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In Vitro Micropropagation and Callus Induction of Mulateiro (Calycophyllum Spruceanum) Seeds Collected from the Amazon Basin

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Abstract:
An in vitro micropropagation and callus induction protocol was developed from seeds of an important medicinal plant Calycophyllum spruceanum (Benth.) known as "mulateiro". This protocol provides an alternative and rapid mean for improvement and multiplication of C. spruceanum through germination and tissue culture using different concentrations of auxin 2,4-D. Different concentrations of 2,4-D auxin alone can produce callus, thus, concentrations of 2,4-D (1 mg.L-¹ and 2 mg.L-¹) were the most effective for callus induction of the medicinal plant C. spruceanum. Mulateiro has high commercial value and is used in the preparation of several cosmetic products and is used in poultice form as an anti-inflammatory, antifungal, healing and rejuvenating agent. Efficient micropropagation of C. spruceanum under in vitro conditions is an important step toward improvement of this neglected but important medicinal plant. Therefore, it is necessary to report an efficient and reproducible protocol for rapid and large-scale propagation of mulateiro by in vitro micropropagation through tissue culture and callus induction. Thus, this study describes a successful in vitro regeneration system of mulateiro for timber and medicinal purposes.

Key words: *Medicinal plant, auxin 2,4-D*

1. Introduction

The mulateiro (*Calycophyllum spruceanum* (Benth.) Hook. F. Ex K. Schum, Rubiaceae), is a species of medium to large size (about 35 m) and rapid growth, typical of primary and secondary forests of humid or temporarily flooded in the Amazon basin. Mulateiro has high commercial value and is used in the preparation of turned products, frames, planks, rafters, firewood and medicinal products (Bandeira *et al*., 2010). Calycophyllum is a small genus with only about six species spread through tropical America; this particular species is indigenous to the Amazon basin in Brazil, Peru, Bolivia, Colombia and Ecuador (Estella, 1995). It is called mulateiro or pau-mulato in Brazil, and capirona in Peru. Mulateiro is noted for its ability to completely shed and regenerate its bark on a yearly basis, making harvesting the bark a totally renewable and sustainable enterprise. The bark is smooth (as if polished) and changes colour throughout the year as it mature - going from a green tone to a brownish tone (Leslie, 2006). Vegetable species belonging to the Rubiaceae family are important as a source of economic and therapeutic value (Di Stasi, 2002). In the Amazon the "mulateiro" bark is used in poultice form as an anti-inflammatory, antifungal, healing and rejuvenating agent (Almeida, 2003). In Peru it is used to treat eye infections. In Paraguay it is used to treat diabetes and in Colombia it is used against parasites and skin diseases (Revilla, 2001). Studies performed with isolated secoiridoids from ethanol extract of mulateiro presented anti-trypomastigote in vitro activity (Portillo & Villa, 2001). Plant cell culture and callus induction is a potentially useful technique for micropropagation and improvement of plants with biotechnological purposes (Debnath *et al*., 2006). Traditional methods like vegetative reproduction or seeds germination have been mainly employed to multiplicate the mulateiro tree. Therefore, it is necessary to report an efficient and reproducible protocol for rapid and large-scale propagation of mulateiro by *in vitro* micropropagation through tissue culture and callus induction. Thus, this study describes a successful *in vitro* regeneration system of mulateiro for timber and medicinal purposes.

2. Material and Methods

2.1 Plant Propagation

Seeds of *C. spruceanum* were collected from a rural area of Rio Branco city, Acre - Brazil. Seeds were treated with ethanol 70% for 30 sec followed by 10% commercial bleach for 10 min and then rinsed twice with distilled water. Germination was achieved by placing the seeds in sterile vessels containing Murashige and Skoog (MS) medium supplemented with 30 g L^{-1} sucrose, 1 mg L^{-1} naphthalene acetic acid (NAA) and 1 mg L^{-1} 6- benzyl amino purine (BAP) and maintained in a growth chamber at 18-22°C with fluorescent light and 16 hrs light and 8 hrs dark photoperiod until germination.

2.2 Callus Induction

In vitro Plantlets large enough to handle were transferred to sterile Petri dishes and cut into 1-2 mm pieces using sterile forceps and scalpel blade. The cut tissues were plated onto callus induction medium containing MS salts, vitamins, myo-inositol (100 $mg.L^{-1}$), sucrose (30 gl^{-1}), phytagel (3.0 gl^{-1}), BAP (2.0 mg L^{-1}), 2,4-D, adjusted to pH 5.8 and incubated at 20 - 24 °C in a dark room. The induced callus mass was monitored for 90 days for further use. The experiment used 5 treatments of auxin 2,4-D (0, 1.0, 2.0,3.0,4.0 and 5.0 $mg.L^{-1}$) with 5 replicates (vessels) of each treatment containing 5 explants.

3. Results and Discussion

3.1. Plant propagation

In order to obtain germination of *C. spruceanum* (mulateiro), seeds were scarificated with sulphuric acid for two hours before sterilization and incubation in MS medium. Because of the lack of previous protocols on *in vitro* growth of *C. spruceanum* in the literature, it was decided to test a standard MS media for plants to obtain the best conditions to grow, propagate and root the germinated plants (see Material and Methods). This germination treatment has given significant results (Fig. 1) similar to those described by Sixtus *et al*. 2003. Induction of shoot regeneration started after one week of culture with initiation of shoot meristems followed by growth of shoot buds thereafter. Excellent results were obtained with this standard media. Among them, plants showed the typical small leaves with rapid growth and similar phenotypes comparing to those obtained for plants grown in soil (Fig. 1). Based on these results, it is recommended to grow and propagate the mulateiro plants in this medium to obtain the explants required for further regeneration assays.

