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### ABSTRACT

*Arrowcane (Gynerium sagittatum Aubl.) (Poaceae) is the fiber source for Colombian most famous handicrafts; however, there are no commercial crops and natural population have decreased jeopardizing artisan activity and associated ecosystems. To provide a*

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mechanism for plant material supply for planting, micropropagation of three arrowcane cultivars was evaluated. Explant from "Criolla", "Martinera" and "Costera" Cvs were in vitro established and multiplied using different BAP concentrations, followed by in vitro rooting in medium with several NAA levels and transfer to Ex vitro conditions. Treatments were distributed with a complete randomized design, data were analyzed with an ANOVA and means separated with Tukey test. BAP statistically increased multiplication rates. NAA promoted adventitious root formation while decreased root length. Complete Ex vitro adaptation occurred for all three cultivars indicating the development of an efficient micropropagation protocol to provide large quantity of high quality arrow cane plants.

## RESUMEN

La Caña flecha (*Gynerium sagittatum* Aubl.) (Poaceae) es la fuente de fibra para las más famosas artesanías colombianas; sin embargo, no existen cultivos comerciales y las poblaciones naturales han disminuido, amenazando la actividad artesanal y los ecosistemas asociados. Con el fin de proveer un mecanismo que suministre plantas para el establecimiento de cultivos, la micropropagación de tres cultivares de caña flecha fue evaluada. Explantes de los cultivares "Criolla", "Martinera" y "Costera" fueron establecidos in vitro y multiplicados usando diferentes concentraciones de BAP, seguido por el enraizamiento in vitro en presencia de diferentes niveles de ANA y transferidos a condiciones Ex vitro. Los tratamientos fueron distribuidos con un DCA, los datos analizados con ANOVA y los promedios separados con la prueba de Tukey. BAP incremento estadísticamente las tasas de multiplicación. ANA promovió la formación de raíces adventicias al tiempo que redujo la longitud de las raíces. Las plantas de los tres cultivares se adaptaron totalmente a las condiciones Ex vitro, indicando el desarrollo de un eficiente protocolo de micropropagación para proveer grandes cantidades de plantas de alta calidad.

## RESUMO

A cana-do-rio ou cana-flecha (*Gynerium sagittatum* Aubl.) (Poaceae) é a fonte de fibra para os mais famosos artesanatos colombianos; no entanto, não existem cultivares comerciais e as populações naturais estão diminuindo, Ameaçando a actividade artesanal e os ecossistemas associados. Para estabelecer um mecanismo que fornece plantas para o estabelecimento das culturas, foi avaliada a micropropagação de três cultivares de cana-de-flecha. Explantes das cultivares "Criolla," Martinera "e" Coastal "foram estabelecidos in vitro e multiplicados usando diferentes concentrações de BAP, posteriormente submetidos a enraizamento in vitro na presença de diferentes níveis de ANA e transferidos para condições Ex vitro. Os tratamentos foram distribuídos em delineamento inteiramente casualizado, os dados analisados com ANOVA e as médias separadas pelo teste de Tukey. O BAP aumentou estatisticamente as taxas de multiplicação. ANA promoveu a formação de raízes adventícias enquanto reduzia o comprimento das raízes. As plantas das três cultivares foram totalmente adaptadas às condições Ex vitro, indicando o desenvolvimento de um protocolo de micropropagação eficiente para fornecer grandes quantidades de plantas de alta qualidade.

## KEYWORDS:

Brave cane, Propagation, In vitro multiplication, In vitro rooting, Ex vitro.

## PALABRAS CLAVE:

Caña Brava, Propagación, Multiplicación in vitro, Enraizamiento in vitro, Ex vitro.

## PALABRAS - CHAVE:

Cana brava, Propagação, Multiplicação in vitro, Enraizamento in vitro, Ex vitro.

