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ORIGINAL PAPER

Azospirillum brasilense enhances in vitro rhizogenesis of *Handroanthus impetiginosus* (pink lapacho) in different culture media

Ezequiel E. Larraburu · Berta E. Llorente

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Abstract
• ***Context*** *Handroanthus impetiginosus* is an ornamental tree used in urban forestry programs and landscaping, produces good timber, and has medicinal qualities. Clearing of natural forest has been increased and motivates to find techniques that optimize rhizogenesis to ex situ conservation and reforestation.
• ***Aims*** This study evaluated *Azospirillum brasilense* as an enhancer of in vitro rooting of *H. impetiginosus* under different indole-3-butyric acid (IBA) pulse inductions and media formulations.
• ***Methods*** In vitro shoots were induced with 0 to 50-μM IBA pulse for 3 days, transferred to two half-strength media (Murashige and Skoog salts with Gamborg's vitamins [MSG] and woody plant medium), and inoculated or not with *A. brasilense* Cd or Az39 strains.
• ***Results*** The statistic response surface methodology determined that both *A. brasilense* strains decreased auxin requirement for rooting up to 49 % in half-strength MSG. In this medium, Cd strain with 30-μM IBA pulse produced the 98 % of rooting shoots 21 days before uninoculated plants induced with the same IBA pulse. Also, the biometric parameter index increased from 127 to 286 % in shoots inoculated and induced with 0 to 30 μM IBA, relative to uninoculated.
• ***Conclusion*** Our results show that biofertilization with *A. brasilense* significantly promotes in vitro rooting of *H. impetiginosus* decreasing auxin requirements and micropropagation costs.

Keywords *Azospirillum brasilense* · Bignoniaceae · *Handroanthus impetiginosus* · Micropropagation · Plant tissue culture · Rhizobacteria

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Contribution of the co-authors Ezequiel Larraburu and Berta Llorente conceived and designed research and then analyzed the obtained data. Ezequiel Larraburu conducted experiments. Berta Llorente supervised the work and wrote the manuscript. Both authors read and approved the manuscript.

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1 Introduction

Pink lapacho [*Handroanthus impetiginosus* (Mart. ex DC) Mattos (=*Tabebuia impetiginosa*)], an ornamental tree in the Bignoniaceae family, is used in urban forestry programs and landscaping for its abundant pink blooms in the spring (Justiniano et al. 2000). It is also a timber and medicinal resource. The stem bark has anti-inflammatory, anti-microbial, diuretic, and anti-carcinogenic properties, and the leaves have been used as external astringents and anti-septic agents. Since several *Handroanthus* species are under threat as a result of increased crop production and grazing in their biome, ex situ conservation of *H. impetiginosus* is essential for preserving its biological diversity (Justiniano et al. 2000; Pijut et al. 2012).

Seedlings are the traditional means of *H. impetiginosus* propagation, but adverse soil and environmental conditions and variable seed production make it necessary to develop techniques to propagate this ecological and economically important species (Pijut et al. 2012). The vegetative production of forestry species by in vitro culture provides material that aids in the preservation of genetic resources, the cryopreservation of endangered and important woody plants for

reforestation, and solves the seasonal supply problems associated with the rooting of stem cuttings (George et al. 2008).

It has been reported that in vitro conditions, such as high relative humidity, low light intensity, large diurnal fluctuations in CO_2 concentration, and excessive nutrient provision, can produce some modifications in micropropagated plantlets such as hyperhydric tissue and underdeveloped root systems, which reduce plant performance. These problems require the use of alternative technologies, such as the inoculation of plant growth-promoting rhizobacteria (PGPR), to eliminate many of the difficulties and alterations associated with the in vitro rooting of woody plants. The ability of PGPR of the genus *Azospirillum* has been examined in herbaceous plants and horticultural crops, whereas this has been poorly studied in woody species (Bashan and de-Bashan 2010; Russo et al. 2012). These bacteria exhibit plant growth regulatory activities and induce defense responses in the host. Growth promotion by *Azospirillum* spp. was mainly related to the production of auxin, cytokinin, and gibberellin (Perrig et al. 2007; Russo et al. 2012). The bacterial growth regulators interact with phytohormones to modify metabolism and plant morphology and, in consequence, improve water and mineral absorption (Bashan and de-Bashan 2010). Another mechanism involved in the positive response of plants to inoculation was the ability of *Azospirillum* spp. to fix nitrogen under microaerophilic conditions although the increase in total plant nitrogen was highly variable (0 to 80 %) (Bashan et al. 2004). It has been proposed that the promotion of plant growth by *Azospirillum* spp. involves a combination of many mechanisms dependent on plant species, *Azospirillum* strain, and environmental conditions prevailing during the interaction (Bashan and de-Bashan 2010).

The effect of different strains of *Azospirillum brasilense* on micropropagation has been studied in jojoba, photinia, soybean, and rootstocks of apple and plum (Carletti et al. 1998; Larraburu et al. 2007, 2010; Cassán et al. 2009; Vettori et al. 2010; Llorente and Larraburu 2013). These studies showed beneficial influences on rhizogenesis through growth promotion and fewer alterations during in vitro culture. In this sense, *A. brasilense* Sp245 produced an increase in stem length and node number (37 and 42 %, respectively), compared to the control on the micropropagation of plum rootstocks and higher vigor (Vettori et al. 2010). In jojoba, *A. brasilense* Cd reached 86 % rooting and showed a shorter time of root initiation than controls which only rooted 43 % (Carletti et al. 1998). Photinia shoots inoculated with *A. brasilense* Cd significantly increase fresh and dry weights of shoots (32 and 62 %, respectively) and roots (105 and 137 %, respectively), root surface area (65 %), and rooting percentage (56 %), compared to control (Larraburu et al. 2007; Llorente and Larraburu 2013). In addition, anatomical and morphological studies showed that inoculated photinia plants have better development than control and were similar to ex vitro plants. Also, the inoculation with Az39 strain caused a significant increase in shoot length, root and shoot dry weight, and early growth of soybean seedlings compared to control (Cassán et al. 2009). Cd and Az39 strains produce growth-promoting substances that affect morphogenesis (Perrig et al. 2007; Bashan and de-Bashan 2010), so these were selected to study the effect on in vitro rooting of a pool of *H. impetiginosus* clonal lines.