Figure 1: In vitro germination of C. spruceanum, germinated seedlings with well-developed root system

3.2 Callus induction

Callus cultures are extremely important in plant biotechnology. *C. spruceanum* provides a powerful tool to produce healthy and vigorously planting material, which can also help to produce plants genetically similar to their parent plants. Callus induction behavior of *C. spruceanum* explants used in this experiment showed variation under the influence of different 2,4-D plant hormone concentrations. The callus was produced from the explants after 4 weeks of culture in the dark. It was previously reported that callus induced in the dark condition is beneficial for callus growth in comparison of light condition (Hamideh *et al*, 2012). The results of the present study shows that, 2,4-D auxin alone can produce callus and concentrations of 2,4-D (1 $mg.L^{-1}$ and 2 $mg.L^{-1}$, fig. 2) were the most effective for callus induction of the medicinal plant *C. spruceanum*.

These results are in line with other research, same as many other plants, because 2,4-D is the primary auxin which is used for the callus induction. It was previously reported that equal concentrations of auxin and cytokinen induced large and fragile callus which had better potential for regeneration (Hitmi et al, 1998), therefore, further tests should be carried out for *C. spruceanum* using these two hormones. Manipulation of the auxin in the medium can lead to the development of shoots, roots, or somatic embryos from which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies.

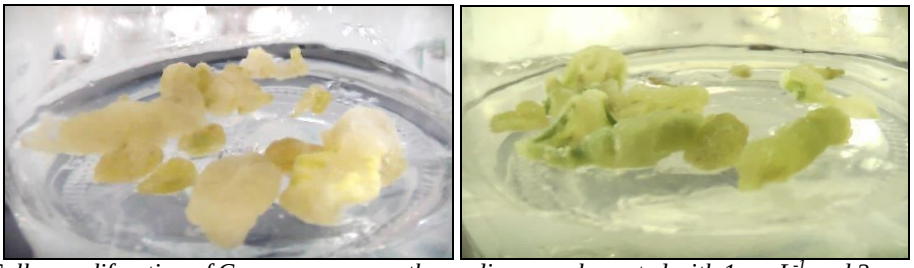

Figure 2: Callus proliferation of C. spruceanum on the medium supplemented with 1 mg.L^{-1} and 2 mg.L^{-1} of 2,4-D

4. Conclusion

C. spruceanum can be easily reproduced *in vitro* by using a standard MS medium and callus induction is well achieved by using concentrations of 1 mg.L^{-1} and 2 mg.L^{-1} of the auxin 2,4-D. Based on these results, it is concluded that regeneration of *C. spruceanum* could provide an effective tool to establish diverse strategies which might potentiate the biotechnological use of this medicinal plant. It could also provide and environmentally friendly, renewable and alternative supply for reforestation, timber, genetic improvement and for future use in secondary metabolite production *in vitro.*

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6. References

1. Estrella E. (1995). Plantas Medicinalis Amazonicas: realidad e perspectivas. Manaus: Editora TCA.
2. Di Stasi L.C., Hiruma C.A. (2002). Plantas medicinais da Amazônia e na Mata Atlântica. 2 ed. São Paulo: Editora Unesp.
3. Leslie Taylor, ND. (2006). Technical Data Report for Mulateiro or Capirona (Calycophyllum spruceanum). 3579 Hwy 50 East, Suite 222, Carson City, NV. 89701.
4. Almeida M.C. (2003). Aspectos ecofisiológicos da germinação de sementes de mulateiro (Calycophyllum spruceanum Benth) - Rubiaceae - Instituto de Biociências da Universidade Estadual Paulista. Available at http:// www.biblioteca.unesp.br. Acessed on 20 September 2013.
5. Revilla J. (2001). Plantas da Amazônia: oportunidades econômicas e sustentáveis. Manaus: co-edição Sebrae/INPA.
6. Portillo A., Villa R. (2001). Antifungal activity of Paraguayan plants used in traditional medicine. J Ethnopharmacol 88: 93-98.
7. Debnath, M., Malik, C.P., Bisen, P.S. (2006). Micropropagation: A tool for the production of high quality plant-based medicines. Current Pharmaceutical Biotechnology, vol. 7, no. 1, p. 33-49.
8. Bandeira J.R., Queiroz J.B.N., Ferreira E.J.L., Nascimento J.F., Carvalho A.L., Mendonça C.C. (2010). Possibilidades para a exploração do mulateiro (calycophyllum spruceanum) em um fragmento florestal secundário da área de proteção ambiental Raimundo Irineu Serra, Rio Branco, Acre. 62ª Reunião Anual da SBPC. Available at: http://www.sbpcnet.org.br/livro/62ra/resumos/resumos/5295.htm. Acessed on 20 September 2013.
9. Sixtus, C.R., Hill, G.D., Scott, R.R. (2003). The effect of temperature and scarification method on gorse (Ulex europaeus L.) seed germination. New Zealand Plant Protection, vol. 56, p201-205.
10. Hitmi A, Barthomeuf C, Sallanon H (1998). Rapid mass propagation of Chrysanthemum cinerariaefolium Vis. by callus culture and ability to synthesise pyrethrins. Plant Cell Rep., 19: 156-160