In vitro MORPHOGENESIS OF *Syngonanthus mucugensis* GIUL. SUBSP. *mucugensis*[1]

Morfogênese *in vitro* de *Syngonanthus mucugensis* Giul. subsp. *mucugensis*[1]

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ABSTRACT

Syngonanthus mucugensis Giul. subsp. *mucugensis* is an herbaceous plant with significant economic value in the ornamental dry flower business. The restricted occurrence of the municipality Mucugê-BA, Brazil, exclusively associated with extractive exploitation, has considered this species as endangered. The objective of this work was to evaluate the organogenic potential of three different types of *S. mucugensis* subsp. *mucugensis* explants to promote the development of an alternative method to the propagation of the genetic resources of this important plant. The morphogenetic capacities of the leaf, stem and root this species was tested using Murashige and Skoog culture medium at half salt concentration and different concentrations of growth of regulators *benzylaminopurine* - BAP (0.00; 2.22 and 4.44 µM), and *naphthalene acetic acid* - NAA (0.00; 1.34 and 2.68 µM). The morphoanatomic events that lead to formation of shoots were described. Stems proved to be the best source of explants, showing 58.75% regeneration of shoot by direct organogenesis in the absence of growth regulators, and 32.18 and 47.55% of shoot regeneration by indirect organogenesis in the presence of 2.22 and 4.44 µM BAP, respectively. As for leaves, there was callus formation, but without regenerating shoots. Morphogenesis was not observed when roots were used as explants. The histological analyses showed that shoot regeneration in *S. mucugensis* subsp. *mucugensis* occurred both indirectly, by unorganized tissue differentiation, and directly through returning to merismatic activity in differentiated mature cells and preexisting bud proliferation.

Index terms: Tissue culture, organogenesis, micropropagation, straw-flowers.

RESUMO

Syngonanthus mucugensis Giul. subsp. *mucugensis* é uma herbácea com grande potencial de utilização no comércio de flores secas ornamentais. A ocorrência restrita ao município de Mucugê-BA, Brasil, associado à exploração extrativista tem levado essa espécie ao risco de extinção. Neste estudo, objetivou-se avaliar o potencial organogênico de três diferentes tipos de explantes de *S. mucugensis* subsp. *mucugensis* visando ao desenvolvimento de um método alternativo para a sua propagação. A capacidade morfogênica de caule, folha e raiz foi testada utilizando o meio de cultura Murashige e Skoog com metade da concentração salina e diferentes concentrações dos reguladores de crescimento benzilaminopurina – BAP (0,00; 2,22 e 4,44 µM) e ácido naftaleno acético – ANA (0,00; 1,34 e 2,68 µM). Os eventos morfoanatômicos que levaram a formação dos brotos foram descritos. O caule demonstrou ser a melhor fonte de explante, apresentando 58,75% de regeneração de brotos via organogênese direta, em meio livre de regulador de crescimento; e 32,18 e 47,55% de regeneração de brotos por organogênese indireta na presença de 2,22 e 4,44 µM de BAP, respectivamente. Para folha, foi obtida a formação de calos sem regeneração de brotos, não sendo observada morfogênese, quando se utilizou raiz como explante. As análises histológicas mostraram que a regeneração de brotos em *S. mucugensis* subsp. m*ucugensis* ocorreu tanto por via indireta, pela diferenciação do tecido calogênico desorganizado, quanto por via direta pela retomada da atividade meristemática em células maduras diferenciadas e proliferação de gemas pré existentes.

Termos para indexação: Cultura de tecido, organogênese, micropropagação, sempre-viva.

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INTRODUCTION

Chapada Diamantina, in Bahia State, Brazil, has an extremely diversified flora, including many ornamental species with significant economic value. Many of these species are endemic and have been aggressively and destructively harvested - resulting in a loss of genetic resources.

Syngonanthus mucugensis subsp. *mucugensis* is an herbaceous species. It grows approximately 40cm, their leaves are united in a compact rosette, and the inflorescences are monoecious, united in a capitulum that

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retains its natural aspect even after collecting and drying—giving it considerable value as a dry ornamental flower. Its distribution is limited to municipality of Mucugê, located in Chapada Dimantina, where it is commonly known as *"sempre-viva de Mucugê"* (Giullieti et al., 1996; Cerqueira et al., 2008).

