European Journal of Medicinal Plants

31(11): 49-56, 2020; Article no.EJMP.59346
ISSN: 2231-0894, NLM ID: 101583475

Induction of Callus in Leaf Explants of *Crinum americanum* L. (Amaryllidaceae)

Kicia K. P. Gomes-Copeland[1*], Izulmé R. I. Santos[2], Amanda G. Torres[1], João V. D. Gomes[1], Fabrício T. C. de Almeida[3], Christopher W. Fagg[4], Sueli M. Gomes[5], Dâmaris Silveira[1] and Luiz A. Simeoni[1]

[1]*Faculdade de Ciências da Saúde, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Asa Norte, Brasília, Distrito Federal, CEP 70910-900, Brasil.*
[2]*Embrapa Recursos Genéticos e Biotecnologia, Asa Norte, Brasília, Distrito Federal, CEP 70770-917, Brasil.*
[3]*Faculdad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.*
[4]*Faculdade de Ceilândia, Universidade de Brasília, Ceilândia, Distrito Federal, CEP 72220-275, Brasil.*
[5]*Departamento de Botânica, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Asa Norte, 70910-900, Brasília-DF, Brasil.*

Authors' contributions

This work was carried out in collaboration among all authors. Authors KKPGC and AGT contributed to in vitro culture. Author CWF contributed in collecting plant sample and identification. Author SMG performed the histochemical tests and contributed to the discussion and made the final revision of the manuscript. Authors FTCA, IRIS and JVDG contributed to critical reading of the manuscript. Authors KKPGC, DS, LAS designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2020/v31i1130298
Editor(s):
(1) Dr. Elena Maria Varoni, University of Milan, Italy.
(2) Marcello Iriti, University of Milan, Italy.
Reviewers:
(1) Deboja Sharma, University of Science and Technology, Meghalaya, India.
(2) Grace Asiko, University of Nairobi, Kenya.
Complete Peer review History: http://www.sdiarticle4.com/review-history/59346

Original Research Article

Received 22 May 2020
Accepted 28 July 2020
Published 06 August 2020

ABSTRACT

Amaryllidaceae include plant species that present alkaloids with analgesic, anti-cancer, anti-bacterial, anti-viral, anti-fungal and anti-malarial activities. Due to this pharmacological value, several species of this family have been widely studied and among them is White lilly, *Crinum*

**Corresponding author: E-mail: kiciagomes@gmail.com;*

americanum. The objective of this work was to induce callogenesis on leaf explants of *C. americanum* cultivated *in vitro* for future production of alkaloids. Leaf explants were grown on a culture medium (solid) Murashige and Skoog (1962) supplemented with different concentrations and combinations of plant growth regulators, auxin 2,4-dichlorophenoxyacetic acid and cytokinin 6-benzylaminopurine and their effect on callogenesis assessed for percentage oxidation and explants responsive to callus induction. Callus formation started 10 days after hormone inoculation, and within 30 days after inoculation the best callogenesis and callus biomass growth were observed in medium containing 2.5 mg L^{-1} of 2,4-dichlorophenoxyacetic acid and 10 mg L^{-1} of 6-benzylaminopurine. The lowest percentage of oxidation was observed on explants cultivated on medium containing 5 mg L^{-1} of 6-benzylaminopurine and 2.5 mg L^{-1} of 2,4-dichlorophenoxyacetic acid. The calli obtained were compact and embryogenic. This work contributes not only to future studies on *in vitro* callogenesis of this species, but also to a possible protocol for the production of alkaloids of interest from cell suspension cultures produced *in vitro*. This is the first report of callus formation in *Crinum americanum* explants.

Keywords: Alkaloids; callogenesis; growth regulators; tissue culture; environmental impact.