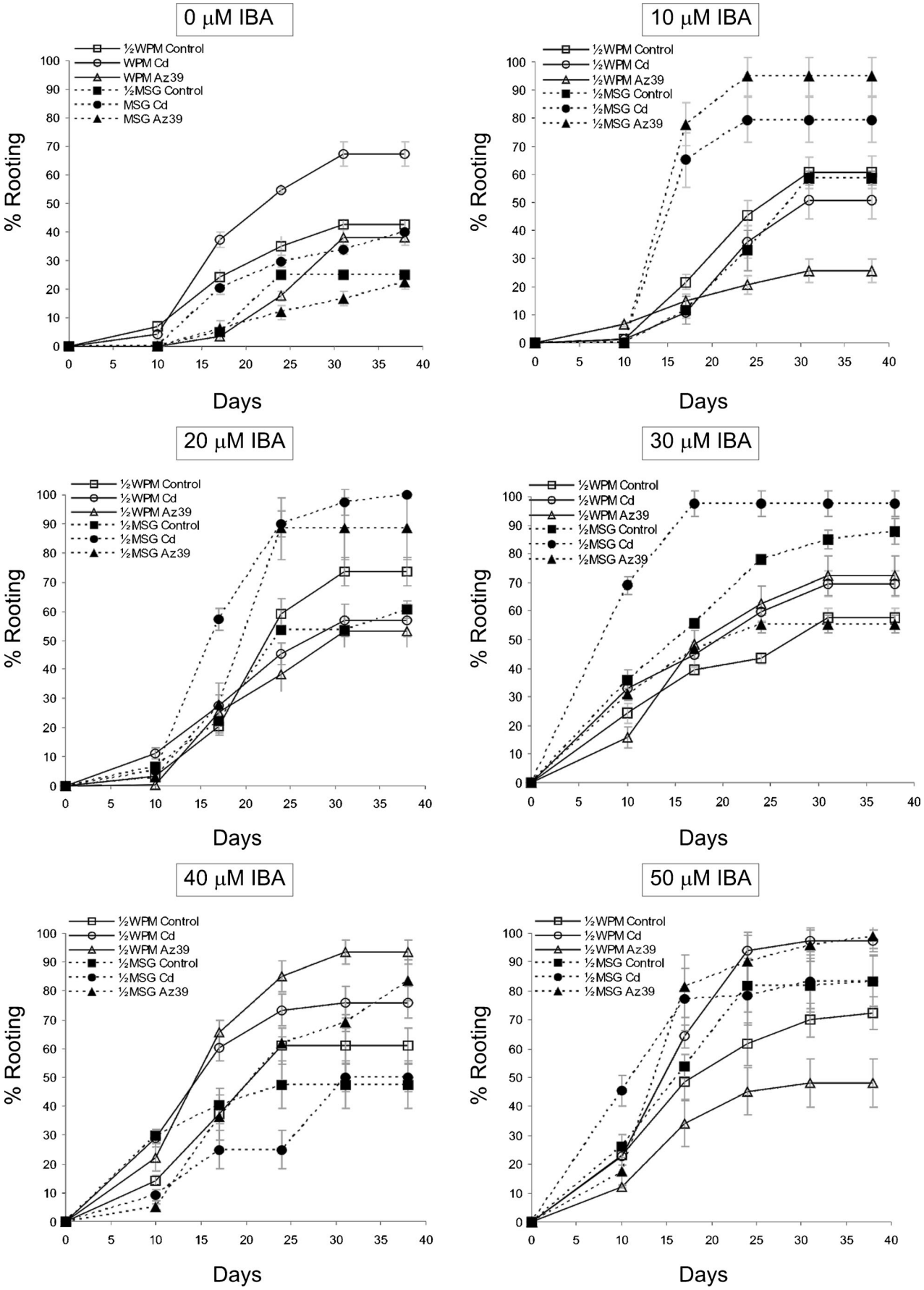
Plant hormonal balance, exogenous auxin concentration, chemical and osmotic medium composition, and individual species requirements are among the factors affecting root initiation (George et al. 2008). Root growth and differentiation have been inextricably linked with plant hormones, particularly auxin, and rhizobacteria may produce modifications in hormonal balance and enhance rhizogenesis. Quantifying the effect of *A. brasilense* inoculation on shoots induced with different indole-3-butyric acid (IBA) concentrations allows evaluating if bacterial hormonal production may replace total or partially the auxin exogenous requirement. In the present study, we tested the hypothesis that inoculation with *A. brasilense* Cd and Az39 may promote in vitro rooting of *H. impetiginosus* under different IBA pulse inductions using two plant culture media formulations.

2 Materials and methods

2.1 Plant material and culture conditions

Microcuttings from several pink lapacho *(H. impetiginosus)* clones obtained by in vitro culture according to Larraburu et al. (2012) were used. In brief, nodal segments (1–1.5 cm) were placed in woody plant medium (WPM; Lloyd and Mc Cown, 1980) supplemented with 1 μM IBA and 20 μM 6-benzylaminopurine for shoot proliferation. Seven shoots per glass flask (350 mL) were cultured in 60 mL of medium. In vitro rooting induction was assayed with 0, 10, 20, 30, 40, and 50 μM IBA for 3 days. Root development was achieved in two auxin-free half-strength culture media: 1/2 WPM and 1/2 Murashige and Skoog salts (1962) with Gamborg's vitamins (Gamborg et al. 1968) (MSG). The osmotic potentials obtained using van't Hoff's equation (Taiz and Zeiger 2010) for 1/2 MSG and 1/2 WPM were −0.264 and −0.200 MPa, respectively. All media were supplemented with 100 mg L^{-1} myo-inositol, 20 g L^{-1} sucrose, and 6 g L^{-1} agar (Britania,

Fig. 1 Rooting percentage as a function of time for in vitro shoots of *Handroanthus impetiginosus* considering the IBA concentration (0, 10, 20, 30, 40 or 50 μM), the culture medium (1/2 MSG or 1/2 WPM), and the bacterial inoculation level (uninoculated or inoculated with *Azospirillum brasilense* Cd o Az39 strains). *1/2 MSG* half-strength Murashige and Skoog (1962) salt with Gamborg's vitamins (1968), *1/2 WPM* half-strength woody plant medium (Lloyd and McCown 1980)


0 μM IBA
10 μM IBA
20 μM IBA
30 μM IBA
40 μM IBA
50 μM IBA
½WPM Control
½WPM Cd
½WPM Az39
½MSG Control
½MSG Cd
½MSG Az39
WPM Control
WPM Cd
WPM Az39
MSG Control
MSG Cd
MSG Az39
% Rooting
Days

Argentina), and the pH of the media was adjusted to 5.8 before autoclaving. Flat bottom glass tubes (100×25 mm) containing 15 mL of treatment media were used for the final rooting stage. The media were autoclaved at 121 °C for 20 min. All cultures were incubated in a growth chamber at 25±2 °C with 55–60 % relative humidity under Phillips fluorescent daylight tubes (50±5 μmol m^{-2} s^{-1}) with a 16-h photoperiod.

2.2 Bacterial strains and culture conditions

A. brasilense Cd (ATCC 29710) and Az39 (locally isolated) were used for the experiments. Bacterial inocula were grown in 250-mL Erlenmeyer flasks with 150-mL liquid medium (Okon et al. 1977). Strains were incubated at 32±1 °C on an orbital Sontec™ shaker (140 rpm) for 72 h. Viable cell counts were evaluated by dilution plate counts on Red Congo (RC) medium (Rodríguez-Cáceres 1982) supplemented with 2 % agar (Britania, Argentina). Plaques were cultured at 37 °C for 48 h, and 2×10^8 colony-forming units (c.f.u.) per milliliter were obtained. The *A. brasilense* Cd strain used in the experiments was kindly provided by Dr. Yaacov Okon (Faculty of Agriculture, the Hebrew University of Jerusalem, Israel), and *A. brasilense* Az39 was from the Institute of Agricultural Microbiology and Zoology (IMIZA), Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Buenos Aires, Argentina.

