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In vitro direct organogenesis in roots of *Ipomoea batatas*

Guillermo E. Delgado-Paredes[1]*, Consuelo Rojas Idrogo[1], Jorge Chanamé-Céspedes[1], Eny I. S. Floh[2] and Walter Handro[2]

[1]*Facultad de Ciencias Biológicas, Universidad Nacional Pedro Ruiz Gallo, Ciudad Universitaria, Juan XXIII N° 391, Lambayeque, Perú*
[2]*Laboratório de Biología Celular de Plantas (BIOCEL), Instituto de Ciências Biológicas, Departamento de Botânica, Universidade de São Paulo, SP 11461, Brasil*

ABSTRACT

In vitro culture is currently used to produce plant material for micropropagation, genetic manipulation and ex situ conservation of commercial species. The organogenic potential of root explants derived from cultured both apical and lateral buds of cuttings of Ipomoea batatas (sweet potato) cv. UNPRG-358 and cv. IN-180 was investigated in response to different incubation conditions such as gelled and liquid culture medium, size of explants (2.0 and 5.0 cm in length with and without apex), type of vessels, different root portions (proximal, medial and distal) and the cytokinins BAP (6-benzyladenine), ZEA (Zeatin) and 2iP (Isopentenyl adenine). The most adventitious buds developed from explants cultured in liquid media under a 16-h photoperiod, and the use of BAP and ZEA promoted the development of more adventitious buds than 2iP. Organogenesis in I. batatas occurred via a direct pathway, which was confirmed by anatomical studies. These histological analyses showed that adventitious buds originated directly from the proliferation of cortex cells, and also at the base of the lateral root formation, and that the lateral roots were regenerated directly from the proliferation of perycicle cells opposite the poles of the primary xylem.

Key words: Indirect organogenesis, *Ipomoea batatas*, Plant propagation, Root apex, Root segments

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INTRODUCTION

Ipomoea batatas (L.) Lam. belong to the family Convolvulaceae, order Solanales. The order consists of 9 families and about 5000 species, and more than four-fifths of the species belong to only two large families, the Solanaceae (2800) and Convolvulaceae (1500)[1]. In the system proposed by the Angiosperm Phylogeny Group, the family Convolvulaceae is placed with Hydroleaceae, Montiniaceae, Solanaceae and Sphenocleaceae, in the same order, Solanales, Core Euasterids I[2]. The Convolvulaceae comprise nearly 1650 predominantly tropical species, and the genus *Ipomoea*, with approximately 500-600 species, comprises the largest number of species within the Convolvulaceae[3].

Among 50 genera of this family, only *I. batatas* is a crop plant whose large, starchy, sweet tasting tuberous roots are an important root vegetable. It has remarkable pro-vitamin A qualities and an important staple source of calories and proteins, and consumed by all age groups[4]. *I. batatas* also have great potential for the production of biomass in biofuel production[5, 6], and in addition, purple-coloured sweet potatoes developed in Japan inthe1990s and several reports have indicated that the anthocyanins displayed antioxidative or radical-scavenging activity and exerted several health-promoting functions in humans[7, 8].

Sweet potato [*I. batatas* (L.) Lam.] is among the world's most important, versatile and underexploited food crops that ranks fourth among the food crops after rice, potato and wheat and seventh in the world in terms of total production[9]. After Columbus introduced the sweet potato to Spain then spread to India, Africa, Asia and Oceania[10]. According to the FAO, 115 countries produced 106.6 Million tonnes (Mt) of sweet potato in 2010,

however supply remains very concentrated 82.3% of global production being in Asia, primarily produced in China (81.2 Mt); Africa contributes up to 14% of global production with more than 14.2 Mt, and Latin America produced 1.97 Mt, i.e. a little more than 2% of global supply: Brazil holds first place on the continent, followed by Cuba and Argentina[11, 12].

Sweet potato is normally field propagated by the use of stem cuttings and scarcely by storage roots; however, under *in vitro* conditions a wide range of methods are available for micropropagation and plant regeneration, for instance, propagation by meristem and single node cuttings, propagation in liquid culture (stem segments), propagation by plantlet regeneration (direct and indirect organogenesis), somatic embryogenesis and protoplasts. In the past, several scientists have been able to successfully regenerate plants of sweet potato from cultured stems, petioles, roots and leaf discs[13], and in all cases the first step was the formation of callus at the cut surface; however, the main disadvantage of using this method as a standard propagation system is that callus-derived plantlets are likely to have undergone minor or major genetic aberrations during the callus stage[14, 15], thus, the regenerated plantlets would not be genetically the same as the original genotype[16]. It is possible that direct organogenesis, without callus formation, does not lead to somaclonal variation.