1. INTRODUCTION

White lilly (*Crinum americanum* L.) is a bulbous plant species of the Amaryllidaceae botanical family. This family comprises 85 genera and at least 1100 species of bulbous plants distributed mainly in tropical and subtropical areas of the world [1]. The genus, Crinum, contains over 130 species widely distributed in tropical and temperate regions of both North and South hemispheres [2]. Crinum species are traditionally cultivated as ornamentals due to the large, conspicuous, beautiful umbels of lily-like flowers, but they have been known to be medicinal herbs. Flowers, leaves, sliced or crushed bulbs and stalks of these plants have been used, for centuries, in folk medicine to cure ailments and diseases [3]. Due to their medicinal properties, Crinum species attracted the attention of the general public, plant gatherers, herbalists and medical science researchers. The contained alkaloids were tested and were found to be responsive in a variety of biological assays, exhibiting analgesic, anticancer, antibacterial, antiviral, antifungal, antimalarial and anti-rheumatic activities [3-5]. Among the many different alkaloids detected in Amarillydaceae, lycorine and galantamine, were major. Lycorine has antiviral, antimalarial and cell division inhibitory activities [6,7]. Galantamine inhibits the acetylcholinesterase (AChE) enzyme and is already used for Alzheimer's disease treatment [8]. Studies have also revealed antiproliferative action of these alkaloids against different cancer cell lines [9].

Even though much has been reported on the pharmacological value of Crinum, only an estimated 20% of the species are represented in these phytochemical analyses [3]. The increased demand for Crinum products to treat a variety of ailments has led to intensive collecting by gatherers to supply traditional medical practices and the pharmaceutical industry, increasing pressure on natural populations and raising concern for the conservation of these plants. In this scenario, the development of sustainable strategies for mass propagation of Crinum plants has become imperative to ensure the steady supply of high quality plant material for medicinal use, scientific research and industrial scale production of the pharmacologically important alkaloids present in this plant and, most importantly, the preservation of Crinum natural populations and biodiversity. *In vitro* plant tissue culture is a well-established and reliable technology for sustainable, controllable and environmentally friendly mass propagation of plants, free of contaminants and diseases, protected from the vagaries of climate, using minimal space, and can be established for most plant species by utilizing appropriate culture medium, culture conditions and appropriate levels of phytohormones [10].

These techniques have been employed for the propagation of a variety of medicinal plant species and could be a valuable tool for large scale production of Crinum plants. Recently, plant tissue culture techniques have been used in commercial production systems for synthesis of high-value plant secondary metabolites, with several advantages over conventional whole plant extraction, like sustainable production with constant and reliable supply of pharmaceutical active compounds free of contamination and diseases, free of the limitations faced by field plant production, closer relationship between

supply and demand or the high cost associated with complex chemical synthesis [10,11].

Friable callus is most suitable to initiate cell suspension cultures because it shows fast and uniform cell growth, and cell suspension cultures can be generated by adding clumps of lose cells of the callus to liquid medium. Callus growth and morphology is explant and species dependent but can be altered by modification of plant growth regulators in the growth medium to promote growth, differentiation, and metabolite production *in vitro* [12]. Plant growth regulators can direct explant metabolism to the desired morphogenetic process, but their effect might vary according to the explant type, plant genotype and environmental growth conditions [13]. Addition of auxins and cytokinins to the culture medium is in some cases essential to stimulate cell proliferation. To maximize production, it is desirable to initiate the callus from the plant part that is known to be the higher producer of the compound of interest. Amaryllis species respond differently to hormonal composition [14]. However, to date, there are no reports of callus induction in *Crinum americanum*. Thus, the objective of this work was to induce callogenesis on leaf explants of *C. americanum* cultivated *in vitro* for future production of alkaloids.

2. MATERIALS AND METHODS

2.1 Plant Materials

The explants used in the experiments conducted in this research were isolated from young leaves of *Crinum americanum* collected from plants growing in their natural habitat in an area located in Asa Sul, Brasília, Distrito Federal, Brazil (15°49'08.190S; 47°55'15.000W), which is within the boundaries of the Cerrado biome. The species was identified by Prof. Dr. Christopher William Fagg (University of Brasília) and a dried sample was deposited in the Herbarium of University of Brasília (UB) (voucher number Fagg 2474).