## INTRODUCTION

*Gynerium sagittatum* Aubl. (Poaceae) is a perennial, giant, rhizomatous, reed grass with erect stems basal clothed with bladeless sheaths while the upper part has the unfolded leaf blades with an open fan-shaped form. Leaves are bright green 160 to 230 cm long and 8 to 14 cm wide; a culm can form as much as 200 leaves during lifetime with 19 to 28 fresh blades at any time [1,2]. For centuries, in the flatlands of Córdoba and Sucre Departments (Colombia), indigenous Zenú communities have used arrow cane central nerve to make distinctive handicrafts worldwide known because of their beauty and tradition; one of the best known is the Sombrero Vueltiao established as Colombian cultural symbol; however, increasing demand of arrowcane products has negatively impacted arrow cane natural populations affecting not fiber availability but also associated wetland ecosystems. The growing handicraft market will require enough fiber supply to sustain increasing sales, to reduce the negative impact on the environment and to maintain the Zenú artisan legacy.

Arrow cane seeds produced in humid Caribbean are unviable; therefore, establishing an efficient clonal plant propagation system for large production of plant material has been a challenge. Cutting treatments such as source (aerial, underground), length, position (vertical, horizontal), substrate and hormone addition for rooting has been evaluated; however, most results showed the need for large cutting portions (<30 cm) making inefficient large-scale propagation using this method [3,4]. Micropropagation instead has been proved to be an efficient technique to produce large quantities of high quality plants from arrow cane Cv "Criolla" using explants with pre-existing meristems [4,5,6, 7,8,9], but there are several other cultivars used by growers and artisans based on fiber softness, resistance and availability. In the present work, the micropropagation of arrow cane cultivars "Criolla", "Costera" and "Martinerá" were evaluated as a way for providing an efficient plant propagation system for establishing commercial arrow cane crops for fiber supply, for craftsmanship industry and to contribute to lower artisan activity impact on the environment.

## METHOD

The study was done at the Plant Biotechnology Lab of the Biotechnology Institute of the Colombian Caribbean

at the Agricultural Sciences School of the Universidad de Córdoba, Montería, Colombia, located at Latitude 8° 31' North and Longitude 75° 58' to Greenwich Meridian.

### Plant material

Explants were isolated from *in vitro* established arrow cane cultivars "Criolla", "Martinerá" and "Costera" cultured in 250 cm<sup>3</sup> flasks containing 30 mL of semisolid Murashige and Skoog (MS) medium [10] with (in mg L<sup>-1</sup>) myo inositol (100), sucrose (30.000), thiamine HCl (0,4) and TC agar (8.000) (Sigma Co.). Flasks were covered with two layers of heavy-duty aluminum foil, sealed with Nescofilm<sup>®</sup> and maintained at 20 °C with 12 h photoperiod (40 μmol m<sup>2</sup> s<sup>-2</sup>). Plants were maintained for more than a year with transfer to fresh medium of the same formulation every four weeks.

### Shoot multiplication

Explants, consisting of clusters with three shoots isolated from *in vitro* growing "Criolla", "Costera" and "Martinerá" plants, were established into 200 cm<sup>3</sup> flasks similar conditions to establishing stage except for addition of four benzylaminopurine (BAP) concentrations (0,0; 0,5; 1,0 and 1,5 mg L<sup>-1</sup>). The experiment was a 2-way factorial (3 cultivars x 4 BAP levels) with 12 treatments and 10 replicates per treatment; the experimental units (120) were distributed using a complete randomized design. After six weeks in culture, the variables number of new shoots per explant and the average shoot length were registered. Data were analyzed using an ANOVA based on the model:

$$Y_{ijk} = \mu + T_i + \beta_j + (T\beta) + \epsilon_{ijk} \quad (\text{Ec. 1})$$

where  $\mu$  was the general mean,  $T_i$  was the effect of cultivar,  $\beta_j$  was the effect of BAP level and  $\epsilon_{ijk}$  was the experimental error; means were separated with Tukey test ( $\alpha = 0,05$ ).