Roots have been successfully used for *in vitro* mass propagation of many plants to circumvent certain difficulties inherent to conventional propagation methods and are easy to maintain and manipulate[17]. For instance, propagation of *Swertia chirata* (Gentianaceae) through seeds is hampered by poor seed germination rate (only 2 to 4%), low seed viability and long gestation period, however a procedure for regeneration of complete plantlets via indirect organogenesis (callus induction, multiplication and adventitious shoot regeneration from callus surface), in MS medium supplemented with 13.32 µM BAP in combination with 0.92 µM 2,4-D, has been described[18]. In *Tylophora indica* (Asclepiadaceae), an importante indigenous medicinal plant found in restricted localities in the India subcontinent, *in vitro* plants regeneration using adventitious roots has been studied in MS medium with various auxins such as 2,4-D, IBA and NAA at different concentrations[19]. In *Passiflora cincinnata*, an edible species with resistance to the bacterial blight caused by bacterium *Xanthomonas campestris* f. sp. *passiflorae*, different concentrations of BA were used *in vitro* to induce buds in three types of explants: leaf discs, roots segments and the seedling itself obtained from *in vitro* seed germination, and the 0.5 mg/L^{-1} BA concentration was the most suitable for all the three explants, however, bud formation time and means (direct/indirect) were different for each type of explant[20]. Likewise, the organic potential of root explants derived from cultured seedlings of *Bixa orellana* (Bixaceae) was investigated in response to different incubation conditions and either 4.44 µM BA, 4.54 µM TDZ, or 4.56 µM ZEA[21], and in *Caesalpinia bonduc* (Fabaceae), a threateaned wood legume, an *in vitro* regeneration protocol has been standardized via direct and indirect methods from excised root explants, in MS medium supplemented with 17.75 µM BAP and 2.46 µM IBA,which induced a mean of 3.40 shoots directly from the surface of excised root explant[22]. In general, plant regeneration from cultured root explants has also been reported for other species including *Solanum melongena*[23], *Melia azederach*[24], *Cassia angustifolia*[25], and *Centaurea ultreiae*[26].

In the other hand, transgenic plants have been obtained after *Agrobacterium rhizogenes*-mediated transformation of 89 different taxa, representing more than 79 plant species, and more transformed plant taxa[27]. For instance, spontaneous shoot regeneration was obtained from the hairy roots of *Rehmannia glutionosa* in liquid woody plant medium, and roots of pRi-regenerants accumulated similar (harpagoside, isoverbascoside), higher (catalposide, aucubin, harpagide, verbascoside) or lower (catalpol, loganin) levels of the metabolites in comparison with untransformed *R. glutinosa* plants[28]; likewise, hairy root cultures of *Plumbago indica*[29] or *Tylophora indica*[30] demonstrated higher level of secondary metabolites than untransformed plants.

The objective of the present was work is to investigate the regenerative potentialities of roots explants of *I. batatas* and to develop a reliable system for mass propagation without an intervening callus phase.

MATERIALS AND METHODS

Plant materials

Two sweet potato cultivars, UNPRG-358 and IN-181 (Native Introduction), were obtained from the Sweet Potato Germplasm Bank of the Universidad Nacional Pedro Ruiz Gallo, Lambayeque (Peru) and International Potato Center (CIP), Lima (Peru), respectively. Stem cuttings (30 to 50 cm length) of three to four months old, in excellent health conditions and free of lateral buds, were placed in plastic pots containing a mixture of sand and soil at a ratio of 2:1 and grown in a greenhouse with shade screen and permanent irrigation. The stem cuttings were treated with fungicide Orthocide 80 at 0.2% for 5 minutes, and afterwards rinsed away by washing the steem in running water.

Desinfestation procedure and experimental design

Both apical and axillary buds (1-2 cm) were washed with detergent under running tap water for 30 minutes and posteriorly were immersed in 70% etanol for 60 second, followed by 0.5% sodium hypochlorite (w/v) and Tween 20 (one or two drops per 100 mL) for three minutes, and then washed 4-5 times with sterilized distilled water. Shoot tips of 0.5 to 1 mm in length were cut from the sterile explants under light microscope, placed in 15x120 mm test tubes and incubated onto MS[31] semisolid medium supplemented with 2.0% sucrose (w/v), 0.6% (w/v) agar and 0.22 mg/l BAP in combination with 0.18 mg/l NAA.

In the micropropagation step, after 90-120 days of culture, the plantlets were cut into segments of approximately 1.5 cm and placed in 18x150 mm test tubes containing 5 ml of semisolid MS medium with 3.0% sucrose, 0.6% agar and without plant growth regulators. In the direct organogenesis step, full radicular system or root segments of 2.0 and 5.0 cm in length, from the 30-and 180-days-old cultures,were aseptically excised and placed in MS medium supplemented with the auxins IAA, NAA and IBA, IAA-BAP or the cytokinins BAP, 2iP and ZEA, in various culture conditions: gelled and liquid medium, proximal, medial and distal region in roots of six cm in length, shape and size of vessels, several subcultures and with or without root apex.

In all experiments, the basal MS medium was supplemented with the vitamins myo-inositol 100 mg/l and thiamine.HCl 1.0 mg/l and 0.6% agar (w/v).

Culture conditions

The pH of all media was adjusted to 5.8±0.1, with KOH and HCl, prior to autoclaving at 121 ^{o}C at 105 kPa for 20 min. Each treatment comprised 15 to 25 explants and was performed twice. The experiments were evaluated every 30 days. The plant material was kept in a growth room with a controlled temperature of 26±2 ^{o}C, a relative air humidity of approximately 80%, photoperiod of 16 hours light and luminous intensity of 60 µmol.m^{-2}.s^{-1} via cool white fluorescent light. Other treatments were established in total darkness.