2.2 *In vitro* Establishment and Callogenesis Induction

Prior to inoculation *in vitro*, leaves washed manually under running tap water to remove dirt particles. Leaves were then immersed in a flask containing a solution of commercial grade sodium hypochlorite (2.5 % active chlorine) supplemented with 1.0 mL of Tween 20 for 15 min. This solution was discarded and leaves were washed with 70% ethanol for 1 min. The ethanol was discarded and leaves were rinsed three times with distilled water sterilized by autoclaving (1.5 atm, 121 ºC, 15 min). After these treatments, the flask containing the leaves was taken to a laminar flow chamber and a second decontamination treatment was carried out. This treatment consisted of immersion in a solution of 50% (v/v) commercial sodium hypochlorite for 10 min, followed by three rinses with distilled water sterilized by autoclaving (1.5 atm, 121 ºC, 15 min). Explants were then excised from the leaves and inoculated into Petri dishes (90 x 15 mm) containing basic MS culture medium [15] supplemented with 30 g L^{-1} sucrose and 6% agar or the same medium supplemented with the following concentrations and combinations of 6-benzylaminopurine (BAP) and 2.4-dichlorophenoxyacetic acid (2,4-D): 5.0 mg L^{-1} BAP + 2.5 mg L^{-1} 2,4-D (T1); 5.0 mg L^{-1} BAP + 5.0 mg L^{-1} 2,4-D (T2); 5.0 mg L^{-1} BAP + 7.5 mg L^{-1} 2,4-D (T3); 5.0 mg L^{-1} BAP + 10.0 mg L^{-1} 2,4-D (T4); 10.0 mg L^{-1} BAP + 2.5 mg L^{-1} 2,4-D (T5); 10.0 mg L^{-1} BAP + 5.0 mg L^{-1} 2,4-D (T6); 10.0 mg L^{-1} BAP + 7.5 mg L^{-1} 2,4-D (T7); 10.0 mg L^{-1} BAP + 10.0 mg L^{-1} 2,4-D (T8). After inoculation, the cultures were kept in a growth room at a temperature of 25 ± 2°C, in the dark, for 30 days. The oxidation percentage, callogenesis response and callus biomass were evaluated at 30 days after inoculation.

2.3 Experimental Design

The experimental design was completely randomized in a 2 x 4 factorial scheme, with two concentrations of BAP (5.0 and 10.0 mg L^{-1}) and four concentrations of 2,4-D (2.5, 5.0, 7.5 and 10.0 mg L^{-1}), totaling eight treatments with three replicates, containing four explants each. Oxidation of callus, explants responsive callogenesis and characteristics of formation of the callus were evaluated.

2.4 Statistical Analysis

Data were submitted to analysis of variance (ANOVA) and the means of the quantitative treatments were evaluated by regression analysis by the statistical analysis program Sisvar [16]. The data expressed in percentages were previously subjected to arcsine transformation (x/100) 0.5.

3. RESULTS AND DISCUSSION

3.1 Effects of the Plants Growth Regulators in the Oxidation and Callus Formation

The disinfection treatment tested in *C. americanum* foliar explants was satisfactory since the clean culture percentage was 94.8%. This is an important step and a significant contribution of this work since this treatment allowed inoculation and growth under aseptic conditions of the foliar explants.

The presence of compact calli on leaf explants of *C. americanum* was first detected 10 days after inoculation (DAI, Fig. 1a) and 30 DAI *in vitro* there was a visible increase in callus biomass (Fig. 1b). This is the first report of callus induction in *C. americanum*. The calli were compact, with translucent and elongated cells (Fig. 1a-b). Calli growth occurred on the four cut sides of the explants, which had exposed internal tissues, but did not occur on the epidermal surfaces of the explants (Fig. 1b). Callogenesis occurred in all hormonal treatments tested. Regardless of the concentration and combination of growth regulators in the treatment, no differences in shape, color and cellular structure were observed in the formed calli.

According to the analysis of variance of the data, there was significant interaction ($p > 0.05$) of the 2,4-D and BAP concentrations with the variable oxidation with the level of oxidation observed in explants (Table 1).

The effect of 2,4-D and BAP concentrations on callus oxidation was analyzed and the results observed 30 DAI are presented in Fig. 2 and Table 1. There was a quadratic behavior of callus oxidation percentage as a function of combined 2,4-D and BAP concentrations. The lowest oxidation percentage (62.5%) was observed for foliar explants inoculated in medium containing 5 mg L-1 BAP and 2.5 mg L-1 of 2,4-D, and the highest percentage of oxidation was observed on explants growing in medium containing a combination of 5 mg L-1 of BAP and 7.3 mg L-1 of 2,4-D. The differences in oxidation percentages observed on foliar explants growing in treatments containing 10 mg L-1 BAP combined with different concentrations of 2,4-D were not significant quote the level. In line with these results, callus yellowing in *Lycopodiella inundata* increased as 2,4-D and NAA (naphthalene acetic acid) concentrations increased in the culture medium [12].