In the 1980s, this species represented an important economic source for Chapada Diamantina, being traded as an ornamental dry flower, especially in the USA and Europe (Giulietti et al., 1988). Although IBAMA prohibited its harvest, predatory exploitation continues, which has placed it in danger of extinction (Cerqueira et al., 2008).

Attempts at conventional propagation of this species by seeding have generated little success, due to the extremely high mortality rate of the seedling during the initial stages of development. Studies on propagation of *S. mucugensis* subsp. *mucugensis* limited for germination and *in vitro* plant growth (Paixão-Santos et al., 2003). As such, there is a real necessity to develop alternative techniques that can assure propagation on a commercial scale—which could relieve the threat of extinct and also generate income for local human populations.

Tissue culture has been appointed as an alternative to propagation and conservation native species that in danger of extinction and do not propagate easily through conventional methods, such as *S. mucugensis* subsp. *mucugensis*.

Organogenesis is the most used method for plant propagation in tissue culture because of its high multiplication rate (Grattapaglia & Machado, 1998). When the explant already has competent cells, there will be direct organogenesis; if there is an intermediate phase before competent cells, there will be indirect organogenesis (Peres, 2002).

Direct organogenesis produces plants that are genetically identical to the matrix plant, and can be used for industrial shoot production, manipulated plant regeneration and *in vitro* plant multiplication preserved *in vitro* (Pence, 1999; Arrabal et al., 2002). Indirect organogenesis, on the other hand, can be an important means for genetic improvement due to the genetic variability potential associated with callus formation (Nikan & Shitole, 1999).

Organogenesis is a complex process, involving endogenous and exogenous variables. Among the factors that affect *in vitro* plant regeneration are: explant source and physiological condition, genotype, type and concentration of growth regulators in culture medium, and environmental conditions (Sugiyama, 1999; Luciani et al., 2006).

In vitro organogenic events can be better understood with the use of anatomy, which is an auxiliary tool in studies of plant tissue culture. The monitoring of the histological events that occur during regeneration of plants is import not only to determine the path of regeneration, but also identify the type of morphogenic competent cells (Mello et al., 2000).

The objective of this paper is to compare the morphogenic capacity of three types of explants of *S. mucugensis* subsp. *mucugensis*, and verify the regeneration means of shoots through histological analyses.

MATERIALS AND METHODS

Plant Material. Seeds of *S. mucugensis subsp. mucugensis* were collected in the Mucugê *Municipal Park* (12º59'83''S and 42º20'91''W). They were disinfected in 70% ethyl alcohol (1 minute), and 2.5% sodium hypochlorite (15 minutes), washed four times in sterile distilled water, and sewn into 250mL flasks containing 50 mL ½ MS culture medium (Murashige & Skoog, 1962) supplemented with 15 g L^{-1} sucrose and solidified with 6 g L^{-1} of agar.

One hundred and eighty days after *in vitro* germination these plants were used as explant source.

Culture conditions. All experiments were carried out at a temperature of 26±2º C, a photoperiod of 16 h, and an active photosynthetic irradiance of 60 mmol m^{-2} s^{-1}.

The tissue culture medium employed was ½ MS supplemented with 15 g L^{-1} sucrose, solidified with 7 g L^{-1} of agar. It pH was adjusted to 5.8 before autoclaving (121º C per 15 min). Explants were inoculated into test tubes (150x15 mm) containing 15 mL of this culture medium.

Morphogenesis induction. The organogenic capacity of the leaves, stem, and roots were tested by inoculating them individually into ½ MS culture medium supplemented with different concentrations of BAP (0.00; 2.22 or 4.44 µM), and NAA (0.00; 1.34 or 2.68 µM). The experiment was considered a 3 x 3 x 3 factorial arranged (BAP x NAA x explant type) as completely randomized design, with ten repetitions of four explants per treatment. The experiment was repeated twice.

Sixty days after inoculation, the percentages of explants that formed shoots, only callus, or callus with later differentiation from shoots, were recorded. All shoots obtained were counted and measured.

