Formación in vitro de rizomas en caña flecha (*Gynerium sagitatum* Aubl.) y recuperación de plantas

Arrow cane (*Gynerium sagitatum* Aubl.) in vitro rhizome formation and plantlet recovery

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RESUMEN

La caña flecha *Gynerium sagitatum* Aubl. (Poaceae) es una especie de gran importancia ambiental, cultural y económica para ciertas comunidades indígenas del Norte de Colombia, empleando su fibra en la fabricación apreciables artesanías. La cosecha de la planta y la no reposición de esta, viene contribuyendo en la disminución de poblaciones naturales de la esta especie. La micropropagación ha emergido como una de las la única posibilidades de producir de forma eficiente material vegetal para el establecimiento de cultivos y restaurar zonas afectadas. Con el fin de mejorar la eficiencia económica del protocolo de micropropagación, estructuras del tipo rizomas fueron inducidas in vitro a partir de explantes consistentes de plantas stablecidas in vitro bajo tres niveles de sacarosa, cuatro de benzilaminopurina – BAP y cuatro de ácido abscísico – ABA en MS con (en mg L^{-1}) myo-inositol (100), tiamine HCL (0,4) y solidificados con Phytagel® (3.000). Los tratamientos (48) fueron distribuidos con un diseño completamente al azar con seis repeticiones por tratamiento. Los cultivos fueron mantenidos durante ocho semanas a 25 °C con 12 h de fotoperíodo (40 µmol fotones m^{-2} s^{-1}) suministrada con lámparas de luz fría fluorescente. Diferencias estadísticas se observaron con respecto al número de rizomas formados y longitud de los rizomas como efecto de la interacción de los tres factores. La recuperación de las plantas ex vitro ocurrió en mayor número (6,0) cuando los rizomas se desarrollaron en medios suplidos con 263000 µM de sacarosa combinado con 4,44 y 8,88 µM de Benzilaminopurina – BAP. Los resultados evidencian la posibilidad de inducir in vitro rizomes de caña flecha para ser utilizados en conservación y propagación de esta especie.

Palabras clave: Caña flecha, micropropagación, in vitro, formación de rizomas, RCV.

ABSTRACT

Gynerium sagitatum Aubl. (Poaceae) is an important environmental, cultural and economic species for aboriginal communities in Northern Colombia, in which the centrale central nerve is used to make precious craftsmanship products. The massive use of the plant with no restoration has dangerously decreased natural populations. Micropropagation has emerged as a way to efficiently produce planting material for cropping and restoration of affected areas. To improve cost efficiency of the micropropagation protocol, in vitro rhizome structures were induced using in vitro-maintained plants as explants under three quantities of sucrose, four of benzyl aminopurine - BAP and four of abscisic acid - ABA supplied in MS with (in mg L^{-1}) myo-inositol (100), thiamine HCL (0.4), and solidified with Phytagel® (3.000). Treatments (48) were distributed using a complete randomized design with six replicates per treatment. Cultures were stored during eight weeks at 25 °C with 12 h photoperiod (40 µmol photons m^{-2} s^{-1}) provided by with cold fluorescent lamps. Statistical differences were observed with respect to number of rhizomes and rhizome length as a result of three factor interaction. Ex vitro plantlet recovery occurred at highest percentage from rhizomes developed in media supplied with sucrose at 263000 µM combined with 4.44 and 8.88 µM BAP. Data evidenced the possibility of inducing in vitro rhizome growth from arrow cane explants to use them as a way for propagation and plant conservation.

Key words: Arrow cane, micropropagation, in vitro, rhizome formation, PGR.

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INTRODUCCIÓN

Habitat is always associated with low wetlands and water strain where favors shore protection and reduce erosion habitat is always associated with low wetlands and water strain where favors shore protection and reduce erosion. Plants are used by Indians communities for house construction, arrows to haunt and fish, animal feeding from leaf tissue and as medicinal products; flavonoids and isoflavonoids such as (2R,3R)-2,3-trans-7,4′-dimethoxydihydroflavonol,(2R,3S,4S)-2,3-trans-3,4-cis-7,4′-dimethoxy-3,4flavandiol,6-hydroxy-7,4′-dimethoxyflavone,6,7,4′-trimethoxyflavone,ferreirin,dihydrocajanin, dalbergioidin, dihydrobiochanin A and biochanin has been isolated from different organs of the plant (Benavides et al. 2007). Studies reported the use of G. sagitatum plants as a mechanism to detoxify soils contaminated with Hg, Pb, Cr and Cd via phytoremediation of landfill leachate (Ortega-Ortega et al. 2011; Madera-Parra et al. 2015 a,b).