### *In vitro* rooting

Three-shoots clusters isolated from *in vitro* multiplied shoots of "Criolla", "Costera" and "Martinerá" plants were transferred onto 30 mL semisolid MS with similar conditions to establishing stage except for addition of and independently supplied with four (0,0; 0,5; 1,0 and 1,5 mg L<sup>-1</sup>) naphthalene acetic acid (NAA). After four weeks in culture, the number of cultures with

roots, the number of roots per culture and the average root length per culture were registered. Collected data were analyzed with ANOVA based on the model:

$$Y_{ik} = \mu + T_i + \beta_j + (T\beta)_{ij} + \epsilon_{iik} \quad (\text{Ec. 2})$$

where  $\mu$  was the general mean,  $T_i$  was the effect of cultivar,  $\beta_j$  was the effect of NAA level and  $\epsilon_{iik}$  was the experimental error.

Means were separated with a Tukey test ( $\alpha = 0,05$ ).

### Transfer to *Ex vitro* conditions

*In vitro* rooted and rootless shoots from multiplication stage were transferred to *Ex vitro* conditions in order to evaluate plant survival rate. In both cases, cultures were extracted from the flasks, washed with distilled water to remove medium remains, and planted in 72-plug trays filled with peat as substrate. A single culture was placed in every plug, sprayed with distilled water and the tray covered with a plastic transparent cover. Trays were placed in a shadehouse (50% light) at 28°C with fog irrigation every 4 h during 1 min. After 3 days, covers were removed and irrigation frequency was changed to one every 8 hours. After 40 days, the number of plants that survived was registered.

## RESULTS

### Shoot multiplication

New shoot growth from established explants was observed for all treatments. Growing shoots emerged from pre-existing meristems present in the original established explant. Cultures from all cultivars showed short stems with small leaves and dark green color as evidence of well adaptation to *in vitro* conditions (Figure 1).

The ANOVA allowed to detect statistical differences ( $Pr < 0,05$ ) among mean results of new shoot formation rate as a result of the effect of cultivar, BAP concentrations in the medium and the interaction between both, cultivar versus BAP concentrations. The collected data showed that the highest multiplication rate (129 new shoots per explant) occurred when explants of "Martinera" were cultured in medium supplied with 1,0 mg L<sup>-1</sup> BAP; in contrast, the lowest number of new shoots per explant (8,2) were observed in the same cultivar when explants

were cultured in absence of BAP (Table 1). The Tukey test showed that for all three cultivars, the addition of BAP in the culture medium statistically increased, (>3x), the multiplication rate compared to non BAP-supplied media (Table 1). For cultivars "Criolla" and "Costera" multiplication rate were not different among BAP-supplied treatments; however for "Martinera", 1,0 mg L<sup>-1</sup> BAP in the medium induced a higher number of new shoots than other BAP treated explants (Table 1). For all cultivars, it was observed that a 1,5 mg L<sup>-1</sup> BAP supply in the media lowered the formation of new shoots.

For the variable shoot length, the ANOVA showed that length of shoots were statistically different ( $Pr < 0,05$ ) as a result of the cultivars, BAP treatments and the interaction between both, BAP levels and cultivars. For all cultivars, the longest shoots grew in media deprived of BAP while those cultured in the 1,5 mg L<sup>-1</sup> BAP were the shortest (Table 1). There was an evidenced gradual reduction in shoot length as BAP levels were increased. The longest shoot average were registered in "Criolla" shoots cultured

Figure 1. Shoot multiplication of "Costera", "Martinera" and "Criolla" Cultivars.

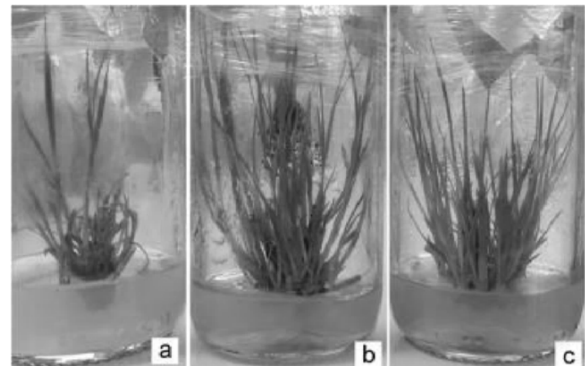


Table 1. Effects of different BAP levels on *in vitro* arrow cane shoot multiplication.