Microscope simple preparation and anatomical characterization

After 15 days of culture in regeneration medium, root segments at different developmental stages were collected and fixed in FAA-50[32] solution (Formalin-Acetic Acid-Alcohol). Fixed samples were dehydrated in a gradedbutanol series (tertiary butyl alcohol) and embedded in paraffin. Cross and longitudinal sections (thickness, 12 µm) were obtained using a manual microtome and stained using safranin and fast green. The specimens were mounted on glass slides. Photographs were taken using a light microscope.

Statistical analysis

In order to compare the treatment means, the results were processed and analyzed by ANOVA and Tukey's HSD multiple-range test ($p \leq 0.05$). All the statistical analyses were performed using the software package Statgraphics Plus®, versión 5.0 (StatPoint, Virginia, USA).

RESULTS AND DISCUSSION

Effect of IAA-BAP in gelled and liquid culture medium

Direct organogenesis of *I. batatas* plants from cultured root explants was obtained. The root explants (2.0 cm length) excised from the plantlets of *I. batatas* when cultured on MS culture medium supplemented with 0.1 mg/l IAA in combination with 2.0 mg/l BAP or MS culture medium without plant growth regulators induced 100 and 50%, respectively of adventitious buds, after 30 days of culture (Fig 1a) (Table 1); however, cultures in liquid medium did not induce adventitious buds formation. Shoot regenerates directly without the intervention of callus formation, but only in gelled medium, at a rate of one shoot per explant. Treatments with auxins IAA, IBA and NAA (0.1 and 1.0 mg/l) did not induce shoot formation (data partially shown in table). In another experiment, a full radicular system in liquid media with 2.0 mg/l BAP resulted in the highest rate of adventitious bud formation for this species with 100% in the third subculture. In this system, the percentage of adventitious buds formation increased from the first subculture (64%) to the third subculture (100%), and significantly declined in the fourth subculture (40%) (Fig 1b); in gelled medium in the full radicular system only 40% of adventitious buds was obtained (Table 4). Although culture on gelled medium provides good physical contact of the explant with nutrients and support for its development[33], culture in liquid media resulted in the highest rate of adventitious bud formation. In liquid media, intimate contact with the surrounding tissue favors the absorption of nutrients and growth regulators, and can led to better organogenic development[34]; however, the reasons for which some plant species responded better to *in vitro* growth in gelled or liquid media are poorly understood[21]. In *Bixa orellana* the highest numbers of adventitious buds (15.8 to 31.9) from root cultures segments developed using liquid cultures with a photoperiod of 16 h; however, the regenerated shoots were hyperhydric, with thick leaves, and under these culture conditions, the ZEA and TDZ treatments resulted in approximatelly twice the number of buds compared to the BA[21]. In our study the

hyperhydricity phenomenon was not observed. Certainly, the continuous agitation of the medium provides an ample supply of oxygen to the tissue, stimulating the growth of shoots[35]; however, in our study the continuous agitation was not used. In another study in *Boswellia serrata*, liquid medium or very weakly gelled medium (0.2% agar) produced more than 20 shoots (approximately two-fold higher than the control), and the maximum shoot length (2.83 cm) was also recorded on the liquid medium[36]. On the other hand, in root explants of 7-day-old broccoli (*Brassica oleraceae* var. italica) the frequency of direct somatic embryo formation was 100% when root explants were cultured in liquid medium[37].

Table 1. Effect of IAA-BAP on shoot bud formation from root (2.0 cm length) explants of *Ipomoea batatas*, cv. UNPRG-358. Data were recorded after 30 days of inoculation

Treatments (mg/l)		(Gelled medium)			(Liquid medium)		
IAA	BAP	Callus induction[a]	Shoot formation (%)	Roots formation[b]	Callus induction	Shoot formation (%)	Roots formation
0.0	0.0	-	50.0±2.6[b]	+++	-	0.0	+
0.1	0.0	+	0.0	+++	-	0.0	-
1.0	0.0	++	0.0	++	-	0.0	-
0.1	2.0	+++	100.0±3.8[a]	+++	-	0.0	-
2.0	0.1	+++	0.0	+++	-	0.0	-
0.5	0.5	++	30.0±1.8[c]	+++	-	0.0	-

[a]-, *without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explants; +++, callus covers the whole explant.*
[b]-, *without roots formed; +, 1 to 3 roots formed; ++, 4 to 6 roots formed; +++, > 6 roots formed.*
The value of each combination consisted of mean ± SD of 15 replicates
Means followed by the same letter at the same column are not statistically significant by Tukey's test (P<0.05).

Table 2. Effect of root regions (proximal, medial and distal) on shoot bud formation from the root (2.0 cm length) explants of *Ipomoea batatas*, cv. UNPRG-358, in gelled culture medium. Data were recorded after 30 days of inoculation

Treatments (mg/l)		Callus induction[a]	Shoot induction (mean ± SE) (%)			Root formation[b]
IAA	BAP		Proximal	Medial	Distal	
0.0	0.0	-	50.0 ±2.7[b]	20.0±1.2[ab]	10.0±0.3[a]	+++
0.0	2.0	++	30.0 ±1.8[c]	30.0±1.9[a]	10.0±0.6[a]	+++
0.01	2.0	+++	25.0 ±0.5[c]	0.0	0.0	+++
0.1	2.0	+++	100.0 ±0.7[a]	0.0	0.0	+++

[a]-, *without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explants; +++, callus covers the whole explant.*
[b]-, *without roots formed; +, 1 to 3 roots formed; ++, 4 to 6 roots formed; +++, > 6 roots formed.*
The value of each combination consisted of mean ± SD of 15 replicates
Means followed by the same letter at the same column are not statistically significant by Tukey's test (P<0.05).