The data obtained were submitted to variance analysis and averages were compared through Tukey Test at 5% probability, using SISVAR 4.3 program (Ferreira, 2003).

Anatomy. During culturing, samples of plant material were periodically taken out and set at 70% alcohol. Stem was used, inoculated in a medium without plant growth regulator; and callus derived from stems inoculated in a medium with 2.22 or 4.44 µM BAP. The samples were sectioned transversally by hand, using a blade, and cleared in 3% sodium hypochlorite for 15 minutes; afterwards, they were washed in distilled water and then stained with an aqueous solution of 0.1% saffranin, and 0.1% astra blue (Bukatsh, 1972). The sections were observed and photographed using a light microscope (Zeiss-Axioskop 2) with a coupled Olympus digital camera.

RESULTS AND DISCUSSION

In vitro morphogenesis of *S. mucugensis* subsp. *mucugensis* was influenced by the type of explant used as well as by the concentration of plant growth regulators in culture medium.

Shoot regeneration, independent from the plant growth regulator used, was obtained from stem explant only (Table 1). These results can be related to the lesser degree of differentiation of tissue and the presence of more meristematic tissue when compared to leaf and root.

Studies with other herbaceous species have shown the diversity of morphogenic response depending on the type of plant tissue used, showing the importance of explant choice for other *in vitro* cultures (Nhut et al., 2004; Dhar & Joshi, 2005; Pedraza-Santos et al., 2006; Aschough et al., 2007).

No other response was obtained with the use of root explant (Table 1). These results suggest that *S. mucugensis* subsp. *mucugensis* roots are highly differentiated. The greater determination of an explant for a means of development, the lesser will the competence to form another type of organ (Peres, 2002). Similar results were found for *Carthamus tinctorius* L. (Nikan & Shitole, 1999) and four species of *Watsonia* genus (Ascough et al., 2007). However, shoots can be regenerated from the root of some herbaceous species, such as *Saussurea obvallata* (DC.) and *Passiflora cincinnata* Mast. (Dhar & Joshi, 2005; Lombardi et al., 2007).

In general, *in vitro* shoot and root formation depend on adding plant growth regulators in culture medium, especially auxins and cytokinins (Skoog & Muller, 1957). However, the answer to the combination of these growth regulators vary as a function of the species and tissue used, as related to *Cryptanthus sinuosus* L. B. Smith (Arrabal et al., 2002); *S. obvallata* (Dhar & Joshi, 2005), *Vriesea reitzii* Leme and Costa (Rech Filho et al., 2005), Alstroemeria cv. 'Yellow King' (Pedraza-Santos et al., 2006), and four species of Watsonia genus (Ascough et al., 2007). This shows the importance of developing specific protocols for the species, and even for the genotypes of the same species (Grattapaglia & Machado, 1998).

In this study, the simultaneous use of auxins and cytokinins does not induce organogenesis in the explants used. However, the use of medium without plant growth regulator, or containing BAP and NAA, individually, has promoted morphogenesis in stem and leaf (Figure 1 and 2).

The highest average rate of shoot regeneration (58.75%) was obtained via direct organogenesis from stem explant inoculated in a medium without plant growth regulator (Table 1, Figure 2A). These results confirm the ones found for *C. sinuosus* (Arrabal et al., 2002), and show that the endogenous hormonal balance of *S. mucugensis* subsp. *mucugensis* is appropriate to promote the *in vitro* multiplication phase.

Direct organogenesis is a benefic method in terms of genetic stability, and it can be used both for regenerating *in vitro* plants and as an economically viable means for producing plants on a high scale, wherever homogeneity is crucial (Pence, 1999; Arrabal et al., 2002).

The exposition of stem and leaf explants of *S. mucugensis* subsp. *mucugensis* to 2.68 µM NAA without adding BAP resulted in callus formation, with a not friable whitish spongy aspect, without shoot regeneration (Figure 1 and 2B). This shows an inhibitory effect of NAA auxin (in the concentrations used in this work) on the organogenesis of the species under analysis. Similar results were found for *Curcuma zedoaria* Roscoe (Miachir et al., 2004) and four species of the genus Watsonia (Ascough et al., 2007).