2.3 Rooting experiments

Single shoots approximately 15 to 20 mm in length with two to three nodes were excised from 4-week-old proliferating cultures. For root induction, each shoot was cultured for 3 days in tubes containing 1/2 WPM or 1/2 MSG media with 0, 10, 20, 30, 40, or 50 μM IBA and then transferred to 1/2 WPM or 1/2 MSG auxin-free root development medium for 35 days. Inoculation was made at the time of transferring induced shoots to root development medium by adding 0.1 mL Cd or Az39 strain of *A. brasilense* culture at the base of each shoot (2×10^7 c.f.u per shoot). This bacterial concentration was chosen to be between the 10^6 and 10^8 c.f.u.mL^{-1} assayed previously, because it permitted the growth of both shoots and bacteria. Treatments omitting bacterial inoculation were controls.

The shoots were considered rooted if one or more macroscopically visible roots (≥0.5 cm long) emerged after 38 days of culture. Emerging time (weekly) of the first root and rooting percentage were determined. The presence or absence of a basal callus and hyperhydricity was scored visually. The fresh and dry weights of shoots and roots, number of leaves and roots, main shoot and root length, and root surface area as milligrams of $Ca(NO_3)_2$ adhered to the root (Carley and Watson 1966) were recorded.

In order to determine the optimal IBA concentration and the time required for maximum rooting, the response surface methodology for each bacterial inoculation and medium combination was applied using the R 2.15.2 statistical software (R Core Team 2013). This methodology allowed to determine that the relationship between the rooting percentage (RP), culture time (T), and different IBA concentrations (H) was satisfactorily described by the equation $RP=aH^2+bT^2+cT+dH+eT+f$, where lowercase letters represent the coefficients of the corresponding terms. Shoot acclimatization of each treatment was performed according to the method described by Larraburu et al. (2012).

2.4 Bacterial colonization and viability

Randomly selected roots from the inoculated and uninoculated treatments were subjected to an ascending gradient of ethanol (30°, 50°, 75°, 100°), dried using the critical point method, mounted, and metalized with gold–palladium (60:40). Bacteria localization was observed and photographed using a Philips XL-30 SEM (SEM Service, MACN, Buenos Aires, Argentina). Bacteria growing in the culture media were observed and photographed by environmental scanning microscopy (ESEM Service, CITEFA, Buenos Aires, Argentina).

After assaying, the roots of control and inoculated plants were shaken for 3 days in Okon's liquid medium, and suitable dilutions were plated on RC medium supplemented with 2 % agar (Britania, Argentina) to test bacterial viability and colony characteristics.

2.5 Experimental design and statistical analysis

Experiments were performed on the basis of a completely randomized design with three replicates per experiment and 15 to 20 shoots per treatment. A full factorial analysis with three factors (culture medium, IBA concentration, and bacterial inoculation) was used for biometric parameters, and a full factorial with repeated measures using the same factors was performed for rooting percentage. Data were statistically analyzed by means of analysis of variance (ANOVA), and means were compared using Tukey's multiple range tests ($p\leq0.05$). When required, values for parameters were transformed for normality using natural log or arcsine of the root. All data were evaluated using SPSS® v.12.0 (SPSS Inc. 2003). To analyze global effects for factors and their interactions, an index was constructed using all the biometric parameters (biometric parameter index (BPI)) as follows:

$$\mathrm{BPI}(t) = \sum_{i=1}^{9} \left(\mathrm{CM}(i,t) - M(i)\right) \Big/ \mathrm{SG}(i)$$

CM(i,t): Cell mean for biometric parameter i in treatment t
GM(i): Grand mean for biometric parameter i
SG(i): Overall standard deviation for biometric parameter i

Table 1 Coefficient of polynomial equation and optimum values for rooting percentage (RP), culture time (*T*), and different IBA concentrations obtained by response surface methodology for each combination of bacterial inoculation (inoculated or not with the Cd and Az39 strains of *Azospirillum brasilense*) and cultivation medium (1/2 MSG and 1/2 WPM)

	Culture medium	f^a	e^a	d^a	c^a	b^a	a^a	p^a	R^2			Optimum	
										RP (%)	Residual standard error	Time (days)	IBA (μM)
Control	1/2 WPM	51.28	18.29	7.40	−0.56	−7.03	−1.07	$4.9\ 10^{-11}$	0.90	73.68	7.71	32.78	79.89
	1/2 MSG	55.19	16.58	15.25	−0.09	−6.28	−5.25	$2.5\ 10^{-7}$	0.78	77.04	13.54	34.19	50.01
A. brasilense Cd	1/2 WPM	52.91	19.32	12.92	0.15	−8.66	7.24	$1.3\ 10^{-11}$	0.91	99.94	8.83	31.98	51.23
	1/2 MSG	81.86	17.75	8.93	−3.06	−10.54	−13.31	$1.0\ 10^{-3}$	0.55	90.10	24.3	29.16	29.17
A. brasilense Az39	1/2 WPM	54.41	16.4	13.44	1.72	−7.01	−8.59	$2.0\ 10^{-6}$	0.74	71.04	15.01	33.89	40.81
	1/2 MSG	71.27	21.95	12.58	2.78	−12.38	−6.19	$1.6\ 10^{-4}$	0.62	90.38	23.94	31.27	46.71

R^2 coefficient of determination, *1/2 MSG* half-strength Murashige and Skoog (1962) salt with Gamborg's vitamins (1968), *1/2 WPM* half-strength woody plant medium (Lloyd and McCown 1980)

[a] Regression coefficients for the model $RP = aH^2 + bT^2 + cT + dH + eT + f$ based on response surface analysis: *H* indol-3-butyric acid (IBA) concentration, *T* days of culture, *RP* rooting percentage