BAP (mg.L <sup>-1</sup> )	Cultivars					
	Criolla		Martinera		Costera	
	Shoot	Shoot length	Shoot	Shoot length	Shoot	Shoot length
0,0	12,3d*	133,6a	8,2d	113,0ab	9,9d	93,7b
0,5	108,6ab	52,4cd	78,0ab	55,9c	34,9c	32,8ef
1,0	121,6ab	32,7ef	129,3a	38,7de	31,8c	27,6f
1,5	71,4b	21,1f	71,4b	33,6ef	23,8c	17,2g

\*Numbers with same letter are not different according to Tukey test ( $\alpha 0,005$ )

in medium with non-BAP supply while the shortest shoots developed from “Costera” explants cultured in medium supplied with 1,5 mg L<sup>-1</sup> BAP (Table 1).

### **In vitro rooting**

Adventitious root formation was observed in all cultures; however, statistical differences were detected ( $P < 0,05$ ) based on the ANOVA analysis with respect to the number and length of adventitious roots as a result of the effect of cultivar and NAA concentrations in the medium (Figure 2).

For cultivars “Criolla” and “Martinera”, shoots cultured in NAA-supplied media formed a significant higher number of roots than those cultured in absence of NAA (Table 2); while for “Costera”, the number of adventitious roots increased at least 4x in shoots cultured in NAA-supplied media compared to those cultured in simple media (Table 2). For shoots of all cultivars, the number of emerging adventitious roots increased as the NAA concentration in the medium was increased, registering the highest number of roots (73) for “Martinera” shoots cultured in medium supplied with 1,5 mg L<sup>-1</sup> NAA and the lowest number (5,4) of adventitious roots in “Costera” shoots cultured in medium without NAA (Table 2).

Average length of adventitious root formed *in vitro* showed statistical differences ( $P < 0,05$ ) as a result of the effect of cultivar, NAA concentrations and the interaction of both factors. Although, for all cultivars, the longest roots grew in media without NAA, only roots produced from “Martinera” shoots were statistically larger than roots grown from shoots cultured in any NAA supplied media (Table 2). “Criolla” and “Martinera” shoots developed roots that were shorter as NAA concentration in the culture medium was increased, while for “Costera” shoots, root length showed no statistical different at any NAA treatment (Table 2); “Martinera” adventitious roots registered the longest (36,5 cm) and shortest (15,9 cm) average root length.

### **Plant adaptation to Ex vitro conditions**

Transplanted *in vitro* rooted plants from all cultivars, as well as rootless shoots from multiplication stage, showed a complete (100%) survival and full adaptation to *Ex vitro* conditions. Four weeks after transplant, plants were healthy, green colored and actively growing with no dead tissues, necrotic areas or adven-

titious growth (Figure 3). Performance of both, rooted and rootless shoots, on field conditions evidenced that *in vitro* rooting is not necessary for arrowcane micropropagated plants to adapt to *Ex vitro* conditions; this shows that *Ex vitro* transfer from multiplication stage can be performed with no implications for plant survival, but instead time for plant production can be shortened and costs reduced.

Micropropagation is a clonal plant propagation technique to produce plants in aseptically closed recipients with artificial media and stored under controlled environmental factors, that has proved to be very efficient for producing large quantities of high quality plants in comparative short periods of time and reduced space [11, 12, 13]. Arrow cane micropropagation as well as other biotechnological techniques has been performed on “Criolla” cultivar [9]. Artisans prefer Cv “Criolla” flexibility and resistance fiber to make hats; however, fiber from other cultivars such as “Martinera” and “Costera” are precious for their softness and plant availability to make shoes, necklace, bracelets, rings and purses. However, in the present research *in vitro* multiplication, rooting and *Ex vitro* adaptation of “Martinera” and “Costera” to “Criolla” plants were evaluated.