Table 1. Callus oxidation in *C. americanum* leaf explants at 30 days after inoculation in culture media containing BAP ($p > 0.05$)

2,4-D mg.L^{-1}	BAP 5 mg.L^{-1}	BAP 10 mg.L^{-1}
2.50	62.50	71.67
5.00	84.17	80.00
7.50	95.00	75.00
10.00	82.50	68.00

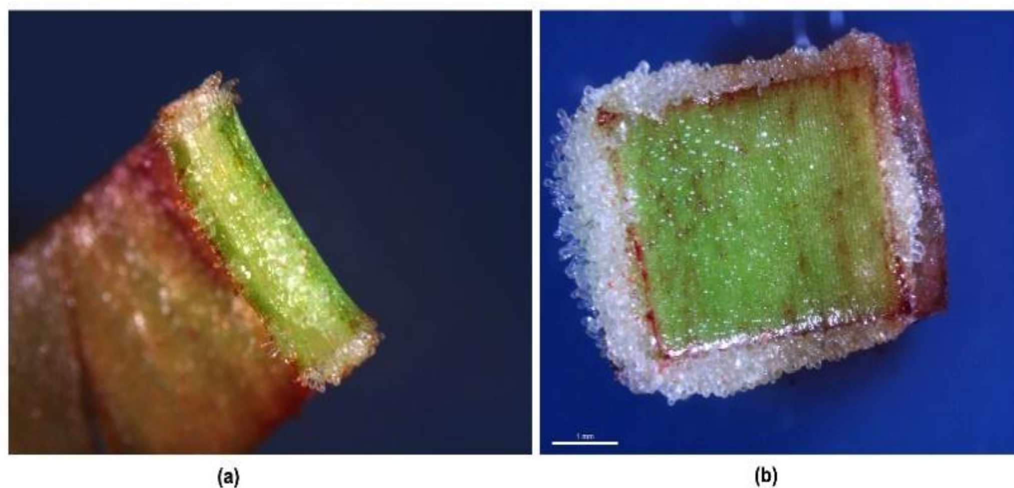

Fig. 1. Callus produced on *C. americanum* young leaf explants with translucent, compact and elongated cells: (a) at 10 days of *in vitro* culture (Bar = 0.1 mm); (b) after 30 days of *in vitro* culture (Bar = 1 mm)

3.2 Callus Biomass

According to the analysis of variance of the data, there was no significant interaction ($p > 0.05$) of the 2,4-D and BAP concentrations with the variable callus biomass. Regarding the effect of 2,4-D and BAP concentrations on callogenesis induction, it was observed in the present study that 30 DAI, the percentage of callogenesis-responsive explants varied as a function of the type and concentration of the growth regulators present in the culture medium (Fig. 3). It is generally reported that 2,4-D is the best auxin to induce and enhance callogenesis *in vitro*. The results observed in the present work corroborate the stimulating effect of 2,4-D on callogenesis induction. Concerning the effect of 2,4-D on callogenesis in leaf explants of *C. americanum*, it was observed that the highest callus induction percentage (45%) and callus biomass occurred in treatments containing 2.5 mg L^{-1} of 2,4-D (Fig. 3a).

A regression analysis of the data showed a decreasing model with a quadratic behavior for 2,4-D concentrations 2.5; 5.00; 7.50 and 10.00 mg L^{-1}, getting 45.00; 13.30; 16.70 and 28.30%, respectively (Fig. 3a). In culture mediums supplemented only with the cytokinin BAP, the best callus induction (33%) was observed in leaf explants growing in medium containing 10 mg L^{-1} of BAP, and the difference in callogenesis response was statistically significant (Fig. 3b). The calli formation in catingueira (*Caesalpinia pyramidalis* Tul.) explants were observed with addition of the highest concentrations of cytokinins [17].

