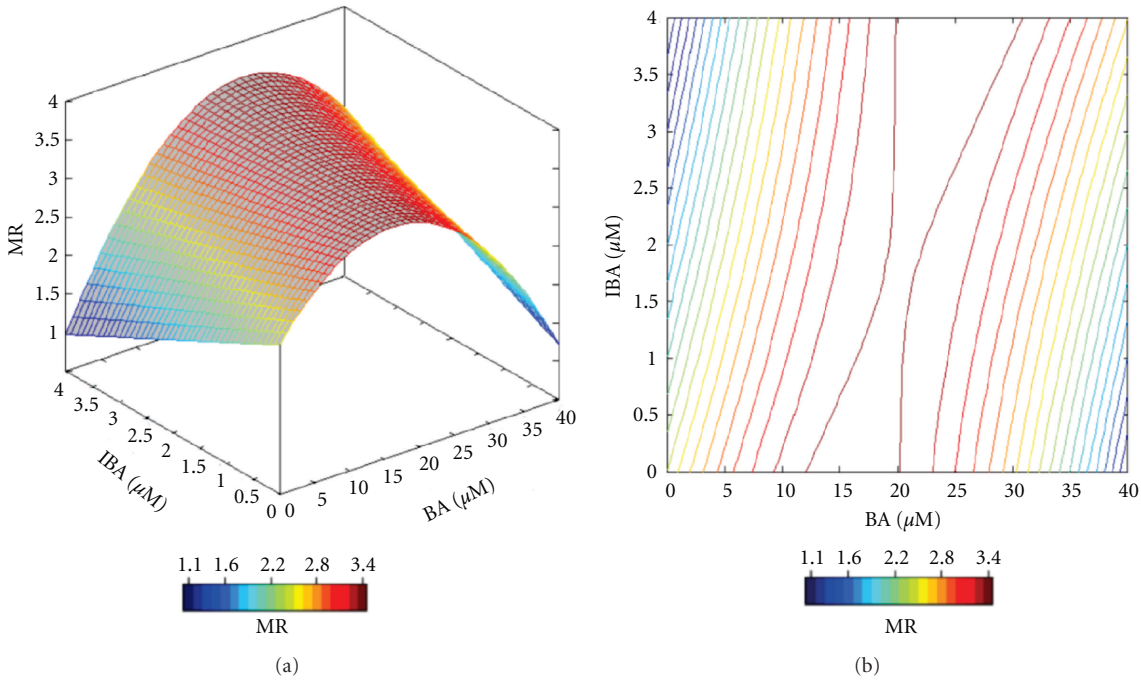

Figure 2: Response surface plot of *Handroanthus impetiginosus* multiplication rate as a function of benzyladenine (BA) and indole but-yric acid (IBA) concentration on Woody Plant Medium [10] after 30-day culture. (a) 3D response surface graph; (b) contour plot of multiplication rate.

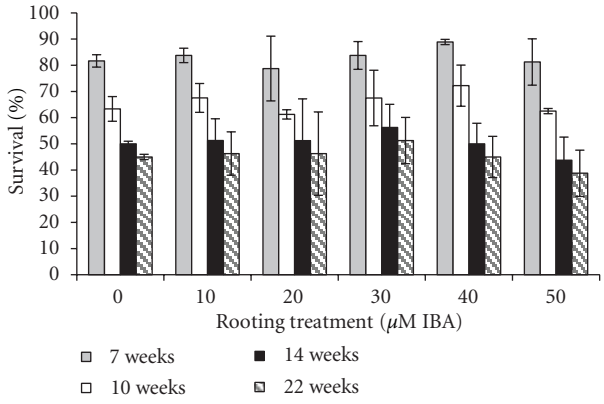

Figure 3: Effect of indole butyric acid (IBA) concentration used in *in vitro* rooting of *Handroanthus impetiginosus* on plant survival percentage during acclimatization stage.

internode elongation were produced in WPM. This result is consistent with observations in *Tabebuia donnell-smithii*, wherein shoot induction in MS salts is reduced nearly 40% compared to shoot induction in WPM medium [14]. The response could be influenced by differences in the micronutrient composition of both basic culture media. For example, WPM contains cupric sulphate, which is absent in the MS composition, and tissue culture studies in different species have shown that high cupric concentrations could be effective in enhancing the morphogenic responses of cultures [22]. Moreover, MS medium contains high nitrate concentrations, which may induce additional metabolic stress on the cultures [10].

In previous studies, BA was shown to enhance *H. impetiginosus* shoot proliferation more than media supplemented with other cytokinins (kinetin and isopentenyl adenine) [23]. Moreover, BA has been used successfully to induce axillary shoot proliferation in many species because it overcomes apical dominance and promotes shoot formation [24, 25]. In previously reported data for *T. rosea* [15], the shoot number in MS medium showed an increasing relationship with BA concentration, whereas, in *T. donnell-smithii*, BA was not as effective [14]. In *H. impetiginosus*, BA affected shoot number in a dose-dependent manner in WPM, but no significant differences were observed in MSG (Table 3).