*In vitro* multiplication based on culture of pre-existing meristems is promoted by adding cytokines to the culture medium in order to disrupt apical dominance and induce a repetitive axillary shoot growth [14, 15,16,17,18,19,20,21,22]. The results of the multiplication stage evaluation showed that supplying BAP in the culture medium induced an increase in shoot multiplication rate for all cultivars compared to non-supplied treatments; effects previously reported in different plant species [22, 23]. BAP supply association to increased shoot multiplication for arrow cane micropropagation has been reported for different cultivars and alternative media formulation. A previous study reported significant increase in new shoot formation for cultivars “Criolla”, “Criolla 1” and “Martinera” when cultured with different BAP concentrations in double phase (liquid/semisolid) medium system; indicating that cytokine effects on arrow cane *in vitro* multiplication are independent of cultivar and culture system Previous reports on arrow cane micropropagation using semisolid system [4].

Despite the significant increase in BAP presence, shoots of cultivars “Criolla” and “Martinera” multiplied at least 4x more than “Costera” shoots when cultured

in a medium supplied with 1,0 mg L<sup>-1</sup> BAP. This indicates significant genotype dependent response to culture conditions and BAP supply; however, even for “Costera” shoots, a multiplication rate of >30 shoots per multiplication round means potentially producing >2 million plants in less than a year.

High quality shoot multiplication stage must complain not only for high multiplication rate but also for good size shoots that are able to develop new organs specially when transferred to *Ex vitro* conditions [24, 25, 26]. In the present research, shoots reduced >50% their length when cultured in media supplied with BAP and decreasing as BAP levels increased; however, the shortest shoots were >10 mm. Usually, micropropagated shoots shorter than 10 mm are prone to die when transferring to *Ex vitro* conditions due to shortage in energetic reserve supply [27, 28]. The collected data showed that when shoots were cultured in the presence of 0,5 mg L<sup>-1</sup> long was at least 33 mm, maintaining a larger standard for plant survival after transferring to *Ex vitro* conditions. Root formation is a critical step for micropropagated plant to be able to take water and minerals [29, 11, 30]. Usually, *in vitro* cultured plants are able to grow without roots since media formulation and environmental conditions protect plants from dehydration and minerals diluted in the medium are easily adsorbed by plants tissues [31]. In contrast, when transferred to *Ex vitro* conditions, roots are necessary for both, water and nutrient uptake [32, 33]. Adventitious root growth from *in vitro* cultured shoots is induced by supplying

Figure 2. Root formation with (a) 0,0 - (b) 1,5 - (c) 1,0 and (d) 1,5 mg L<sup>-1</sup> NAA in the medium.

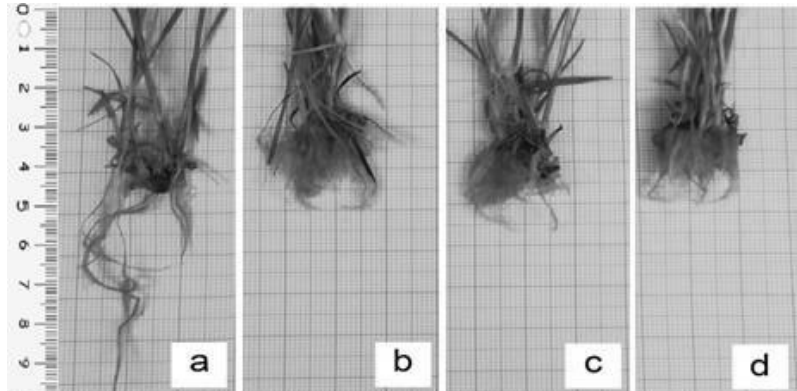


Figure 3. *Ex vitro* adaptation of micropropagated arrow cane plants cultivars “Criolla and “Martinera”.



Table 2. Rooting of three arrow cane cultivars in media with different levels of NAA.