Table 3. Effect of 0.1 mg/l IAA - 2.0 mg/l BAP on shoot bud formation from root (2.0 cm length) explants of *Ipomoea batatas*, cv. UNPRG-358, using various glass containers, in gelled culture medium. Data were recorded after 30 days of inoculation

Shape and size of vessels	Morphogenic responses		
	Callus induction[a]	Shoot formation (%)	Root formation[b]
Test tubes (25x150 mm)	-	0.0[c]	-
Glass jars (90x40 mm)	-	46.7±3.5[a]	+
Petri dishes (90 mm Ø)	-	22.0±2.8[b]	++
Erlenmeyers flasks (125 ml)	-	0.0[c]	+

[a]-, *without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explants; +++, callus covers the whole explant.*
[b]-, *without roots formed; +, 1 to 3 roots formed; ++, 4 to 6 roots formed; +++, > 6 roots formed.*
The value of each combination consisted of mean ± SD of 15 replicates
Means followed by the same letter at the same column are not statistically significant by Tukey's test (P<0.05).

Table 4. Effect of 2.0 mg/l BAP and subculture numbers on shoot bud formation from full radicular system explants of *Ipomoea batatas*, cv. UNPRG-358. Data were recorded after 30 days of inoculation

Treatments (mg/l)	Subculture numbers	Shoot induction (mean ± SE) (%)	Callus induction[a] (%)	Root formation[b]
BAP				
0.0 (Liquid medium)	-	0.0	-	+++
2.0 (Liquid medium)	First	64.0 ±2.4[bc]	-	+++
2.0 (Liquid medium)	Second	72.0 ±2.6[b]	-	+++
2.0(Liquid medium)	Third	100.0±3.5[a]	-	+++
2.0 (Liquid medium)	Fourth	40.0 ±1.9[d]	-	+++
2.0 (Gelled medium)	-	40.0 ±2.2[d]	-	+++

[a]-, *without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explants; +++, callus covers the whole explant.*
[b]-, *without roots formed; +, 1 to 3 roots formed; ++, 4 to 6 roots formed; +++, > 6 roots formed.*
The value of each combination consisted of mean ± SD of 15 replicates
Means followed by the same letter at the same column are not statistically significant by Tukey's test (P<0.05).

Table 5. Effect of several cytokinins (BAP, 2iP and ZEA) on shoot bud formation from root (2.0 and 5.0 cm length) explants of 30 days old, of *Ipomoea batatas*, cv. IN-181 and cv. UNPRG-358, in gelled medium. Data were recorded after 30 days of inoculation

Treatments (mg/l)			IN-181 (%)			UNPRG-358 (%)		
BAP	2iP	ZEA	Roots with apex (2.0 cm)	Roots without apex (2.0 cm)	Roots without apex (5.0 cm)	Roots with apex (2.0 cm)	Roots without apex (2.0 cm)	Roots without apex (5.0 cm)
A. With treatments in darkness (3 weeks)								
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0±1.3^{f}
2.0			10.0±0.9^{c}	0.0	0.0	0.0	0.0	10.0±0.7^{f}
	0.5		0.0	0.0	0.0	0.0	0.0	60.0±3.1^{b}
		2.0	10.0±1.1^{c}	0.0	0.0	0.0	0.0	50.0±2.6^{c}
B. Without treatments in darkness								
0.0	0.0	0.0	0.0	20.0±2.1^{a}	10.0±0.4b	0.0	0.0	20.0±1.3^{e}
2.0			30.0±3.3^{b}	10.0±1.4^{b}	10.0±0.9^{b}	20.0±2.2^{a}	0.0	60.0±3.5^{b}
	0.5		0.0	0.0 c	0.0	0.0	30.0±^{a}	70.0 ±3.8^{a}
		2.0	60.0±3.8^{a}	20.0±2.2^{a}	100.0±4.5^{a}	0.0	0.0	30.0 ±1.8^{d}

The value of each combination consisted of mean ± SD of 25 replicates
Means followed by the same letter at the same column are not statistically significant by Tukey's test (P<0.05).