Callus formation without shoot regeneration was also observed in 12.35% of leaf explants submitted to 4.44 µM BAP; the callus obtained showed friable consistence with a yellowish coloration (Table 1, Figure 2C). These results corroborate Nikam & Shitole's (1999) who obtained callus without shoot regeneration in *Safflower* L. cv. Bhima leaves.

Indirect organogenesis was observed in stem explant inoculated in a culture medium containing BAP. The highest average rates for shoot explants were obtained in a medium containing 4.44 µM (47.05%), which differed significantly from 2.22 µM concentration (32.18%), indicating that an increase in BAP concentration is a beneficial to shoot production via indirect organogenesis in *S. mucugensis* subsp. *mucugensis* (Table 1). In these treatments, the calli came out two weeks after explant

Table 1 – Effect of plant growth regulators (PGR) BAP and NAA on the percentage of shoots via direct organogenesis (% of shoots/DO) and indirect (% of shoots/IO) and percentage of callus obtained from stem explants, leaf and root of *Syngonanthus mucugensis* Giul. subsp. *mucugensis.*

PGR (µM)		% of shoots/DO			% of shoots/IO			% of callus		
BAP	NAA	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root
	0	58.75	0	0	0	0	0	0	0	0
0	1.34	0	0	0	0	0	0	0	0	0
	2.68	0	0	0	0	0	0	8.62	19.73	0
	0	0	0	0	32.18	0	0	0	0	0
2.22	1.34	0	0	0	0	0	0	0	0	0
	2.68	0	0	0	0	0	0	0	0	0
	0	0	0	0	47.05	0	0	0	12.35	0
4.44	1.34	0	0	0	0	0	0	0	0	0
	2.68	0	0	0	0	0	0	0	0	0