Callus induction on foliar explants of *C. americanum* occurred similarly to what has already been reported for other plant species. Histology of callogenesis has shown that cell multiplication occurs from parenchyma tissues, which are internal to the leaf, without divisions of the epidermal cells, which is what was observed in the present study. Likewise, the use of the growth regulators proved to be important for callogenesis in explants of *C. americanum*, as previously reported for growth and differentiation of callus on explants of "Catimor" coffee and habanero pepper [18,19]. The effect of growth regulator concentrations on callus consistency was also studied in the ornamental plant *Alocasia micholitziana* Sander (Araceae) and in this species the reduced concentrations of BAP and 2,4-D led to formation of friable calli, while higher concentrations induced non friable callus [20]. A similar pattern was evidenced when promoting *Narcissus confusus* Pugsley non-embryogenic calli [21]. Changes in callus structures occur as endogenous nutrients and hormones become available for the cells, thus defining their histological patterns [22]. This behavior may be attributed to growth regulators, since they can inhibit, promote or modify different physiological responses in plants, regardless of the assigned concentrations, since they control the plant growth and development [23]. The use of auxins in the culture medium is essential for *Leucojum aestivum* calli formations [24].

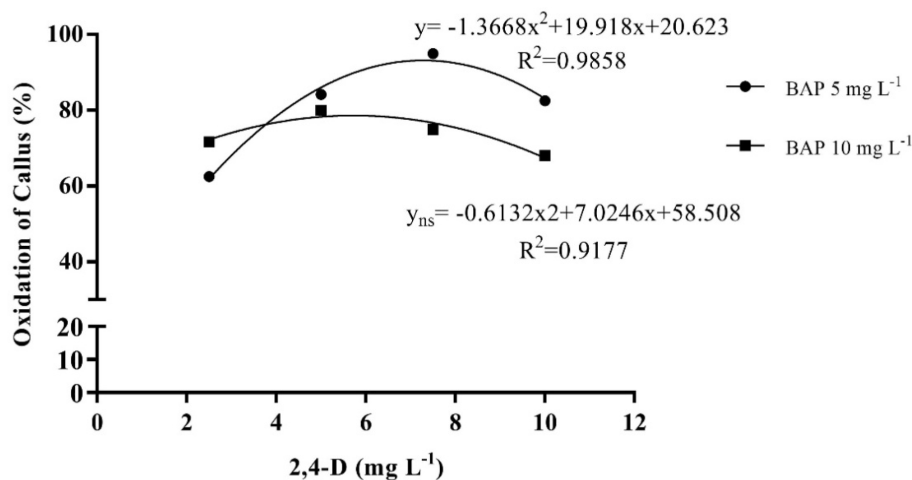

Fig. 2. Callus oxidation in *C. americanum* leaf explants at 30 days after inoculation in culture media containing BAP

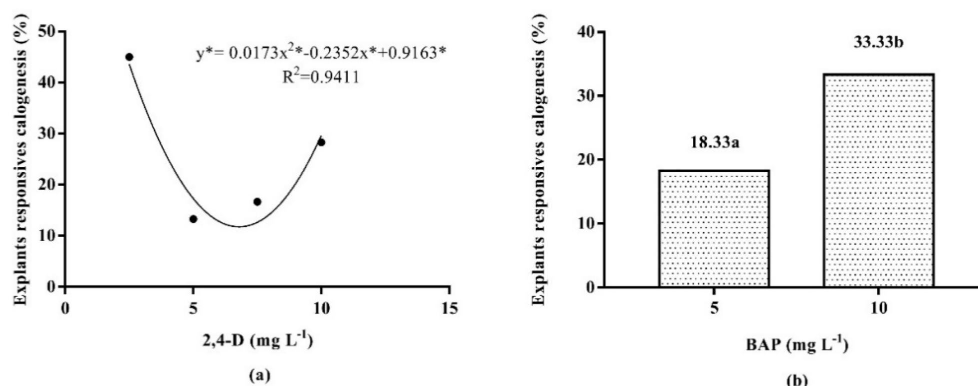

Fig. 3. Percentage of *C. americanum* leaf explants responsive to callus induction. (a) Regression analysis for 2,4-D concentrations at 30 days post inoculation. (b) Tukey test at 5% probability of BAP concentrations at 30 days post inoculation

Concentrations of 2 or 4 mg L^{-1} of 2,4-D were the best to promote callus induction in *Narcissus confusus* [21]. These studies also verified that there was no callogenesis in the absence of auxins. Calli development in most plants usually requires the use of cytokinin and auxin [25,26]. These hormones interact synergistically or antagonistically. Thus, these results indicate that it is necessary to test different cytokinins as well as other concentrations in order to establish a favorable hormonal balance. *Satureja khuzistanica* Jamzad callus biomass increased progressively as BAP concentration increased [27]. In this work, the effect alone of the BAP in the biomass callus was important (Fig. 3).