Table 6. Effect of several cytokinins (BAP, 2iP and ZEA) on shoot bud formation from root (2.0 and 5.0 cm length) explants of 180 days old, of *Ipomoea batatas*, cv. IN-181 and cv. UNPRG-358, in gelled medium. Data were recorded after 30 days of inoculation

Treatments (mg/l)			IN-181 (%)			UNPRG-358 (%)		
			Percent shoot induction (mean ± SE)			Percent shoot induction (mean ± SE)		
BAP	2iP	ZEA	Roots with apex (2.0 cm)	Roots without apex (2.0 cm)	Roots without apex (5.0 cm)	Roots with apex (2.0 cm)	Roots without apex (2.0 cm)	Roots without apex (5.0 cm)
A. With treatments in darkness (3 weeks)								
0.0	0.0	0.0	0.0	0.0	0.0	10.0±0.3^{b}	10.0±0.6^{d}	0.0
2.0			0.0	10.0±0.6^{a}	10.0±1.7^{a}	30.0±2.7^{a}	10.0±0.8^{d}	0.0
	0.5		10.0±0.7^{a}	10.0±1.1^{a}	0.0	0.0	0.0	20.0±1.9^{b}
		2.0	0.0	0.0	0.0	10.0±1.5^{b}	70.0±4.3^{a}	0.0
B. Without treatments in darkness								
0.0			0.0	10.0±1.4^{a}	0.0	0.0	10.0±1.3^{e}	10.0±0.5^{c}
2.0			0.0	0.0	10.0±1.5^{a}	30.0±2.2^{a}	10.0±1.7^{e}	20.0±2.8^{b}
	0.5		0.0	0.0	0.0	0.0	20.0±3.4^{c}	10.0±1.4^{c}
		2.0	0.0	10.0±0.9^{a}	0.0	0.0	30.0±2.8^{b}	80.0±3.9^{a}

The value of each combination consisted of mean ± SD of 25 replicates
Means followed by the same letter at the same column are not statistically significant by Tukey's test (P<0.05).

Effect of the root portions

The root explants (2.0 cm length) excised from the plantlets of *I. batatas* when cultured on MS gelled culture medium supplemented with 0.1 mg/l IAA in combination with 2.0 mg/l BAP induced 100% of adventitious buds, after 30 days of culture, only in the proximal portion (nearest to the stem); however, on MS culture medium without hormones or supplemented with 2.0 mg/l BAP, various percentages (10 to 50%) of shoots induction in all root portions tested (proximal, medial and distal) were observed (Table 2). It is possible to deduce that the greatest adventitious buds formation in the proximal portion is due to the increased accumulation of endogenous plant growth regulators, particularly cytokinins and the greatest maturation of tissues, especially the perycicle and endodermis.

On callus induction and direct shoot regeneration of lettuce (*Lactuca sativa*) three regions (medial, near to petiole and petiole) on cotyledon explants were tested, and the region near of the petiole of cotyledon explants was the best for direct shoot regeneration, showing a high significant response to growth regulators[38]. In Paradise tree (*Melia azedarach*) three different portions (proximal portion, distal portion and rachis of the leaflets) of three developmental stages (folded, young still expanding and completely expanded) of leaves of 10 -15 years old plants were culturedon MS medium supplemented with several plant hormones, and the rachis of the leaflets of the completely expanded leaves was found to be the most responsive tissue[24]. In the regeneration potential of excised aspen (*Populus tremula*) roots cultivated in liquid medium, the position of the isolated roots sections on the main root affected the regeneration, the proximal section further away from the root tip producing the highest number of buds per explant in both BA and TDZ treatments[17].