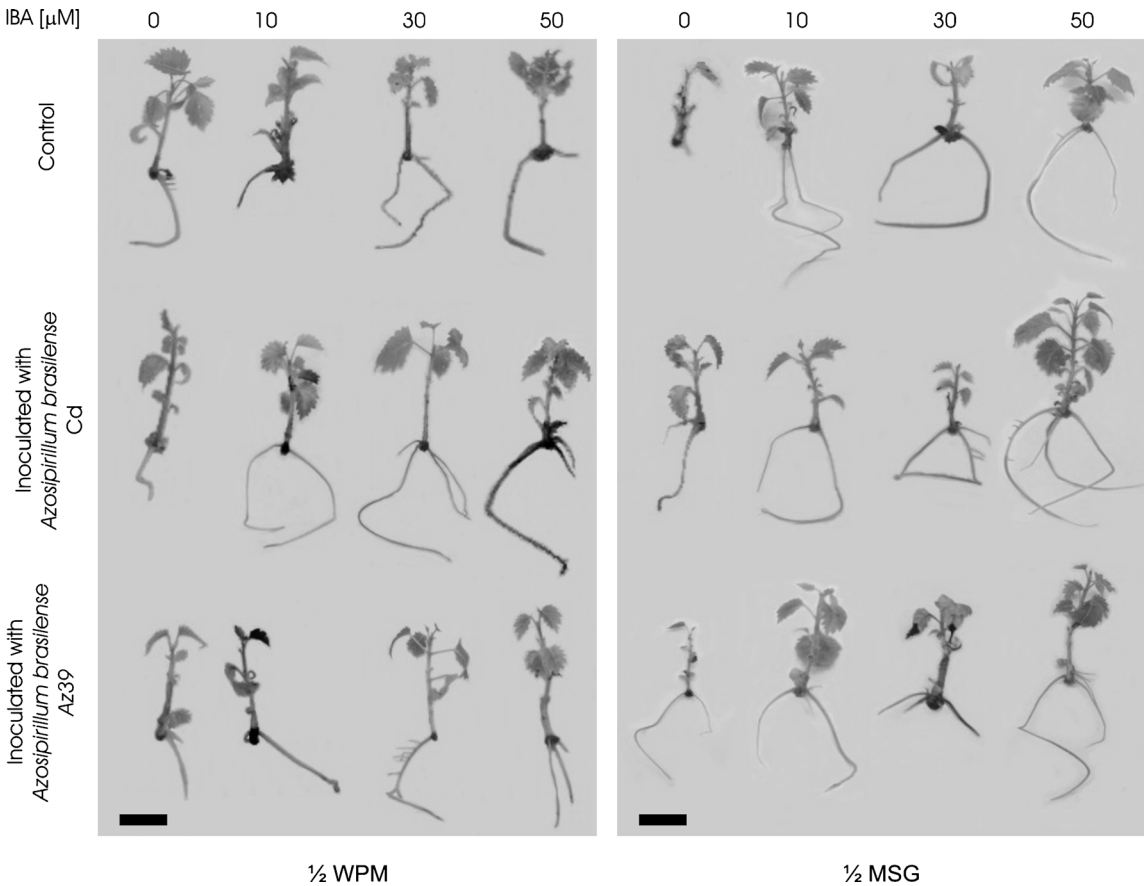


Fig. 2 Typical examples of *Handroanthus impetiginosus* plants inoculated or uninoculated with *Azospirillum brasilense* Cd or Az39 strains, induced with 0, 10, 30, or 50 μM IBA and cultured in two basal media (1/2 MSG or 1/2 WPM). *1/2 MSG* half-strength Murashige and Skoog (1962) salt with Gamborg's vitamins (1968), *1/2 WPM* half-strength woody plant medium (Lloyd and McCown 1980)

Table 2 Effect of basic culture medium (1/2 WPM, 1/2 MSG), *Azospirillum brasilense* bacterization, and 3 days of IBA pulse induction on aerial parameters of in vitro rooting of *Handroanthus impetiginosus* after 38 days of culture

	IBA (μM)	1/2 WPM									1/2 MSG								
		Control			*A. brasilense* Cd			*A. brasilense* Az39			Control			*A. brasilense* Cd			*A. brasilense* Az39		
			Row	Col		Row	Col		Row	Col		Row	Col		Row	Col		Row	Col
Leaf number	0	6.4	AB	a	6.0	ABC	a	4.7	C	a	5.6	BC	ab	8.0	A	a	7.6	A	ab
	10	4.2	B	b	3.7	B	bc	3.9	B	a	4.6	AB	b	5.0	AB	b	6.2	A	abc
	20	4.0	B	b	3.6	B	bc	3.6	B	a	6.7	A	a	5.8	A	ab	8.3	A	a
	30	5.3	A	ab	4.4	AB	ab	3.6	B	a	5.4	A	ab	5.7	A	ab	4.5	AB	c
	40	4.4	ABC	b	2.8	C	c	3.3	BC	a	6.1	A	ab	4.8	AB	b	5.2	AB	bc
	50	4.1	BC	b	3.8	C	bc	4.3	BC	a	6.2	A	ab	7.1	A	ab	5.7	AB	abc
Shoot length (mm)	0	20.2	C	a	22.4	C	a	19.6	C	b	21.1	C	b	36.9	A	a	28.5	B	ab
	10	24.6	B	a	20.9	B	ab	22.0	B	ab	22.3	B	ab	30.2	A	ab	32.5	A	a
	20	21.9	AB	a	21.7	AB	a	20.9	B	ab	23.2	AB	ab	27.6	AB	b	28.6	A	ab
	30	23.9	A	a	22.5	A	a	21.2	A	ab	24.0	A	ab	26.5	A	b	23.9	A	bc
	40	20.9	C	a	17.7	C	b	18.8	C	b	29.0	A	a	27.2	AB	b	19.9	BC	c
	50	21.4	B	a	22.1	B	a	25.7	AB	a	24.8	AB	ab	29.8	A	ab	24.8	AB	bc
Fresh shoot weigh (mg)	0	47.5	AB	a	44.8	B	a	32.5	C	b	42.3	BC	ab	60.3	A	ab	54.1	AB	a
	10	37.2	BC	ab	45.6	ABC	a	35.7	C	ab	35.8	C	b	50.1	A	b	48.6	AB	a
	20	31.3	C	bc	32.9	C	a	32.3	C	b	55.6	AB	ab	51.0	B	b	73.4	A	a
	30	47.4	AB	a	40.3	B	a	46.1	AB	a	50.3	AB	ab	51.5	AB	b	60.1	A	a
	40	29.8	B	bc	35.5	B	a	45.0	AB	a	58.4	A	a	57.3	A	ab	60.3	A	a
	50	28.5	D	c	36.8	CD	a	30.8	D	b	62.0	AB	a	74.3	A	a	47.1	BC	a
Dry shoot weight (mg)	0	10.0	B	a	13.0	A	a	9.2	BC	a	7.4	C	b	9.8	B	a	8.8	BC	a
	10	8.3	BC	ab	11.5	A	a	8.7	ABC	a	6.7	C	b	9.3	AB	a	7.0	BC	a
	20	6.8	BC	bc	9.7	AB	a	9.0	ABC	a	6.3	C	b	8.8	ABC	a	11.9	A	a
	30	10.4	AB	a	11.5	A	a	8.0	AB	a	6.9	B	b	10.2	AB	a	10.4	AB	a
	40	5.9	B	cd	6.9	AB	b	10.9	A	a	5.9	B	b	9.4	AB	a	8.2	AB	a
	50	4.7	B	d	10.1	A	a	8.5	A	a	11.1	A	a	12.7	A	a	8.5	A	a

Different capital letters in the same row indicate significant differences ($p \leq 0.05$, Tukey's test) between culture media with and without bacterization for each pulse IBA concentration. Dissimilar lowercase letters in the same column indicate significant differences ($p \leq 0.05$, Tukey's test) between pulse IBA concentrations for each medium and bacterization combination

IBA indole-3-butyric acid, *1/2 MSG* half-strength Murashige and Skoog (1962) salt with Gamborg's vitamins (1968), *1/2 WPM* half-strength woody plant medium (Lloyd and McCown 1980)

The predicted IBA concentration (H) and time (T) required for maximum rooting percentage (RP) for each combination of culture medium and bacterial inoculation were determined by applying a response surface methodology (RSM) using the R 2.15.2 statistical software (R Core Team 2013).