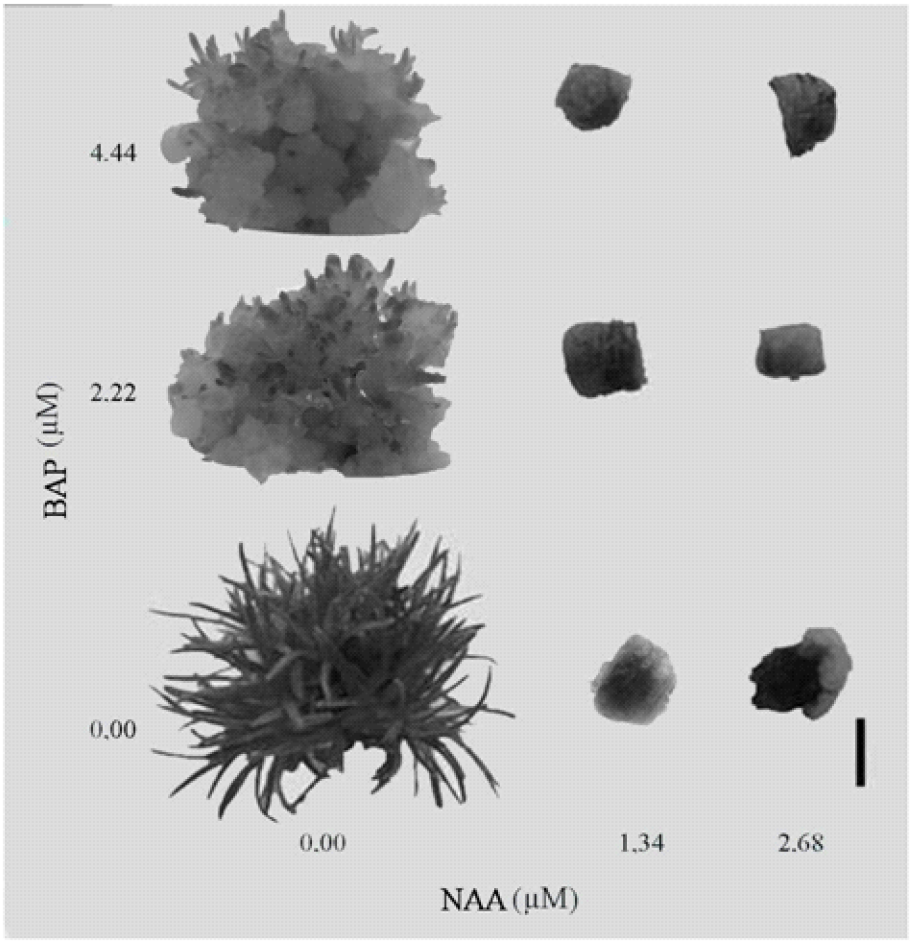

Figure 1 – *In vitro* morphogenesis of stem explant of *Syngonanthus mucugensis* Giul. subsp. *mucugensis* in ½ MS culture medium supplemented with BAP and NAA (bar: 0.5 cm).

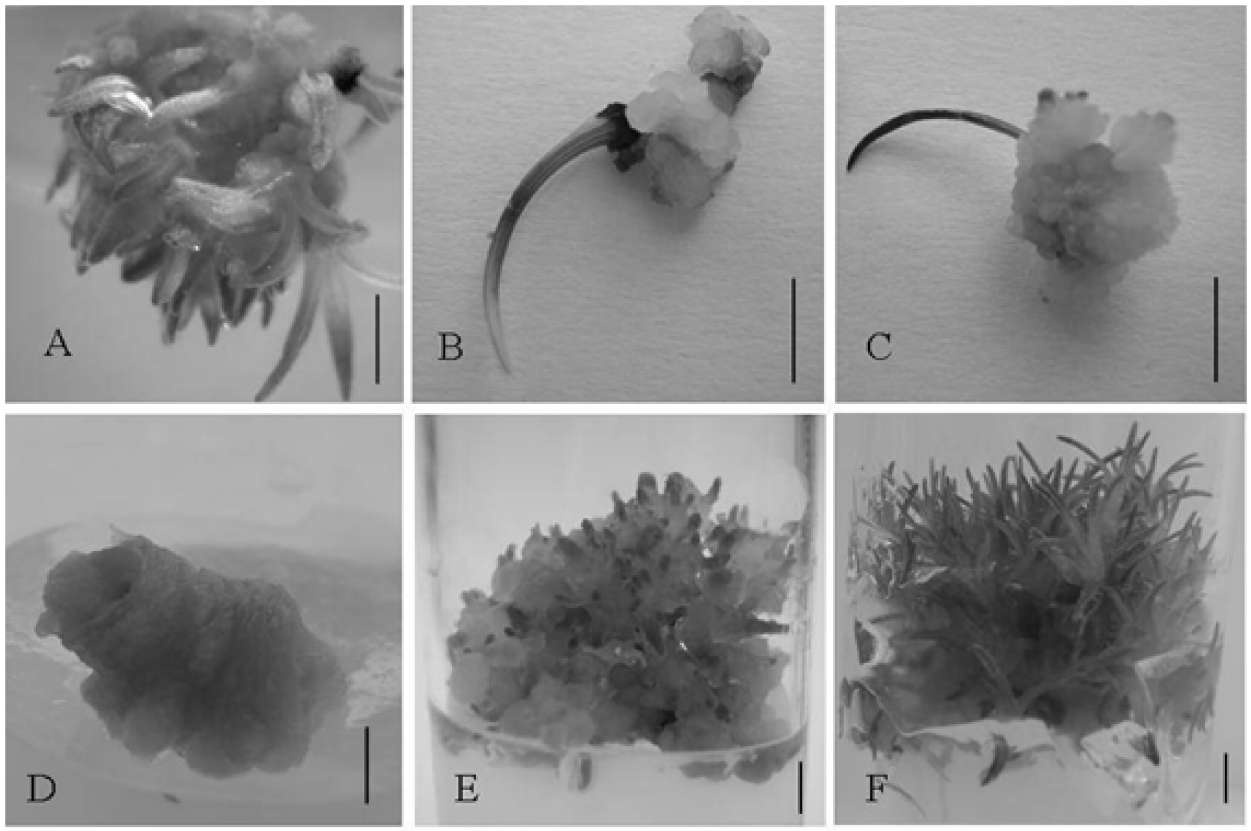

Figure 2 – *In vitro* morfogenesis of *Syngonanthus mucugensis* Giul. subsp. *mucugensis* in ½ MS culture medium. Direct organogenesis in medium without growth regulators (A), callogenesis in inoculated leaf in medium containing 2.68 µM NAA (B) and 4.44 µM BAP (C), and indirect organogenesis in medium containing BAP (D, E and F) (bar: 0.5 cm).