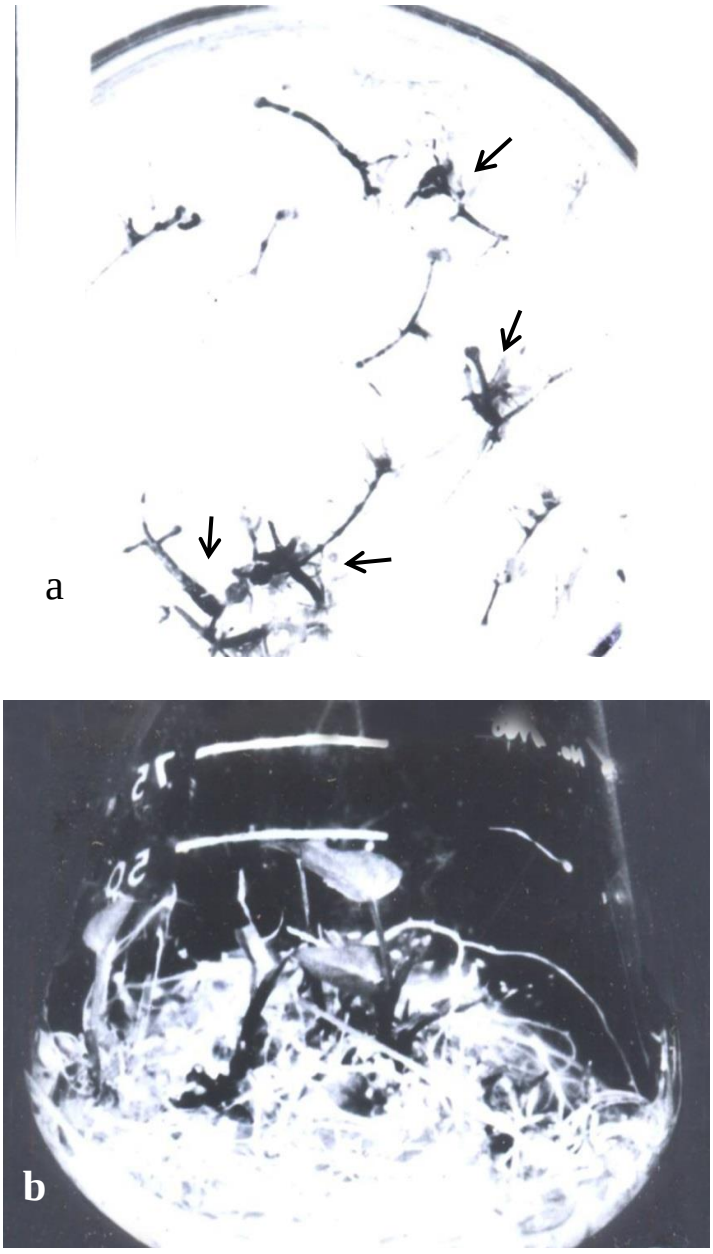

Figure 1. *In vitro* organogenesis of *I. batatas*. a. Adventitious buds formation obtained after culture of isolated root explants (2.0 cm length) in gelled medium for 30 days with a 16-h photoperiod and b. Adventitious buds formation obtained after culture of full radicular system in liquid medium for 30 days with a 16-h photoperiod

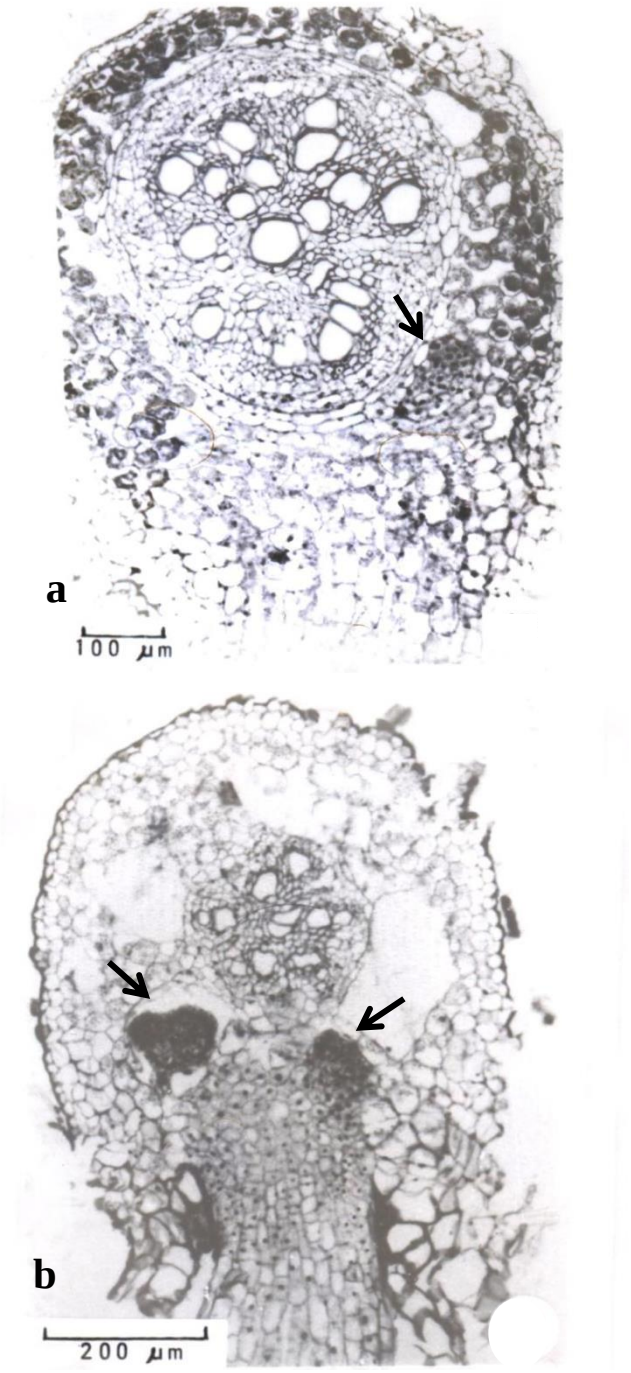

Figure 2. Cross-sections of direct organogenesis from root explants of *I. batatas*. a. Root segment in the initial stage of differentiation. Note the formation and early development of meristems (arrow) and b. Development of two meristem and the cortex cells and lateral root in the pericycle cells opposite the xylem