G. sagitatum plants are canes with up to 10 m length stems and 2-8 cm diameter. Leaves are linear cauline, leaf blades up to 2 m length and 8 cm wide with scabrous margins. Inflorescence is a 1-1.5 m long panicle with 3 mm dioecious flowers in spikelet and 1 mm long brown seeds. Stems grow from rhizomes that develop horizontally up to 10 m from the main stem; a single stem can hold up to 200 leaves in a life cycle that ends when flowers emerge and seed. Propagation occurs by sexual and clonal way; however, under local conditions seeds are highly inviable; therefore, clonal propagation by rhizome originated shoots is the main propagation method in natural conditions; rhizomes grow developing an underground stem net that work as propagation system into new territories (Aramendiz et al. 2005; Clayton et al. 2015). In Colombia, central nerve of G. sagitatum leaves are the main fiber source for traditional Colombian craftsmanship made by Zenu communities in the North Caribbean planes since pre Columbian times, perpetuating legacy and cultural aboriginal traditions.

Production of large quantities of clonal planting material had been a main constrain for commercial cultivation of arrow cane plants; fiber extraction from natural population is endangering the species and sustainability of the craftsmanship activity along with negative impacts on the environment (Aramendiz et al. 2005). Micropropagation is a clonal propagation method used to produce large quantities of disease free planting material in short periods of time (Pati et al. 2006; Kumar et al. 2015). A micropropagation protocol to clonally propagate G. sagitatum using explants with pre-existing meristem have been developed and is currently used for massive plant production (Suárez et al. 2009; Pastrana y Suárez 2009). Lately, a double phase medium strategy has been implemented as a way to increase cost efficiency and plant quality of G. sagitatum micropropagated plants (López 2013). In vitro developing of modified stems such as tubers and rhizomes has been reported as a way for plant micropropagation and germplasm conservation in several plant species (Rayirath et al. 2011; Olivier et al. 2012; Muñiz-García et al. 2014; Badr et al. 2015). Hormone products such as ABA, BAP and increased sugar supply in the medium have been reported as elicitors for in vitro tuber and rhizome formation (Lema-Ruminska 2013; Ncube et al. 2014; Wang et al. 2015). G. sagitatum rhizome formation occurs naturally in the field; however, the in vitro induction and development of rhizome structures as a way for plant micropropagation and plantlet recovery is unknown. In the present research, combined ABA, BAP and sugar treatments on in vitro G. sagitatum rhizome formation and plantlet recovery were evaluated.

MATERIALS AND METHODS

Plant material was obtained from in vitro cultured arrow cane plants cv "Criolla". Plants were subcultured every four weeks, for more than a year, into fresh semisolid multiplication medium consisting of MS (Murashige y Skoog 1962) supplied with (in mg L^{-1}) myo-inositol (100), sucrose (30.000), thiamine HCL (0.4), benzilaminopurine (BAP) (0.5) and solidified with Phytagel® (3.000) (Sigma Co.). Culture conditions were 25 °C with 12 h photoperiod (40 µmol photons $m^{-2}s^{-1}$) provided by with cold fluorescent lamps.

The pH of all media was adjusted to 5.7-5.8 prior to addition of gelling agent. Aliquots of 30 ml were dispensed in 250 ml glass flaks and covered with heavy duty aluminum foil. Media were sterilized in an Sterilof® autoclave (Model 90/1PGRgPr) at 120 °C and 1.2 PSI during 15 min.

Three stem clusters obtained from in vitro maintained plants were established in semisolid MS with in (mg L^{-1}) myo-inositol (100), thiamine HCL (0.4) and solidified with Phytagel® (3.000) (Sigma Co.), and additionally supplied independently with three levels of sucrose (87000, 175000 y 263000 µM) combined with five levels of abscisic acid (ABA) (0.0; 1.89; 3.78 y 7.77 µM) and five level of BAP (0.0; 2.22; 4.44 y 8.88 µM). Cultures were established into 250 ml glass flasks with 30 ml of medium. Flasks were covered with two-layers of heavy duty aluminum foil, sealed with Nescofilm® and maintained at 25 °C with 12 hour photoperiod (40 µmol photons m^{-2} s^{-1}) provided by withe cold fluorescent lamps. After eight weeks, every culture was taken from the flask inside the laminar flow hood and dissected using a SZX7 Olympus® microscope to register the number of developed structures, the type of structure developed and length of each structure.