3 Results

3.1 Rooting percentage

Factorial repeated measures analysis showed that interactions between pulses of IBA induction, medium type, bacterial inoculation, and time significantly affected the in vitro rooting percentage of *H. impetiginosus* shoots ($F=6.439$, $p=0.000$).

The rooting percentage of uninoculated and non-induced pink lapacho shoots was higher in 1/2 WPM (43 %) than in 1/2 MSG (25 %). Conversely, when 30 or 50 μM IBA was used to induce rooting, the shoots grown in 1/2 MSG had higher rooting percentages than those in 1/2 WPM (Fig. 1). Furthermore, Cd strain inoculation increased the rooting with respect to controls in most treatments. The effect was particularly perceptible with 30 μM IBA in 1/2 MSG, in which *A. brasilense* Cd had 98 % rooting 21 days before shoots without inoculation or with Az39 bacteria (Fig. 1).

The coefficients of the equation obtained by response surface statistical analysis of rooting percentage differed

Table 3 Effect of different formulations of basic culture medium (1/2 WPM, 1/2 MSG), *Azospirillum brasilense* bacterization, and 3 days of IBA pulse induction on root parameters during in vitro rooting of *Handroanthus impetiginosus* after 38 days of culture

	IBA (μM)	1/2 WPM									1/2 MSG								
		Control			*A. brasilense* Cd			*A. brasilense* Az39			Control			*A. brasilense* Cd			*A. brasilense* Az39		
			Row	Col		Row	Col		Row	Col		Row	Col		Row	Col		Row	Col
Root number	0	2.2	A	a	1.5	A	b	1.4	A	b	1.6	A	b	1.6	A	b	1.6	A	b
	10	1.8	B	a	1.8	B	b	1.8	B	b	1.8	B	b	2.4	AB	a	3.2	A	a
	20	1.8	B	a	2.1	B	ab	1.8	B	b	1.8	B	b	3.4	A	a	2.5	AB	ab
	30	2.4	A	a	2.5	A	a	1.9	A	b	1.4	B	b	2.5	A	a	2.4	A	ab
	40	2.1	B	a	2.0	B	ab	1.9	B	b	2.0	B	b	2.6	AB	a	3.5	A	a
	50	2.2	B	a	2.6	AB	a	3.2	A	a	3.2	A	a	2.9	AB	a	3.3	A	a
Main root length (mm)	0	15.9	BC	ab	12.2	C	bc	14.8	BC	a	15.3	BC	c	29.4	A	a	19.3	B	a
	10	10.3	B	c	14.7	B	abc	14.1	B	a	50.8	A	a	29.8	A	a	30.3	A	a
	20	11.6	B	bc	12.7	B	bc	11.2	B	a	38.3	A	ab	33.7	A	a	25.6	A	a
	30	19.8	AB	a	24.1	AB	a	17.8	AB	a	27.7	AB	abc	31.5	A	a	14.6	B	a
	40	11.5	C	bc	11.5	C	c	15.0	BC	a	21.6	ABC	bc	23.4	AB	a	29.2	A	a
	50	15.4	C	abc	20.5	BC	ab	14.9	C	a	27.7	AB	abc	35.7	A	a	24.9	ABC	a
Fresh root weight (mg)	0	21.8	A	a	20.8	AB	a	14.8	C	ab	24.7	A	d	16.9	BC	c	14.8	C	b
	10	17.3	C	a	20.1	BC	a	15.4	C	ab	33.0	A	bcd	27.0	AB	bc	27.9	AB	a
	20	12.3	B	b	16.2	B	a	12.3	B	b	30.1	A	cd	33.9	A	ab	31.4	A	a
	30	22.9	B	a	22.4	B	a	20.3	B	a	45.7	A	abc	42.6	A	ab	30.2	AB	a
	40	21.0	B	a	18.9	B	a	17.4	B	ab	57.2	A	ab	36.6	A	ab	49.0	A	a
	50	17.9	C	a	22.2	C	a	15.7	C	ab	65.7	A	a	56.6	AB	a	39.5	B	a
Dry root weight (mg)	0	2.5	B	b	3.8	A	a	3.5	A	a	2.7	B	d	1.5	C	c	1.6	C	d
	10	2.1	B	b	3.7	A	a	3.0	AB	ab	4.4	A	bc	4.2	A	bc	2.9	AB	bc
	20	2.3	C	b	3.6	AB	a	2.2	C	b	3.2	B	cd	4.8	A	cd	3.8	AB	ab
	30	3.4	BC	a	4.6	AB	a	4.0	AB	a	5.8	A	b	4.5	AB	b	2.4	C	cd
	40	2.7	B	ab	3.4	AB	a	3.2	AB	a	2.8	B	d	4.7	A	ab	5.0	A	a
	50	2.4	C	b	3.6	C	a	2.8	C	ab	8.6	A	a	6.7	AB	a	5.4	B	a
Root surface area[a]	0	6.7	C	bc	12.6	B	a	11.7	B	a	19.1	A	a	4.7	D	c	3.9	E	c
	10	7.7	C	bc	10.7	BC	a	8.1	C	a	23.3	A	a	13.9	B	b	25.3	A	ab
	20	5.7	B	c	8.6	B	a	4.8	B	b	20.0	A	a	20.6	A	ab	24.9	A	ab
	30	11.0	CD	a	8.3	D	a	12.2	CD	a	25.9	A	a	23.7	AB	ab	15.0	BC	b
	40	13.3	BC	a	8.2	C	a	9.4	C	a	24.7	AB	a	25.9	AB	ab	37.8	A	a
	50	9.4	B	ab	10.2	B	a	9.8	B	a	29.4	A	a	31.2	A	a	19.4	A	b

Different capital letters in the same row indicate significant differences ($p \leq 0.05$, Tukey's test) between culture media with and without bacterization for each pulse IBA concentration. Dissimilar lowercase letters in the same column indicate significant differences ($p \leq 0.05$, Tukey's test) between pulse IBA concentrations for each medium and bacterization combination

IBA indole-3-butyric acid, *1/2 MSG* half-strength Murashige and Skoog (1962) salt with Gamborg's vitamins (1968), *1/2 WPM* half-strength woody plant medium (Lloyd and McCown 1980)

[a] Expressed as milligram of saturated $Ca(NO_3)_2$ adhered to the root surface

depending on the culture medium and bacterial strain used (Table 1). The proposed equation showed that the IBA concentration needed to obtain maximum rooting in pink lapacho shoots was also dependent on the type of culture medium. In treatments without inoculation, the culture in 1/2 WPM required 79.9 μM IBA for 73.7 % rooting, while in 1/2 MSG, 77 % rooting occurred with 50 μM IBA.