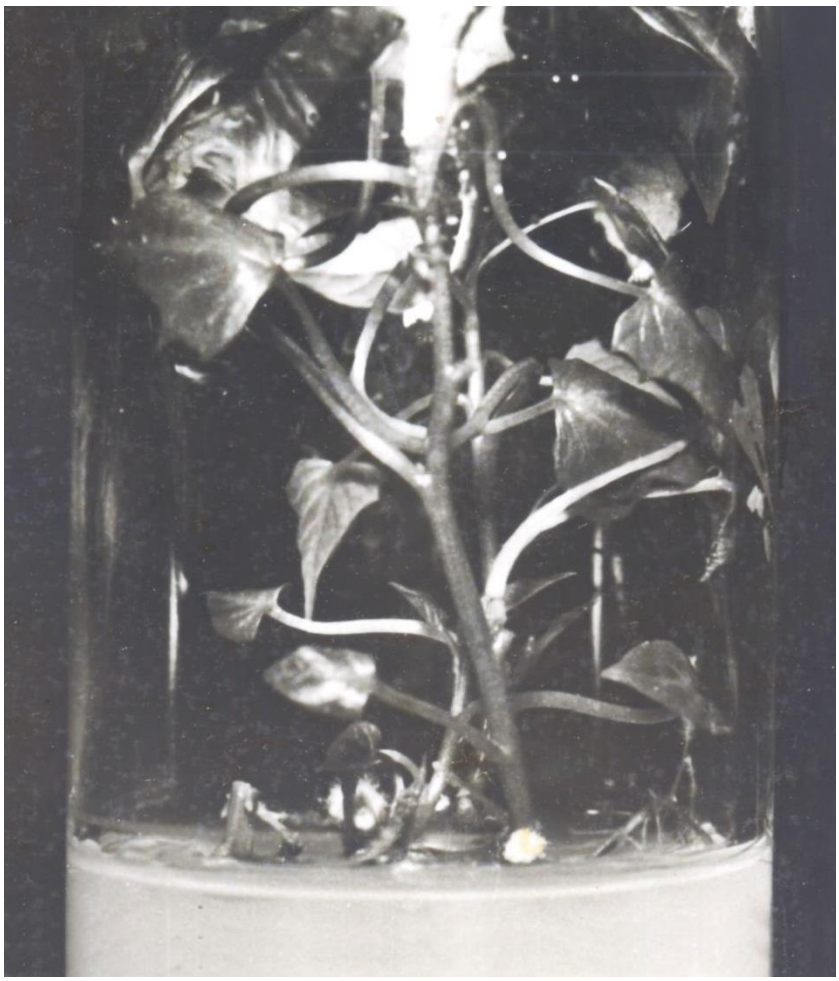

Figure 3. Plant propagation of cv. UNPRG-358 obtained after culture of root explants in gelled medium for 45 days with 16-h photoperiod (MS + sacarose 4%).

Effect of culture vessels

The root explants (2.0 cm length) excised from the plantlets of *I. batatas* when cultured on MS culture medium supplemented with 0.1 mg/l IAA in combination with 2.0 mg/l BAP induced 46.7 and 22.0% of adventitious buds, after 30 days of culture, when glass jars of 90x40 mm, and Petri dishes of 90 mm Ø, respectively, were used. In all other tested containers, test tubes (18x150 mm) and Erlenmeyers flasks, shoot formation not was induced (Table 3).

In plant tissue culture the physical properties of the vessels and caps or closures affected the growth microenvironment of plantlets by the interface between inside and outside environments, and the most important specifications for vessels are to provide uniform and adequate light quality, to isolate contaminating microorganisms and to allow gas exchange[39]. In the germplasm maintenance of four mint (*Mentha* spp.) accesions, cultured in four different culture vessels, the highest weight loss from the media, evapo-transpiration and fresh weight gain were recorded in industrial glass jar and magenta vessel, and the lowest weight loss from the media and fresh weight gain both were found in culture tubes[40].

Effect of subculture numbers

The root explants (full radicular system) excised from the plantlets of *I. batatas* when cultured on MS culture medium supplemented with 2.0 mg/l BAP induced 72.0 and 100.0%, respectively, of adventitious buds, after 30

days of culture, in the second and third subculture, delayed significantly in the fourth subculture with 40%. In the gelled medium the formation of adventitious shoots was 40% (Table 4).

A decrease in multiplication potential during long-term growth and repeated subculturing of shoots on medium of constant hormonal composition was reported in several species as six ornamental species and cultivars of Rosaceae[41], pineapple[42], and several shoots of contemporary fruit rootstocks[43].

Effect of cytokinins, size and age of explant with and without root apex

The root explants (2.0 cm length with and without root apex and 5.0 cm length without root apex) excised from the plantlets of *I. batatas* of 30 days old, when cultured on MS culture medium supplemented with cytokinins BAP, 2iP or ZEA, the highest percentages of buds were induced in roots of 5.0 cm in length without root apex under continuous irradiance. In these roots, the UNPRG-358 genotype showed a better response than the IN-181 genotype; however, in roots of 2.0 cm in length, with or without root apex but always in conditions of continuous irradiance, the IN-181 genotype showed a better response (Table 5). In the other hand, in the root explants (2.0 cm length with and without root apex and 5.0 cm length without root apex) excised from the plantlets of *I. batatas* of 180 days old, when cultured on MS culture medium supplemented with cytokinins BAP, 2iP or ZEA, the highest percentages of buds were induced in roots of 2.0 and 5.0 cm in length without radicular apex and under continuous irradiance. In both cases, the UNPRG-genotype showed a better response than the IN-181 genotype (Table 6). In both genotypes, UNPRG-358 and IN-181, it was demonstrated that cytokinin supplementation was essential for the differentiation of adventitious buds, since molecular signalling from cytokinin is required during the maintenance and differentiation of shoot and root apical meristems and the formation of vascular tissues[44].

Organogenesis *de novo* in tissue cultures has provided useful systems for studying regulatory mechanism of plant development[45]. The physiological analysis on direct and indirect organogenesis *in vitro* was the identification of a predominant role of auxin and cytokinin as chemical determinants in plant development[46]. Mutants impaired in different stages of organogenesis *in vitro* are also useful for dissecting this phenomenon. In this sense, the overall results of phenotypic analysis on various organogenic responses of the temperature-sensitive mutants (*srd1*, *srd2*, and *srd3* mutants) allowed outlines of organogenesis of *Arabidopsis in vitro* to be drawn as follows[47]: IC, incompetent with respect to organogenesis and cell proliferation; CR, competent with respect to root organogenesis and cell proliferation; CSR, competent with respect to shoot and root organogenesis[47, 48].