inoculation and showed friable consistency and a yellowish coloration (Figure 2D). Shoot regeneration from callus initiated in the fourth week of observation, indicating the possibility of callogenesis control through manipulating *in vitro* culture time (Figure 2E and 2F).

The possibility of somaclonal variation during the formation of callus, in indirect organogenesis, suggests that this means of regeneration can be tested to increase the genetic base of *S. mucugensis* subsp. *mucugensis*, as the populations of these species have a very low level of genetic and morphological variability, and a very high endogamy rate (Pereira et al., 2007; Cerqueira et al., 2008).

The increase of genetic diversity for the species under analysis may be beneficial for genetic improvement programs in view of recovering poor areas and bringing out new varieties in the ornamental plant business.

The rates for the number of shoots per explant obtained from stem inoculated in a medium containing 2.22 and 4.44 µM BAP were 28 and 32, respectively. These results did not differ among themselves. However, they were significantly higher than those found for a medium without growth regulators, which was also high proliferative, 22 shoots/explant (Figure 3).

The obtaining of a great number of shoots per explant in a medium without growth regulator was also related for *C. sinuosus* (28) for Arrabal et al. (2002). Nonetheless, various ornamental herbaceous species did not regenerate shoots in a medium without growth regulator, such as *Tagets erecta* L. (Vanegas et al., 2002) and Alstroemeria cv. 'Yellow King' (Pedraza-Santos et al., 2006), or showed a small number of shoots per explant under these conditions, as observed for *Vriesea gigantea* Gaudich (3.14), *Vriesea philippocoburgii* Wawra (5.05) and *V. reitzii* bromeliad (2.3) (Droste et al., 2005; Rech Filho et al., 2005).

The values found in this work for shoot number per explant in a medium containing BAP (28 e 32) are higher

than those obtained for *V. reitzii* (4.5), and *Ananas comosus* var. erectifolius (21) harvested in a medium containing growth regulators (Rech Filho et al., 2005; Pasqual et al., 2008), which shows the capacity for *in vitro* proliferation of the species under analysis.

For shoot length, we observed an inversely proportional relation to proliferative rate; the longest average length of shoots was obtained in a medium without growth regulator (1.1 cm) which differed significantly from the media supplemented with 2.22 and 4.44 µM BAP, 0.58 and 0.50 cm, respectively (Figure 3). These results can be related to competition between the shoots produced, or the presence of BAP in the culture medium.

Although cytokinins are important in the multiplication *in vitro*, this hormone can cause the inhibition of shoot growth (Grattapaglia & Machado, 1998). Similar results were reported for two herbaceous species *Strelitzia reginae* Banks. and *A. comosus* var. *erectifolius* (Paiva et al., 2004; Pasqual et al., 2008)

The histological analyses showed that shoot regeneration in *S. mucugensis* subsp. *mucugensis* occurred both indirectly, by unorganized tissue differentiation, and directly through returning to merismatic activity in differentiated mature cells and pre existing bud proliferation (Figure 4).

The occurrence of different morphogenetic patterns in the same explant demonstrated the importance of the growth regulator in determining the generative means of shoots in the species under analysis, which corroborate the results obtained for *Citrus sinensis* (L.) Osbeck by Almeida et al. (2006).

S. mucugensis subsp. *mucugensis* stem shows a uniserial epidermis with sclefified cells. Transversal sectioning shows an anatomic organization with two very distinct regions: the cortex and the central cylinder separated by endoderm and pericycle. The cortex shows to be homogeneous with isodiametric parenquimatic cells containing thin walls, without intercell spaces; and the central cylinder is composed by parenquimatic cells and vascular bundles disposed in atactostele, common in monocotyledonous (Figure 4A).