A three-factor (sucrose, ABA and BAP) experiment was designed with 48 treatments and 10 replicates per treatment for a total of 480 experimental units. Samples were distributed with a complete randomized design based on the model $Y_{jijkl} = \mu + \tau_i + \beta_j + \gamma_k + (\tau\beta)_{ij} + (\tau\gamma)_{ik} + (\beta\gamma)_{jk} + (\tau\beta\gamma)_{ijk} + \varepsilon_{ijkl}$; were µ was the mean, τβγ were the levels of sucrose, BAP and ABA and ε was the experimental error. Data were analyzed with an ANOVA ($\alpha = 0.05$).

Vegetative structures grown from explant from the different treatments were transferred into 72-plug containers filled with peat as substrate. The containers were placed in a shade house with 20% light penetration and sprinkle irrigation with a frequency of three-2 minutes irrigation a day. Four weeks after transferring to ex vitro conditions, the number of plants was recorded and percentage of plantlet recovery calculated.

RESULTS AND DISCUSSION

Normal plants consisting of 2-3 stem clusters from a single growth point developed from explants cultured in medium used as control treatment (Figure 1a). In contrast, differentiated rhizomes were observed growing from explants cultured in treatments supplied with high sucrose content (263000 µM) (Figure 1b) while no rhizome structures were observed growing from explants cultured, particularly but instead leafy-like growth was observed in treatments supplied with lower levels of sugar (87 mM) and BAP (0.0 and 2.22 µm) (Figure 1c).

A rhizome is a specialized storage plant stem in which the main axis grows horizontally at or just below the ground surface. The structure has nodes and internodes with a leaf-like sheath attached at each node, adventitious roots and lateral shoots grow next to the node, and upright - growing shoots emerge either from the terminal tip or lateral nodes allowing the parent plant to clonally propagate (Li et al. 2014). In the present study, structures

emerging from the explants cultured in media with several sucrose/BAP/ABA combinations were consistent with rhizome characteristics showing lateral growth and emerging shoots at different points although no root formation was observed (Figure 1b). This response is consistent with the presence in the medium of BAP and ABA and their effects, specifically for BAP, on lateral shoot growth promotion from axillary meristems and disruption of adventitious lateral root development, by accumulation, from the medium supply, of high levels of citokinins in cells of the root meristem such as lateral root cap, columnella initials and quiescent center cells resulting in an antagonistic interaction with endogenous auxin repressing its effects (Chan et al. 2013; Antoniadi et al. 2015; Bielach et al. 2012 a,b).

The ANOVA allowed to detect that sucrose (Pr= <0.0001), BAP (Pr= <0.0001), ABA (Pr= <0.0001) levels, and the interaction sucrose/BAP/ABA (Pr= <0.0011) significantly affected the number of rhizome structures formed from explants cultured in the applied treatments (Table 1). The collected data shows that the highest number of rhizomes developed when explants were cultured in media supplied with 263000 µM sucrose combined with 2.22 or 4.44 µM BAP and 0 or 1.89 µM ABA. Complete absence of ABA and BAP, or combination of the highest amount of both PGRs resulted in no rhizome formation. Explants cultured in medium supplied with BAP at 2.22 µM formed structures regardless of the amount of sucrose or ABA (Table 1).

Rhizomes in nature are modified underground stems used by plants to store starches and proteins, and allow herbaceous perennial plants to survive seasonally under adverse environmental conditions. Development of storage organs such as tubers has been extensively studied in species like Solanum tuberosum reporting that photoperiod, phytochrome regulation, flowering and starch accumulation genes, GA and ABA hormones, and several transcriptions factors are involved in tuber formation. Instead, rhizome is a complex poorly understood developmental process that depends on expression of genes controlled environmentally and endogenous factors (Cheng et al. 2013; Fischer et al. 2008; Abelenda et al. 2011; Tjaden et al. 1998; Kuipers et al. 1994). Recent studies have reported that like for tuber formation genes involved in photoperiod pathway (PHYB, CO, GI and FT), starch biosynthesis (SUS, UGPase, GBSS and SSS) and hormone signal transduction (GA, ABA, CTK, auxin, ethylene and JA) are related to rhizome formation and development (Yang et al. 2015). Additionally, studies on Sorghum spp and Miscanthus spp have identified rhizome enriched genes, supporting the gene-controlled developmental process of rhizome development (Jang et al. 2009; Kim et al 2014). On the other hand, sucrose has been observed to increase storage organ formation and size

Table 1. Mean number of in vitro *Gynerium sagitatum* Aubl. rhizome structures developed in media supplied with different sucrose, Benzil Aminopurine (BAP) and Abscisic Acid (ABA) amounts.