Pink lapacho *in vitro* shoots exhibited normal architecture. Little to no hyperhydricity or callus development was observed in WPM, similar to observations described in certain blueberry cultivars [12]. Shoot hyperhydricity in *in vitro* plant cultures generally indicates the presence of several stressing conditions [26]. High osmoticity and ammonium concentration in MSG and an excess of exogenous cytokinin are stressing conditions that have been described in *in vitro* vegetative propagation in several species [25–27].

Some combined treatments in WPM supplemented with BA and IBA stimulated *H. impetiginosus* shoot proliferation and elongation. Although a general auxin-cytokinin balance for *in vitro* propagation of all plants cannot be defined, we can establish an effective hormonal relationship for a particular plant using RSM. RSM has been used for modelling, analysing, and optimising *in vitro* culture of species such as

jojoba, melon, mahogany tree, and *Basilicum polystachyon* [28, 29]. RSM is recommended when 2 or more variables are studied simultaneously because it decreases the number of treatments required to determine functional relationships between factors for obtaining an optimal response. In the present study, BA and IBA concentration effects of the 21 relationships tested were verified to obtain the best model for determining the hormonal ratio required to produce an optimal MR. The model suggests that IBA concentrations below zero would be required to obtain high MR values when using culture media with BA concentrations $\leq 12.0\,\mu$M. This condition could be achieved by using inhibitors of endogenous auxins, and assays are currently under development in our laboratory.

With no auxin pulse, 1/2 WPM was more effective than 1/2 MSG for rooting of *H. impetiginosus* shoots (43% and 25%, resp.), although the difference was not significant. Similarly, hormone-free WPM can sustain spontaneous adventitious rooting (approximately 25%) of *T. donnell-smithii* shoots [14]. However, 1/2 MS medium without any growth regulators failed to induce root formation in *Oroxylum indicum* Vent. (Bignoniaceae) after 8 weeks of incubation in [24], although 52% of *T. rosea* shoots grown in auxin-free MS medium developed adventitious roots [15]. This result shows that the nutrient requirements for root induction are variable even in the same family and that they also depend on the genotype, as indicated in blueberry cultivar micropropagation [12].

The rooting experiments showed that induction with exogenous auxin was essential for increasing *in vitro* development of *H. impetiginosus* shoots in 1/2 MSG but did not affect most of the parameters assessed in 1/2 WPM. Similar effects of auxin were observed in the *in vitro* rooting of *T. rosea* in MS medium with $5.37\,\mu$M naphthaleneacetic acid, which produced 100% of rooting shoots with a high number of adventitious roots [15].

Unlike in other species cultured in media supplemented with high concentrations of auxin [24], the 30 and $50\,\mu$M IBA pulses in *H. impetiginosus* did not produce any callus that could disrupt the vascular tissue continuity between the shoot and root and thus decrease survival.

The root surface area was significantly affected by media type with increases in 1/2 MSG. Improvement in root surface area enhances root water and nutrient absorption, and the lower water potential of 1/2 MSG might stimulate root development as an adaptation mechanism to increase the absorptive surface. Moreover, the deficiency of certain microelements, such as cobalt or iodine, in WPM compared to MSG could explain the differences in root and shoot development in *H. impetiginosus*.

In vitro plantlets grow in defined media under aseptic and controlled environmental conditions. Acclimatisation is a process whereby *in vitro* plants develop mechanisms to survive without external carbohydrate supplementation and in different temperatures, light intensities, and humidities. In general, micropropagated woody species exhibit poor recovery during acclimatisation as described by Suarez et al. [15], who obtained a low, unspecified percentage in *T. rosea*. However, the final survival rate in *T. donnell-smithii* was 92.5% [14]. The methodology described here resulted in a 50% uniform population of healthy micropropagated *H. impetiginosus* individuals with morphological features similar to the parental populations. Alternative approaches, such as the effect of plant growth-promoting rhizobacterial inoculation, are being developed to optimise this stage.

5. Conclusion

Nutrient salt compositions of culture media and their interactions with plant growth regulators affect morphogenic responses in micropropagation. The method described here optimised culture media for the *in vitro* propagation of a clonal pool of *H. impetiginosus* and could be applied to related species, several of which are categorised as vulnerable on the International Union for the Conservation of Nature Red List [30]. The basic protocol produced at least 4600 rooted plantlets from 1 germinated seed after 8 months of culture. This performance was derived using 1 month for germination, 6 months for successive multiplications in WPM supplemented with $20\,\mu$M BA and $1\,\mu$M IBA (MR: 4.37), and 40 d for rooting in 1/2 MSG using $50\,\mu$M IBA induction (83% rooting). The basic protocol provides an effective method for the massive propagation of *H. impetiginosus* and encourages future research on this species.

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