Inoculation with both strains of *A. brasilense* decreased the optimal concentration of IBA for inducing rooting in pink lapacho in both culture media compared to the uninoculated treatments. The use of *A. brasilense* Cd reduced 56 % (from 79.9 to 51.2 μM) the IBA concentrations required for optimal rooting in 1/2 WPM and 71 % (from 50 to 29.2 μM) in 1/2 MSG. *A. brasilense* Az39 inoculation also reduced 96 %

(from 79.9 to 40.8 μM) the IBA concentrations required for optimal rooting in 1/2 WPM and 7 % (from 50 to 46.7 μM) in 1/2 MSG.

3.2 Biometric parameters

Factorial analysis showed that the triple interaction between bacterial inoculation, culture medium, and IBA concentration induction pulse was significant ($p \leq 0.05$) for all aerial and root parameters studied, except leaf number.

In general, plants obtained in all treatments showed suitable development in vitro with little callus formation and no signs of hyperhydricity. One to four roots per shoot with good development and a low frequency of secondary roots were observed (Fig. 2). Mean comparison analysis for each parameter by the Tukey's test ($p \leq 0.05$) showed that several of the 36 treatments belonged to multiple groups, hindering data interpretation. Therefore, it was decided to make comparisons inside each auxin concentration (analysis by row, capital letters) and for all IBA concentrations in each medium–bacterial inoculation combination (analysis per column, lowercase) (Tables 2 and 3). The higher absolute number of leaves and shoot length in controls were observed in 1/2 MSG induced with 20 and 40 μM IBA, respectively, with significant differences ($p \leq 0.05$) with respect to shoots in 1/2 WPM (Table 2, Fig. 2). In uninoculated treatments in 1/2 MSG with 10, 20, or 50 μM IBA pulse, root length, root fresh and dry weights, and root surface area were significantly higher ($p \leq 0.05$) than those in 1/2 WPM with the same IBA pulse. The length of the longest root in controls ranged between 10.3 and 50.8 mm, with the highest value obtained with a pulse of 10 μM IBA in 1/2 MSG medium (Table 2, Fig. 2). In general, in 1/2 WPM, controls showed better results when induced with 30 μM IBA, although the only significant differences ($p \leq 0.05$) with respect to non-induced shoots (0 μM IBA) were observed in dry root weight and surface area (Tables 2 and 3, Fig. 2).

Regardless of the auxin concentration used, the mean comparison analysis generally showed that the bacterial inoculation in 1/2 MSG medium increased the biometric parameters in pink lapacho, producing higher values than those observed in 1/2 WPM. In 1/2 MSG medium compared to uninoculated plants, inoculation with *A. brasilense* Cd yielded significantly higher ($p \leq 0.05$) in leaf number (44 %), shoot length (36 to 75 %), shoot fresh and dry weights (33 to 43 %), and length of the longest root (92 %) at low IBA concentration (0–10 μM) whereas increases in root number (82 to 90 %) and root dry weights (53 to 66 %) were observed with middle IBA levels (20 to 40 μM).

A biometric parameter index (BPI) was developed to categorize the effectiveness of different treatments consisting of the sum of the standardized mean for each parameter analyzed (Fig. 3). The 1/2 MSG treatments generally produced positive BPI in all IBA concentrations, while the BPI was negative for all 1/2 WPM treatments. The Cd strain inoculation in 1/2 MSG increased between 127 and 286 % the BPI in shoots induced with 0 to 30 μM IBA, relative to uninoculated treatment. The Az39 strain raised the BPI of shoots induced with 10, 20, and 40 μM IBA (304, 444, and 113 %, respectively). In addition, *A. brasilense* Cd increased 20 % plant survival rate with respect to controls after 5 month of acclimatization, whereas *A. brasilense* Az39-inoculated plants showed no significant survival increases.

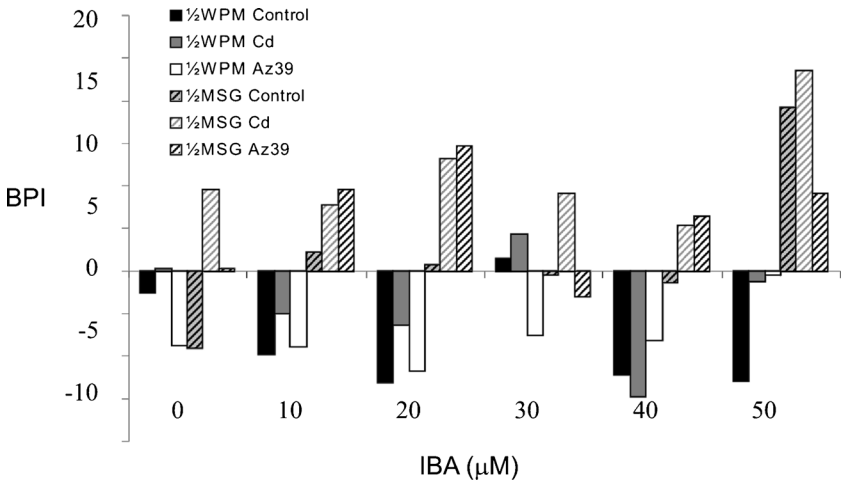


Fig. 3 Biometric parameters index (BPI) consisting of the sum of the standardized mean of all parameters of *Handroanthus impetiginosus* plants analyzed as a function of IBA concentration (0, 10, 20, 30, 40, or 50 μM) for each combination of culture medium (1/2 MSG or 1/2 WPM) and bacterial inoculation level (uninoculated or inoculated with *Azospirillum brasilense* Cd o Az39 strains). *1/2 MSG* half-strength Murashige and Skoog (1962) salt with Gamborg's vitamins (1968), *1/2 WPM* half-strength woody plant medium (Lloyd and McCown 1980)