ANA (mg.L <sup>-1</sup> )	Cultivars					
	Criolla		Martinera		Costera	
	Roots	Root length (cm)	Roots	Root length (cm)	Roots	Root length (cm)
0,0	14,4d	34,5ab	7d	36,5a	5,4d	33,0ab
0,5	44,4bc	24,5bcde	43,8bc	19,7cde	23,0cd	29,0abcc
1,1	53,8ab	18,2de	59,4ab	16,2e	47,2 bc	25,3abcde
1,5	63,6ab	17,7de	73,0a	15,9e	56,7ab	30,1abc

\*Numbers with same letter are not different according to Tukey test (α 0,005)

auxins in the culture medium [34, 35]. In the present research, micropropagated shoots cultured in absence or presence of ANA did form adventitious roots independently of the cultivar, indicating that arrow cane is an easy to root plant species [36]. *In vitro* rooting evaluation is based on both quantity and quality of roots formed.

For non-NAA supplied treatments, “Criolla” shoots formed >10 roots per explant; however, “Martinera” and “Costera” resulted in low root formation. As observed in previous studies, in the present research, shoots cultured in NAA supplied media formed 4X more roots than those transferred to simple media with at least 20

adventitious shoots per explant, and increasing the number of roots as NAA level in the medium were augmented [37, 38, 39, 7, 8]. These results showed that the quantity of adventitious roots formed *in vitro* by arrow cane micropropagated shoots increases when shoots are cultured in the presence of NAA in the medium, independently of the genotype. However, with respect to root quality, length and width of roots considered as indicators [40, 41, 42], Long-thin roots are usually considered inconvenient since they are fragile and transfer to *Ex vitro* may require trimming that opens wounds for microbial contamination under *Ex vitro* conditions. Non-supplied NAA treatments not only induced a lower number of roots but also larger roots than those produced by shoots cultured with NAA-supplied media. The *in vitro* rooting data collected in the present research suggests that NAA supply not only increases the number of adventitious roots but also improves quality of root formation.

*Ex vitro* adaptation is the stage where micropropagated plants are adapted to regular environmental conditions. Two are the major problems for micropropagated plants to survive *Ex vitro* conditions: low control of water loss and low photosynthetic competence [43, 44]. *In vitro* micropropagated plants are cultured in culture media with >90% water inside sealed containers that produce a highly saturated humid environment. Under these conditions, plants do not develop mechanism to control water loss, such as stomata closure and cuticle accumulation; therefore, if transferred directly to outside conditions, tissues will rapidly dehydrate to death. To avoid this situation, *in vitro* maintained plants must be transferred initially to a water saturated environment, usually modified with fog irrigation systems to protect plants from high water loss. On the other hand, *in vitro* cultivated plants grow under low light intensity that results in a deficient chloroplast development and incompetence for photosynthesis activity; therefore, during adaptation to *Ex vitro* conditions sunlight should be increased gradually to avoid plant loss and induce new leaf formation to acquire full photosynthetic competence. Energy required for new leaf development is obtained from energetic reserves store in Shoots organs such as stem and leaves, developed *in vitro* [45, 46, 47, 48, 49, 50]. In the present research, micropropagated shoots, with and without adventitious roots, were transferred to *Ex vitro* conditions in a shadehouse with fog irrigation.

Both conditions were gradually reduced until plants were established under complete sunlight with a single daily irrigation. Independently from cultivar, all plants transferred to *Ex vitro* conditions fully adapted and survived (100% survival). These results confirm once again that direct transfer of arrow cane shoots from propagule multiplication stage to *Ex vitro* adaptation do not have any effect on plant survival rates compared to transferring of *in vitro* rooted shoots but instead reduces costs and time.

## CONCLUSIONS

BAP supply in the medium increased *in vitro* shoot multiplication rates and reduces shoot length for arrow cane micropropagated plants cultivars “Criolla”, “Martinera” and “Costera”. Addition of NAA in the culture medium for root formation increased the number of adventitious roots formation for arrow cane micropropagated plants cultivars “Criolla”, “Martinera” and “Costera”. *In vitro* rooting did not have effect on survival rates of for arrow cane micropropagated plants cultivars “Criolla”, “Martinera” and “Costera” when transferred to *Ex vitro* conditions. The present micropropagation protocol offers the opportunity for large quantity of arrow cane plant production of “Criolla”, “Martinera” and “Costera” cultivars.

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