After explant inoculation in a medium without growth regulator, with some pre existing axillary buds, the formation of a merismatic zone was observed in the sub epidermic region of the cortex; the merismatic cells were distinguished from other cells for their small size and densely colored cytoplasm. From the merismatic zone there was initial leaf and adventitious bud differentiation, ending in shoot differentiation with visible vascular connection with explant tissue, which characterizes the regeneration process as direct organogenesis (Figure 4B). Shoots originated from stem sub epidermic cells were also related for *C. sinuosus* and *Ophiorrhiza prostrata* D. Don (Arrabal et al., 2002; Beegum et al., 2007).

In a culture medium supplemented with BAP, callus formation was observed all over the explants surface from the division of cortex parenquimatic cells near the epiderm (Figure 4C and 4D). In the unorganized callogenic tissue surface, meristemoids organization was observed with subsequent appearance of green areas dots corresponding to bud formation (Figure 4E and 4F), which differentiated in shoots (Figure 4G and 4H). This intermediate phase of the callus features indirect regeneration.

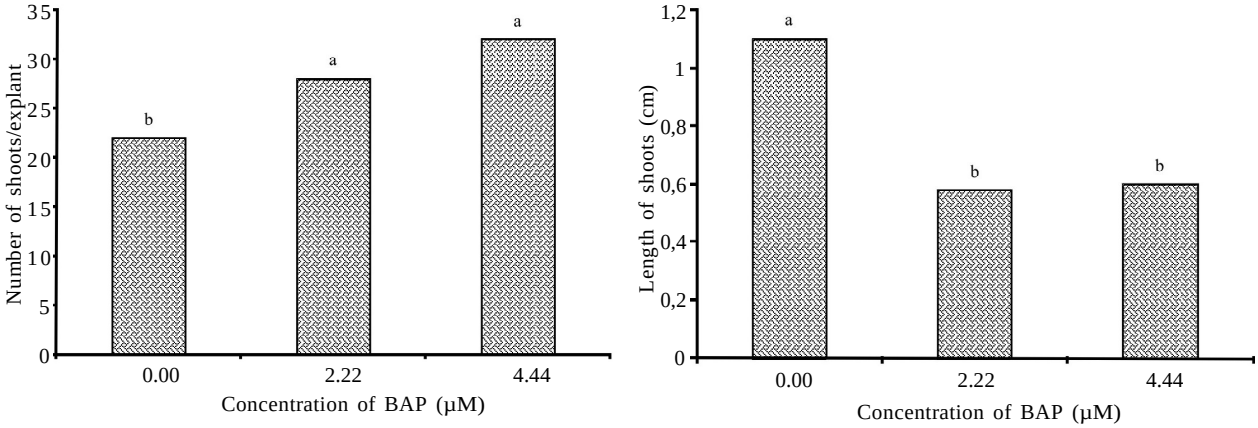

Figure 3 – Number of shoots per explant (A) and average shoot length (B) of *Syngonanthus mucugensis* Giul. subsp. *mucugensis*, as a function of BAP concentration in a culture medium.