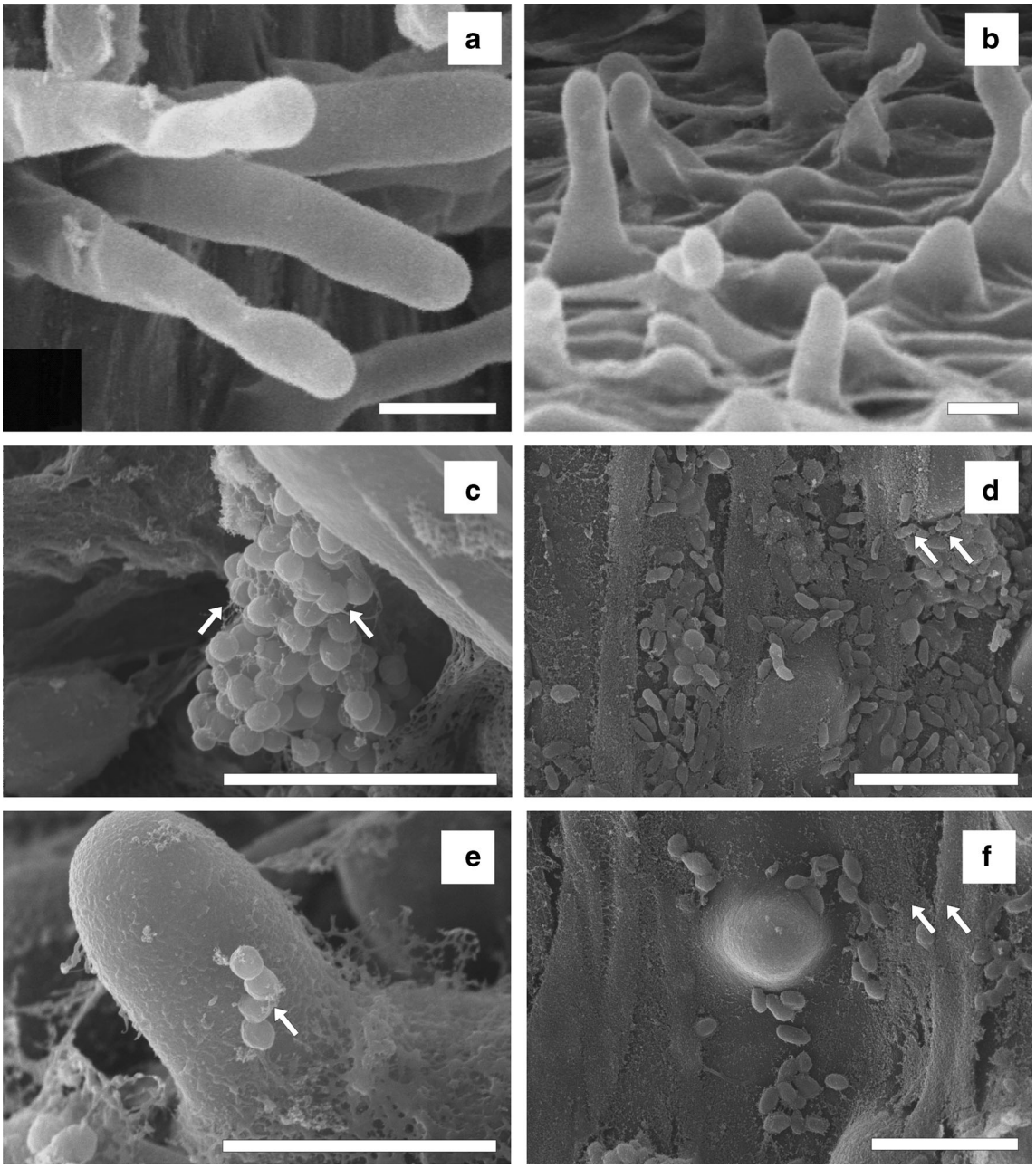


Fig. 4 Scanning electron microscopy examination of the *Handroanthus impetiginosus* root surface of shoots induced with 30 μM IBA, uninoculated or inoculated with *Azospirillum brasilense* Cd. **a**, **b** root surface of uninoculated shoots. **c**, **f** root surface of inoculated shoots. **a**, **c**, **e** 1/2 MSG medium. **b**, **d**, **f** 1/2 WPM medium. Examples of distinct fibrillar material released by bacteria and formation of microcolonies were indicated by *white arrows* in **c**–**f**. *1/2 MSG* half-strength Murashige and Skoog (1962) salt with Gamborg's vitamins (1968), *1/2 WPM* half-strength woody plant medium (Lloyd and McCown 1980). *Bars*=10 μm

3.3 Bacterial localization and survival

SEM examination of root surfaces verified *A. brasilense* Cd adhesion to root hairs and rhizodermis. Clusters of bacteria associated with fibrillar material surrounding the rhizodermis surface were observed (Fig. 4). Although the Az39 strain was not found on the radical surface, at the end of the assay, the viability of both bacterial strains was verified in all inoculated treatments.

4 Discussion

H. impetiginosus shoots cultured in 1/2 WPM without IBA induction showed a higher rooting percentage than in 1/2 MSG, similar to that observed in *Tabebuia donnell-smithii* shoots (González-Rodríguez et al. 2010). However, *Tabebuia rosea* shoots grown in auxin-free developed more adventitious roots (52 %) in 1/2 MS medium (Suarez et al. 2006). These results showed variable nutrient requirements for

rhizogenesis, even within the same genus, as shown in jojoba and blueberry micropropagation (Llorente and Apóstolo 1998; Tetsumura et al. 2008). The differences observed in rooting percentage of *H. impetiginosus* between 1/2 MSG and 1/2 WPM may be explained by differences in the medium components (cobalt and iodide are included only in MS, whereas copper is only in WPM) or changes in their concentration (potassium and nitrogen have higher concentration in MS medium compared to WPM) (George et al. 2008; Tetsumura et al. 2008).

The IBA pulse concentration required to produce the maximum in vitro rooting percentage of *H. impetiginosus* shoots in both culture media was reduced by *A. brasilense* Cd and Az39 strain inoculation (Bashan and de-Bashan 2010). Similar effects were observed in jojoba, photinia, and soybean in vitro rooting (Carletti et al. 1998; Larraburu et al. 2007, 2010; Cassán et al. 2009; Llorente and Larraburu 2013). The Cd strain also increased the pink lapacho rooting percentage without auxin induction, while the Az39 strain only stimulated *H. impetiginosus* induced with specific IBA concentrations. These results could be related to the differential production of plant growth regulators between the bacterial strains. *A. brasilense* Cd, grown in a chemically defined medium (NFB), produces higher levels of IAA, gibberellic acid, and zeatin than the Az39 strain under the same conditions (Perrig et al. 2007).

The increase in *H. impetiginosus* root length was observed in the medium with higher nitrate concentration (1/2 MSG) by applying exogenous IBA such as the reversion of nitrate inhibitory effect observed when maize roots were treated with IAA (Zhao et al. 2007). Also, the root length of the shoots without IBA induction in 1/2 MSG medium was increased by inoculation with Cd and Az39 strains of *A. brasilense*. Therefore, the effect of rhizobacteria on root length may be explained by alteration of endogenous auxin balance, as was previously described in other species (Perrig et al. 2007; Bashan and de-Bashan 2010; Fibach-Paldi et al. 2012; Russo et al. 2012).

Although inoculated treatments in both culture media presented the same (1/2 WPM) or lower (1/2 MSG) root adsorption than controls, several Cd-inoculated shoots showed significant increases ($p \leq 0.05$) in shoot and root dry weight. These increases have been attributed to the positive effect induced by *Azospirillum* spp. on the uptake of NO_3^-, NH_4^+, HPO_4^{2-}, K^+, Fe^{2+}, and various micronutrients. In this sense, *A. brasilense* Cd enhanced the proton efflux activity of soybean and cowpea roots (Bashan et al. 1992) that is directly related to the balance of ions in plant roots, even without proliferation of roots (Bashan and de-Bashan 2010 and cited within). These and other results support the hypothesis that nutrient uptake was more efficient in roots of *Azospirillum*-inoculated plants.

Various studies have suggested that root colonization by several strains of *A. brasilense* was the key factor in successful plant–PGPR interactions. They can colonize root surfaces as well as the root interior depending on the plant species, PGPR strains, and nutritional quality of the substrate (Bashan et al. 2004). The allocation of *A. brasilense* Cd in *H. impetiginosus* was restricted to the root surface in accordance with that observed on photinia (Larraburu et al. 2010). The bacteria formed microaggregates, and there were also fibrillar materials that could be associated with the exopolysaccharide secretion involved in bacterial aggregation and root surface colonization (Bashan et al. 2004; Fibach-Paldi et al. 2012). The Az39 strain was not observed on the root surface using SEM. Since viability assays confirmed that strain Az39 was active at the end of the experiment similar to the Cd strain, their absence could be due to a labile bacterial root adhesion affected by solvents used in sample preparation methods. Further studies, such as immunofluorescence, must be performed to evaluate Az39 root colonization.