The induction of adventitious buds in *I. batatas* was directly influenced by the type of cytokinin present in the induction medium, possibly due to variations in the differential translocation rates[49]. ZEA and BAP promoted the development of more buds than did 2iP, and ZEA was more effective than BAP in the formation and development of morphologically normal shoots. In *Bixa orellana*, this ability of ZEA to induce bud formation was previously reported in rooted hypocotyls, where these of 4.56 µM ZEA resulted in 3 to 4 buds per explant, via direct organogenesis[50]; however, in this same species, the root explants in the concentration of 4.56 µM ZEA resulted in 31.9 buds per explant, also via direct organogenesis, although with 4.44 µM BA the frequency of adventitious buds formation was 85%[21]. Therefore, as well as in *B. orellana*, in our study with *I. batatas* ZEA and BAP proved to be the best cytokininsfor differentiation and development from roots, as it resulted in the highest percentage of morphologically normal, regenerated plants. On the other hand, from the comparison between genotypes of sweet potato, the IN-181 genotype, root segments of 2.0 and 5.0 cm in length without root apex, and a pre-treatment with light for three weeks, showed a greater totipotency than the UNPRG-358 genotype, root segments of 2.0 cm in lenght with root apex and a pre-treatment with darkness, respectively. Other studies have highlighted the role of cytokinins on adventitious buds formation in root explants, for instance, in *Centaurea ultreiae*, a critically endangered species, the best response, 94.3% of root explants producing a mean of 5.6 viable shoots per explants, was observed when explants were incubated on a medium containing 0.55 µM BA, and histological studies have revealed that adventitious shoots were derived from pericycle cells of root explants and parenchymatic cells of callus tissues[26]. In *Cassia angustifolia*, root explants taken from 30-day-old aseptic seedlings were cultured on MS medium supplemented with different cytokinins and the maximum shoot regeneration frequency (90%) was obtained with 2.5 µM BA; the histological sections at different developmental stages of shoot buds revealed the organization of the nodular meristematic zone leading to the orientation and differentiation of shoot buds in large number and thereafter, their conversion into healty shoots[25]. Likewise, eggplant (*Solanum melongena*) was efficiently regenerated from cultured roots of 15-d-old seedlings on MS medium containing 0.45 µM TDZ and 13.3 µM BA[23]. In *Doritaenopsis* the effect of the cytokinins TDZ, BA or ZEA on protocorm-like body (PLB) induction from root tips grown *in vitro* was studied, and TDZ was found to be a more effective cytokinin in the induction of PLBs than BA or ZEA[51]. In the potential regeneration of excised aspen (*Populus tremula*) roots cultivated in liquid medium, TDZ had a marked effect on bud development as compared with BA, inducing a tenfold increase in the number of buds regenerated from various root explants[17]. On the other hand, the explant

age had an important influence on shoot regeneration; however, in regeneration of lettuce (*Lactuca sativa*) the cotyledon explants from 3 and 7-day-old *in vitro*, did not have a significant effect[38].

Histological analysis

Histological studies are essential to confirm direct organogenesis. In *I. batatas* cv. UNPRG-358, the histological analysis showed that shoot buds regenerated directly from the proliferation of cortex cells, near the endodermis, and also at the base of the lateral root formation (Fig 2a), and that the lateral roots were regenerated directly from the proliferation of perycicle cells opposite the poles of the primary xylem (Fig 2b). Similarly to that observed in the histological analysis of *Bixa orellana* initially, pericycle cells divided mostly periclinally, and then in different planes, yielding a group of cells with meristematic features[21]. The meristematic cells were small, compact, with primary cell wall, numerousplasmodemata, and had dense cytoplasm and reduced volumen compared to adjacent, vacuolated cells. In another study in root explants of 7-day-old broccoli (*Brassica oleraceae* var. italica), the histological analysis indicated that somatic embryos were initiated directly from the pericycle cell layers of root explants as early as 1 day after liquid culture[37]. In *Centaurea ultreiae*, a critically endangered species, histological studies revealed that adventitious shoots were derived from pericycle cells of root explants and parenchymatic cells of callus tissues[26]. Likewise, in *Cassia angustifolia*, the histological sections at different developmental stages of shoot buds revealed the organization of the nodular meristematic zone leading to the orientation and differentiation of shoot buds in large number and thereafter their conversion into healty shoots[25], and in *Populus tremula* buds regenerated in close proximity to the site of lateral roots in BA-treated roots, while in TDZ-treated root sections, the buds formed all over the root regardless of the presence of lateral roots; the buds developed from inner cortical and subepidermal cell layers, disrupting the epidermis and the inner layers[17].

CONCLUSION

This study developed an efficient and reproducible regeneration protocol via direct organogenesis from the root explants of *I. batatas*. The explants can be easily obtained from established shoot cultures and do not require disinfection treatment. This protocol will therefore serve as an alternative method for *in vitro* clonal propagation and further biochemical and physiological investigations.

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