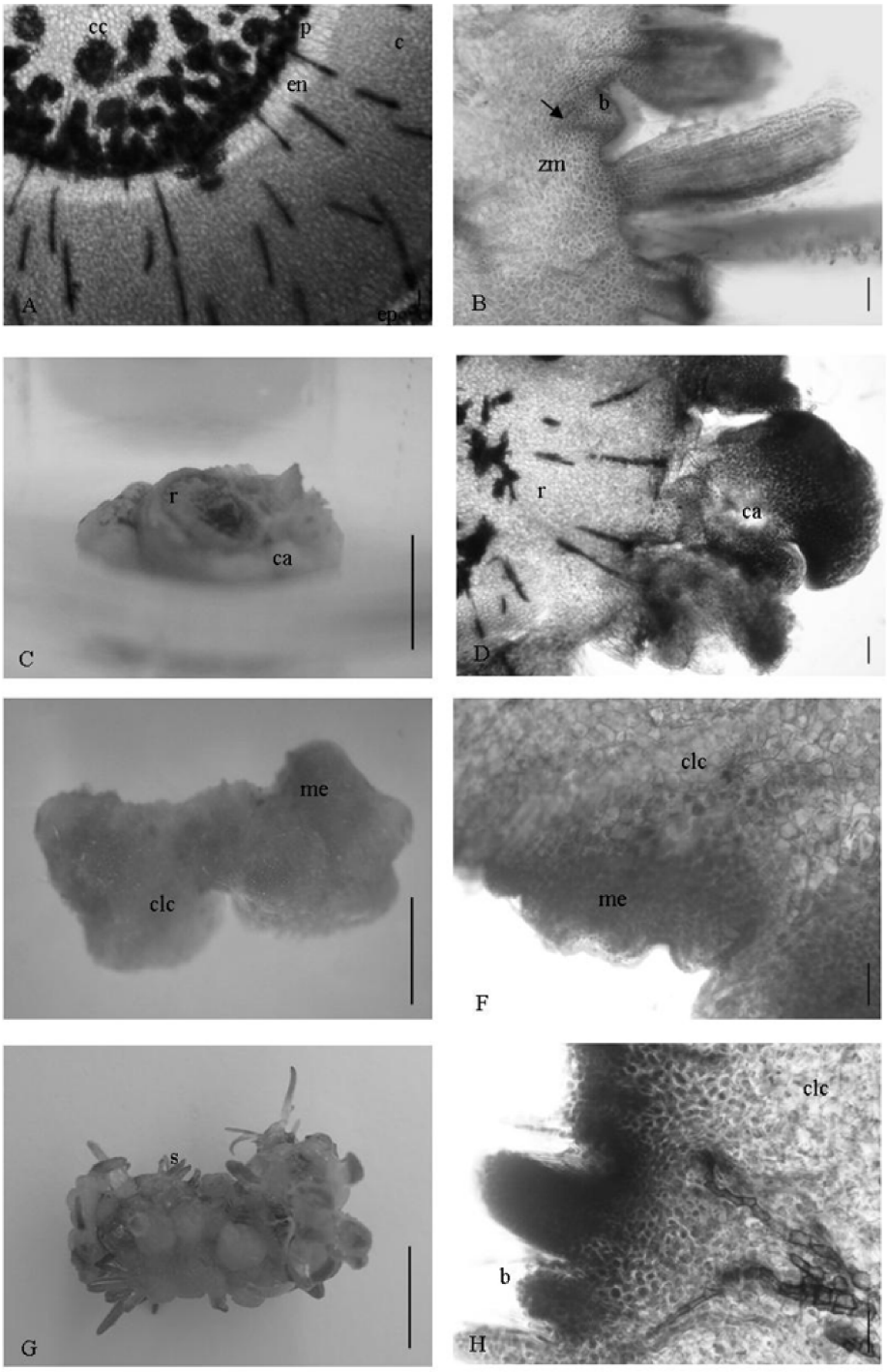

Figure 4 – Stem transversal sectioning of *Syngonanthus mucugensis* Giul. subsp. *mucugensis* (A); buds differentiation and initial leaf in the sub epidermic region of stem (B); Overview and stem transversal sectioning with calli (C and D), calli with meristemoids (E and F), and shoots differentiated from meristemoids formed on the callus surface (G ande H). b-bud, ca-callus, c-cortex, cc-central cylinder, clc-callus cell, en-endoderm, ep-epiderm, me-meristemoids, p-pericicle, r-rizome, s-shoot, zm-meristematic zone .(bars: A, B, D, F e H = 0,1 mm; C, E e G = 0,5 cm).

CONCLUSIONS

Plant tissue culture techniques represent a viable alternative to shoot production of *S. mucugensis* subsp. *mucugensis*.

The stem shown to be the best source of explants to shoot regeneration, both via direct organogenesis on MS medium ½-free regulator and indirect organogenesis, in the presence of BAP.

The leaf and root explants were not able to produce shoots in the presence of regulators BAP and NAA, at the concentrations used.

The increase in the BAP concentration stimulated greater number of shoot proliferation via indirect organogenesis.

We suggest new research to establish *in vitro* propagation and *in vitro* conservation protocols for *S. mucugensis* subsp. *mucugensis*, an important plant genetic resources.

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