		Sucrose (87000 µM)				Sucrose (165000 µM)				Sucrose (263000 µM)			
		BAP (µM)				BAP (µM)				BAP (µM)			
		0.00	**2.20**	**4.44**	**8.88**	**0.00**	**2.20**	**4.44**	**8.88**	**0.00**	**2.20**	**4.44**	**8.88**
	0.00	0.00	2.17	0.00	0.17	0.00	1.00	2.00	4.33	0.00	6.00	4.83	2.50
ABA	1.89	0.00	2.33	3.30	0.00	0.00	1.17	2.50	3.17	0.00	4.00	4.67	3.67
(µM)	3.78	0.00	0.17	0.00	0.00	0.00	2.17	1.00	0.00	0.50	1.67	3.67	0.00
	7.57	0.00	1.33	0.00	0.00	1.00	2.67	0.33	0.00	1.00	2.50	0.50	0.00

Effects (α = 0.05): Sucrose Pr< 0.0001, BAP Pr< 0.0001, ABA Pr< 0.0001, sucrose x BAP x ABA Pr< 0.0001.

in several species. In vitro formed Curcuma longa rhizomes were more numerous and accumulated a higher dry-mass when cultured in medium with increased sucrose supply (El-Hawaz et al. 2015). In vitro cultures of *Dioscorea rotunada* and D. cayenensis developed more tubers when cultured in medium with 233000 µM sucrose compared to those cultured with 87000 µM sucrose (Olivier et al. 2012; Dai et al. 2014). In the present research, the presence of BAP showed to be necessary for rhizome formation and increasing the sucrose level in the medium resulted in a higher frequency of rhizome formation; these results are consistent with previous in vitro rhizome formation reports.

The results of the ANOVA evidenced that sucrose, BAP, ABA and the interaction Sucrose/BAP/ABA statistically affected the size of rhizomes emerging from the explants (Table 2). Explants cultured in medium supplied with 263000 µM sucrose and 4.44 µM BAP induced the longest structures, followed by those formed from explants cultured in 175000 µM sucrose, 2.22 µM BAP and 7.57 µM ABA. The smallest structures occurred when explants were cultured in 87000 µM sucrose combined with 2.22 µM BAP and 3.78 µM ABA.

Starch accumulation has been associated with storage organ enlargement and swelling. In potato, tuber growth was found to be in coordination with accumulation of starch and in lotus rhizome enlargement is highly associated with starch biosynthesis (Abelenda et al. 2011). Starch synthesis initiates with sucrose conversion to fructose and DUPG by the gene SUS; the expression of this gene has been correlated with both potato tuber and lotus rhizome growth (Ogawa et al. 2009). Additionally, AGPase catalyzes the formation of ADP-glucose from G1P in plastids (chromoplasts, chloroplasts and amylopasts) of all starch-synthesizing tissues (Tetlow et al. 2004); lotus AGPases genes NNU_07115 and NNU_25036 were found associated with rhizome growth (Yang et al. 2015). In addition to carbohydrate accumulation, phytohormones such as GA, BAP, ABA, JA and ethylene have coordination with accumulation of starch been reported to play an important role in storage organs formation (Fernie And Willmitzer 2001). ABA has been found to promote tuber formation and reduce stolon length (Guo et al. 2010; Muñiz-Garcia et al. 2014); however, deficient ABA mutants showed ability to form tubers, indicating that ABA is not essential for tuber formation. Instead, ABA seems to play a role as facilitator of other hormone functions and sugar accumulation (Rook et al. 2006; Sharma et al. 2004; Hu et al. 2012). Recently, auxin, ethylene, cytokinins and jasmonic acid related genes were found associated with rhizome formation and enlargement (Yang et al. 2015). In the present research, the collected data showed that increasing sucrose resulted in longer *G. sagitatum* rhizomes.

Table 2. Length mean (mm) of in vitro *Gynerium sagitatum* Aubl. rhizome structuresdeveloped in media supplied with different sucrose, Benzil Aminopurine (BAP) and Abscisic Acid (ABA) amounts.