The callogenesis procedure described in the present work is an important scientific contribution with many applications, the example of the aseptic *ex situ* cultivation and micropropagation *C. americanum*; be first step to enable the establishment cell suspension cultures for C. *americanum*, possibility future use of bioreactor technology for large scale proliferation of these plant cells for industrial alkaloid production, a technology already applied in the production of galantamine and other related alkaloids [24,28]. Moreover, this procedure can be applied in gene banks for the establishment of in vitro collections of germplasm of *C. americanum*, which is a major contribution for the preservation of native populations of this valuable species [29].

Another important contribution of this work is the of an efficient disinfection treatment of leaf explants of *C. americanum*, which allowed inoculation and growth under aseptic conditions of this species. An interesting investigation observed 100% decontamination in *Crinum variabile* (Jacq.) Herb. leaf explants, however, they did not obtain explants regeneration [3]. The establishment of an efficient disinfection procedure is a challenging step in the culture *in vitro* of species of Amaryllidaceae due to the presence of endophytic fungi that are difficult to remove and can prevent the establishment of *in vitro* cultures of these plants.

4. CONCLUSION

This is the first report of callogenesis in *C. americanum* leaf explants. The establishment of efficient disinfection procedures was determined for studied species. The best callus induction and growth response was observed on 2.5 mg L^{-1} of 2,4-D and 10 mg L^{-1} of BAP. It was also observed that the lowest oxidation rate in leaf explants was correlated with lower auxin concentration in the culture medium. Therefore, this work contributes not only to future studies on *in vitro* callogenesis of this species, but also to a possible protocol for the production of alkaloids of interest from cell suspension cultures produced *in vitro*, which is justified by the pharmacological relevance of these plants.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Amaral-Lopes AC, Cavalcanti TB. Brasil *Habranthus* (Amaryllidaceae) of Brasil. Rodriguesia. 2015;66(1):203-220.
2. Verdoorn C. The Genus *Crinum* in Southern Africa. Bothalia. 1973;11(1):27-52.
3. Fennell CW, van Staden J. *Crinum* species in traditional and modern medicine. J Ethnopharmacol. 2001;78:15-26.
4. Nair JJ, van Staden J. Pharmacological and toxicological insights to the South African Amaryllidaceae. Food Chem Toxicol. 2013;62:262-275.
5. Berkov S, Bastida J, Viladomat F, Codina C. Analysis of galanthamine-type alkaloids by capillary gas chromatography-mass spectrometry in plants. Phytochem Anal. 2008;19(4):285-293.
6. Liu J, Hu WX, He LF, Ye M, Li Y. Effects of lycorine on HL-60 cells via arresting cell cycle and inducing apoptosis. FEBS Lett. 2004;578(3):245-250.
7. Bastida J, Lavilla R, Viladomat F. Chemical and biological aspects of *Narcissus* alkaloids. In: The Alkaloids: Chemistry and Biology. San Diego: Elsevier; 2006.
8. Takos AM, Rook F. Towards a molecular understanding of the biosynthesis of amaryllidaceae alkaloids in support of their expanding medical use. Int J Mol Sci. 2013; 14(6):11713-11741.
9. Havelek R, Muthna D, Tomsik P, Kralovec K, Seifrtova M, Cahlikova L, Hostalkova A, Safratova M, Perwein M, Cermakova E, Rezacova M. Anticancer potential of amaryllidaceae alkaloids evaluated by screening with a panel of human cells, real-time cellular analysis and Ehrlich tumor-bearing mice. Chem Biol Interact. 2017;275:121-132.
10. Ochoa-Villarreal M, Howat S, Hong S, Jang MO, Jin YW, Lee EK, Loake GJ. Plant cell culture strategies for the production of natural products. BMB Rep. 2016;49(3):149-158.
11. Wilson SA, Roberts SC. Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. Plant Biotechnol J. 2012; 10(3):249-268.
12. Bienaimé C, Melin A, Bensaddek L, Attoumbré J, Nava-Saucedo E, Baltora-Rosset S. Effects of plant growth regulators on cell growth and alkaloids production by cell cultures of *Lycopodiella inundata*. Plant Cell Tissue Organ Cult. 2015;123(3):523-533.
13. Hussain A, Ahmed I, Nazir H, Ullah I. Plant Tissue Culture: Current Status and Opportunities. In: Leva A, Rinaldi LMR, editors. Recent Advances in Plant in vitro Culture. Intech Open; 2012.
14. Amani S, Zarei H, Azar AM, Mashayekh K. Micropropagation of *Hippeastrum hybridum*. Sci J. 2015;36(4):594-605.
15. Murashige T, Skoog F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant. 1962;15(3):473-497.
16. Ferreira DF. Sisvar: A computer statistical analysis system. Ciênc Agrotec. 2011; 35(6):1039-1042.
17. Silva TDS, Nepomuceno CF, Borges BPdS, Alvim BFM, De Santana JRF. Multiplicação *in vitro* de *Caesalpinia pyramidalis* (Leguminosae). Sitientibus Ser Ciênc Biol. 2013;13:1-6. Portuguese.
18. Santana-Buzzy N, López-Puc G, Canto-Flick A, Barredo-Pool F, Balam-Uc E, Avile´s-Vinãs S, Solís-Marroquín D, Lecona-Guzmán C, Bello-Bello JJ, Gómez-Uc E, Mijangos-Cortés JO. Ontogenesis of the Somatic Embryogenesis of Habanero Pepper (*Capsicum chinense* Jacq.). Hort Science. 2009;44(1):113-118.
19. Menóndez-Yuffá A, García de García E. Morphogenic events during indirect somatic embryogenesis in coffee "Catimor" Protoplasma. 1997(199):208-214.
20. Thao NTP, Ozaki Y, Okubo H. Callus induction and plantlet regeneration in ornamental *Alocasia micholitziana*. Plant Cell Tissue Organ Cult. 2003;73(3):285-289.
21. Sellés M, Viladomat F, Bastida J, Codina C. Callus induction, somatic embryogenesis and organogenesis in *Narcissus confusus*: Correlation between the state of differentiation and the content of galanthamine and related alkaloids. Plant Cell Rep. 1999;18(7-8):646-651.
22. Jimenez VM, Bangerth F. Endogenous hormone concentrations and embryogenic callus development in wheat. Plant Cell Tissue Organ Cult. 2001;67:37-46.