Results obtained in this work show that biofertilization with *A. brasilense* Cd and Az39 significantly promoted in vitro rooting of *H. impetiginosus* decreasing auxin requirements; therefore, they decreased micropropagation costs. The inoculation increased the rooting percentage and biometric parameters producing micropropagated plants without the alterations generally associated to in vitro conditions. In addition, the colonization of in vitro shoots by PGPR may be used as a model system to study other aspect, such as biochemical and anatomical processes during plant–bacteria interactions.

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References

Bashan Y, de-Bashan LE (2010) How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—a critical assessment. Adv Agron 108:77–136. doi:10.1016/S0065-2113(10)08002-8

Bashan Y, Alcaraz-Melendez L, Toledo G (1992) Responses of soybean and cowpea root membranes to inoculation with *Azospirillum brasilense*. Symbiosis 13:217–228

Bashan Y, Holguin G, de-Bashan LE (2004) *Azospirillum*-plant relationships: physiological, molecular, agricultural and environmental advances (1997–2003). Can J Microbiol 50:521–577. doi:10.1139/W04-035

Carletti SM, Llorente BE, Rodríguez Cáceres EA, Tandecarz JS (1998) Jojoba inoculation with *Azospirillum brasilense* stimulates *in vitro* root formation. Plant Tissue Cult Biotechnol 4:165–174

Carley HE, Watson RD (1966) A new gravimetric method for estimating root-surface areas. Soil Sci 102:289–291

Cassán F, Perrig D, Sgroy V, Masciarelli O, Penna C, Luna V (2009) *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed

germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). Eur J Soil Biol 45:28–35. doi:10.1016/j.ejsobi.2008.08.005

Fibach-Paldi S, Burdman S, Okon Y (2012) Key physiological properties contributing to rhizosphere adaptation and plant growth promotion abilities of *Azospirillum brasilense*. FEMS Microbiol Lett 326:99–108. doi:10.1111/j.1574-6968.2011.02407.x

Gamborg OL, Miller R, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158

George EF, Hall M, de Klerk G-J (2008) Plant propagation by tissue culture 3rd Edition. Volume 1. The background. Springer, The Netherlands, pp 65–173. doi:10.1007/978-1-4020-5005-3

González-Rodríguez J, Ramírez-Garduza F, Robert M, O'Connor-Sánchez A, Peña-Ramírez Y (2010) Adventitious shoot induction from adult tissues of the tropical timber tree yellow *Ipé primavera* (*Tabebuia donnell-smithii*; rose [bignoniaceae]). In Vitro Cell Dev Biol Plant 46:411–421. doi:10.1007/s11627-010-9304-9

Justiniano MJ, Fredericksen TS, Nash D (2000) Ecología y silvicultura de especies menos conocidas—Tajibos o lapachos (*Tabebuia* spp). Editora El País, Santa Cruz, Bolivia, p 60

Larraburu EE, Carletti SM, Rodríguez Cáceres EA, Llorente BE (2007) Micropropagation of photinia employing rhizobacteria to promote root development. Plant Cell Rep 26:711–717. doi:10.1007/s00299-006-0279-2

Larraburu EE, Apóstolo NM, Llorente BE (2010) Anatomy and morphology of photinia (*Photinia* × *fraseri* Dress) *in vitro* plants inoculated with rhizobacteria. Trees 24:635–642. doi:10.1007/s00468-010-0433-x

Larraburu EE, Apóstolo NM, Llorente BE (2012) *In vitro* propagation of pink lapacho: response surface methodology and factorial analysis for optimisation of medium components. Int J Forest Res 318258:9. doi:10.1155/2012/318258

Llorente BE, Apóstolo NM (1998) Effect of different growth regulators and genotype on *in vitro* propagation of jojoba. N Z J Crop Hortic Sci 26:55–62. doi:10.1080/01140671.1998.9514040

Llorente BE, Larraburu EE (2013) *In vitro* propagation of fraser photinia using *Azospirillum*-mediated root development. In: Lambardi M, Ozudogru A, Jain S (eds) Protocols for micropropagation of selected economically-important horticultural plants. Methods in molecular biology. Humana Press, New York, pp 245–258. doi:10.1007/978-1-62703-074-8_19

Lloyd GB, McCown BH (1980) Commercially-feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Proc Int Plant Prop Soc 30:421–426

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assay with tobacco tissue cultures. Physiol Plant 15:473–497

Okon Y, Albrecht SL, Burris RH (1977) Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl Environ Microbiol 33:85–88

Perrig D, Boiero M, Masciarelli O, Penna C, Ruiz O, Cassan F, Luna M (2007) Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation. Appl Microbiol Biotechnol 75: 1143–1150. doi:10.1007/s00253-007-0909-9

Pijut PM, Beasley RR, Lawson SS, Palla KJ, Stevens ME, Wang Y (2012) *In vitro* propagation of tropical hardwood tree species-a review (2001–2011). Propag Ornam Plants 12:25–51

R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/. Accessed 10 Oct 2013

Rodríguez-Cáceres EA (1982) Improved medium for isolation of *Azospirillum* spp. Appl Environ Microbiol 44:990–991

Russo A, Carrozza GP, Vettori L, Felici C, Cinelli F, Toffanin A (2012) Plant beneficial microbes and their application in plant biotechnology. In: Agbo EC (ed) Innovations in biotechnology., pp 57–72. doi: 10.5772/31466, InTech, ISBN: 978-953-51-0096-6

SPSS Inc. (2003) SPSS for Windows, Version 12.0. Chicago, SPSS Inc.

Suarez IE, Jarma AJ, Avila M (2006) Development of an *in vitro* propagation protocol for roble (*Tabebuia rosea* Bertol DC). Temas Agrarios 11:52–62

Taiz L, Zeiger E (2010) Plant physiology, Fifth Edition, Sinauer Associates, 782 pages

Tetsumura T, Matsumoto Y, Sato M, Honsho C, Yamashita K, Komatsu H, Sugimoto Y, Kunitake H (2008) Evaluation of basal media for micropropagation of four highbush blueberry cultivars. Sci Hortic 119:72–74. doi:10.1016/j.scienta.2008.06.028

Vettori L, Russo A, Felici C, Fiaschi G, Morini S, Toffanin A (2010) Improving micropropagation: effect of *Azospirillum brasilense* Sp245 on acclimatization of rootstocks of fruit tree. J Plant Interact 5:249–259. doi:10.1080/17429145.2010.511280

Zhao D-Y, Tian Q-Y, Li L-H, Zhang W-H (2007) Nitric oxide is involved in nitrate-induced inhibition of root elongation in *Zea mays*. Ann Bot 100:497–503. doi:10.1093/aob/mcm142