		Sucrose (87000 µM)				Sucrose (165000 µM)				Sucrose (263000 µM)			
		BAP (µM)				BAP (µM)				BAP (µM)			
		0.00	**2.20**	**4.44**	**8.88**	**0.00**	**2.20**	**4.44**	**8.88**	**0.00**	**2.20**	**4.44**	**8.88**
ABA (µM)	0.00	0.00	4.78	0.00	0.52	0.00	2.67	3.28	3.15	0.00	3.90	6.48	1.45
	1.89	0.00	4.45	2.38	0.00	0.00	2.82	4.13	1.90	0.00	2.98	3.31	3.58
	3.78	0.00	0.36	0.00	0.00	0.00	3.70	2.75	0.00	0.50	2.40	5.33	0.00
	7.57	1.00	3.02	0.00	0.00	1.00	5.38	0.40	0.00	1.00	4.25	0.70	0.00

Effects (α = 0.05): Sucrose Pr< 0.0001, BAP Pr< 0.0001, ABA Pr< 0.0001, sucrose x BAP x ABA Pr< 0.0001.

Plants developed only from in vitro grown rhizomes in media supplied with 263000 µM sucrose with 4.4 µM BAP at all ABA levels and with 8.8 µM BAP at 0 and 1.89 µM ABA. Only rhizomes cultured in 4.44 µM BAP were able to regenerate plants at 165000 µM sucrose. Structures formed from explants cultured in medium supplied with 263000 µM sucrose combined with 4.44 µM BAP allowed the highest number of plants recovered; while those cultured in medium with 165000 µM sucrose combined with 4.44 µM BAP and 1.89 µM ABA resulted in the lowest number of plantlet recovered. The collected data showed that increased sucrose levels in the medium resulted in rhizomes with increased capacity for plant recovery (Table 3).

Table 3. Percentage of plantlet recovered from in vitro-developed rhizomes of *G. sagitatum.*

	Sucrose (165000 µM) Benzil Aminopurine (µM)				Sucrose (263000 µM) Benzil Aminopurine (µM)			
	0.00	**2.20**	**4.44**	**8.88**	**0.00**	**2.20**	**4.44**	**8.88**
	0.00	*	*	*	*	*	38.00	33.00
ABA	1.89	*	*	13.00	*	*	*	21.00	36.00
(µM)	3.78	*	*	*	*	*	*	18.00	*
	7.57	*	*	*	*	*	*	30.00	*

*No rhizome transferred to ex vitro conditions.

In vitro propagated plants and organs grown under low light radiation (>60 µmol m^{-1} s^{-1}) are unable to activate photosynthesis, and therefore photoautotrophic nutrition is based on sugar supplied on the medium with starch accumulation occurring basically in plastids (Shin et al. 2009). Chloroplasts from in vitro grown tissues are underdeveloped and electron flow in the thylakoid membrane does not occur becoming incapable to convert light energy into chemical energy; additionally, stomatal conductance are low when plants are exposed to low light intensity (Yamori et al. 2010; Yamori et al. 2015; Huang et al. 2015). Since plants and organ are not photosynthetically active, organs transferred ex vitro conditions must have plenty of energy reserved to support new structures formation (leaves and roots) (Mollo et al. 2011; Badr et al. 2015). Because larger organs have more stored reserves (starch) they have more opportunity to successfully support growth of new emerging plants when transferred to ex vitro conditions.

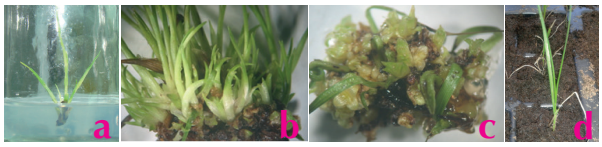

Figure 1. From left to right: a: Plants developed in control treatment, b: rhizomes from high sucrose medium, c: leafy tissues from explants cultured in low BAP and sucrose supplied medium, and d: recovered acclimatized plant.

CONCLUSSIONS

Rhizome structures from in vitro cultivated *Gynerium sagitatum* Aubl. explants is viable and potentially used for plant propagation and in vitro conservation.

Rhizome production from in vitro cultured *Gynerium sagitatum* Aubl. plants is statistically affected by sucrose/BAP/ABA interaction in the medium.

Combined supply of 26300 M sucrose with 4.44 BAP in the culture medium resulted in most numerous larger rhizomes and higher survival rates for ex vitro acclimatized *Gynerium sagitatum* Aubl. plants.

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