23. Vieira EL, Souza GS, Santos AR, Silva JS. Manual de Fisiologia Vegetal. São Luís: EDUFMA; 2010. Portuguese.
24. Ptak A, Simlat M, Kwiecień M, Laurain-Mattar D. *Leucojum aestivum* plants propagated in *in vitro* bioreactor culture and on solid media containing cytokinins. Eng Life Sci. 2013;13(3):261-270.
25. Cooke TJ, Poli D, Sztein AE, Cohen JD. Evolutionary patterns in auxin action. Plant Mol Biol. 2002;49(3/4):319-338.
26. George EF, Hall MA, Klerk GJD. Plant growth regulators II: cytokinins, their analogues and antagonists. In: George EF, Hall MA, Klerk GJD, editors. Plant Propagation by Tissue Culture. Dordrecht: Springer; 2008.
27. Sahraroo A, Babalar M, Mirjalili MH, Moghaddam MRF, Ebrahimi SN. *In-vitro* Callus Induction and Rosmarinic Acid Quantification in Callus Culture of *Satureja khuzistanica* Jamzad (Lamiaceae). Iran J Pharm Res. 2014;13(4):1447-1456.
28. Ivanov I, Georgiev V, Georgiev M, Ilieva M, Pavlov A. Galanthamine and related alkaloids production by *Leucojum aestivum* L. shoot culture using a temporary immersion technology. Appl Biochem Biotechnol. 2011;163(2):268-277.
29. Pilatti FK, Aguiar T, Simões T, Benson EE, Viana AM. *In vitro* and cryogenic preservation of plant biodiversity in Brazil. In Vitro Cell Dev Biol Plant. 2010;47(1):82-98.

© 2020 Gomes-Copeland et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/59346