



Effect of Phytohormones on Leaf Explants of *Strychnos potatorum* L. – An Endangered Medicinal Plant

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ABSTRACT

Tissue culturing of medicinal plants is widely used to produce active compounds for herbal and pharmaceutical industries. Conservation of genetic material of many threatened medicinal plants also involves culturing techniques. This work reviews *in vitro* micropropagation techniques and gives examples of various commercially important medicinal plants. Fresh leaves of *Strychnos potatorum* plants collected from natural growing populations was sterile inoculated on media. For an efficient callusing a range of 18 variant of basal MS (Murashige - Skoog) media formulation was tested, with different growth regulators combinations. The best rate of callus production was achieved after 3 weeks of culture on media supplemented with 0.4 mg/l KIN and 3 mg/l 2,4-D. The established protocols offer a valuable micropropagation method that could be useful as a starting point for *in vitro* conservation of this endangered plant, or for mass propagation of species for pharmaceutical importance.

Keywords: Loganiaceae, *Strychnos potatorum* L., endangered plant, plant growth regulators, callusing.

INTRODUCTION

The World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicine¹ resulting into the increasing demand for medicinal plants. Among around 45000 plant species in India near 1500 Plant species are listed as rare and threatened in India². High demand of Medicinal plants leads over exploitation, loss of natural habitats, Environmental pollution etc. are responsible for disappearing the species from a natural habitat³. Many modes have been applied for regeneration/multiplication of the Medicinal plants. Micro-propagation focuses on development of various Medicinal plants by tissue culture techniques. Hormonal treatment for propagation of the plants leads fast multiplication of the plant species⁴. Advances in biotechnology, especially in the area of *in vitro* culture techniques and molecular biology provide some important tools for improved conservation and management of plant genetic resources⁵⁻⁶.

An approach to *ex situ* conservation includes methods like seed storage in seed banks, field gene banks, botanical gardens, DNA and pollen storage⁷. *In vitro* culture includes some techniques involving the growth under sterile conditions and constant environmental factors of plant germplasm on artificial culture media. Explants are mostly shoot, leaf, flower pieces, immature embryos, hypocotyls fragments or cotyledons⁸. Generally, younger and more rapidly growing tissues are suitable. The criteria for a proper quality explants are normal, true-to-type donor plant, vigorous and disease free⁹. As a rule, fragile tissues including meristems, immature embryos, cotyledons and hypocotyls requires less exposure to sterilizing agents than seeds or lignified organs⁸. Explants

may be obtained from seedlings grown from sterilized seeds. Biotechnology offers avenues for maintenance, genetic improvement and efficient use of endangered plant resources and products¹⁰. Tissue culture is used for conservation of biological diversity by multiplication of plant species that have extremely small populations, for species with restricted reproductive capabilities and for recovery and reintroduction¹¹. The main areas of research in plant tissue culture viz. micropropagation, anther and microspore culture, somaclonal variations and mutagenesis, protoplast culture and somatic hybridization are some of the effective tools for regeneration and conservation of endangered plants¹⁰.

Strychnos potatorum L. (Fam: Loganiaceae) is a moderate sized tree found in southern and central parts of India, Sri Lanka and Burma¹². The *Strychnos potatorum* L. is recognized as endangered and vulnerable plant species by Indian Forest Department, MOEF circular dt. 4.10.2000, P.R. Sinha & G.S. Rawat 2008, Alliance for Natural Health International, 2011¹³. The root cures all kind of leucoderma. The ripe fruit is emetic, diaphoretic, alexiteric, cures inflammation, anemia, jaundice¹⁴. The seeds are used in hepatopathy, nephropathy, gonorrhoea, leucorrhoea, gastropathy, bronchitis, chronic diarrhoea, dysentery, renal and vesicle calculi, diabetes, burning sensation, dipsia, conjunctivitis, scleritis, ulcers and other eye diseases¹⁵. Phytochemical studies revealed the presence of diaboline (major alkaloid) and its acetate¹⁶, triterpenes and sterols¹⁷, mannogalactans¹⁸. The seeds are reported to have various activities like antidiabetic¹⁹, antihypercholesterolemic activity²⁰, diuretic²¹, anti diarrhoeal²², hepatoprotective²³ and antiulcer²⁴.

MATERIALS AND METHODS

Tissue culture techniques

There are many types of tissue culture techniques available for micropropagation and plant regeneration. Some commonly used are listed in this section:

Sterilization of explants

The *Strychnos potatorum* L. plants were grown at the Botanical garden of, Institute of Science, Civil Lines, Nagpur, India. The young fresh leaf explants collected from *Strychnos potatorum* L. plant were washed under running tap water for 30 minutes and was carried out surface sterilization according to our previously published report²⁵. The sterilized explants were then excised into small pieces and then inoculated into flask and bottle containing 60 ml basal MS (Murashige - Skoog)²⁶ medium, and sealed under aseptic condition. Cultured flask and bottle were maintained at 22°C with a 14 hr photoperiod (3000 lux).

Explant source

Explant is material used as initial source of tissue culture. Tissue culture success mainly depends on the age, types and position of explants²⁷ because not all plant cells have the same ability to express totipotency²⁸⁻³⁰. The most commonly used explants are shoot tips, nodal buds and root tips. Large explants can increase chances of contamination and small explants like meristems can sometimes show less growth³⁰⁻³¹.

Sterilization

Microbial contamination of plant tissue culture is a common problem³¹⁻³³. Common bacterial contaminants are *Bacillus*, *Pseudomonas*, *Staphylococcus* and *Lactobacillus*³³⁻³⁵. Preventing of microbial contamination of plant tissue cultures through sterilization is crucial to successful micropropagation of plant. The identification of common contaminants of the explants may proved to be effective means for preventing contamination by adding small quantity of fungal and / or bacterial specific antibiotics in the cultures media³⁶. Microbes multiply and compete with growing explant for nutrients, while releasing chemicals which can alter culture environments e.g. pH and inhibit explants growth or cause death³⁰⁻³³. Explants are cleaned by distilled water and sterilized using 2% bovistin, 0.1% Sodium hypochlorite, and ethyl alcohol^{32, 37-38}. Sterilization of laboratory instruments is carried out by autoclaving, alcohol washing, baking, radiations, flaming and fumigation³⁹. A considerable decrease in bacterial contamination was seen by using ultrasonic sonicator⁴⁰.

Tissue culture Media

Culture media contains vital nutrients and elements for *in vitro* growth of plant tissues. Choosing the right media composition is important for successful tissue culturing^{27,41-42}. Medium contains a carbon source (sucrose), macro and micro nutrients, vitamins, hormones

and other organic substances^{31, 41}. A wide range of media are available for plant tissue culture, but MS²⁹ medium is commonly used^{27, 29, 41}. Other media used are Linsmaier-Skoog (LS)⁴³, Schenk and Hilderbrandt (SH)⁴⁴, WPM (Woody plant medium)⁴⁵, and the Nitsch and Nitsch (NN)⁴⁷. Agar is not essential media component but is used as gelling agent^{39, 41}. It prevents death of cultured cells due to submerging and lack of oxygen in liquid medium. The pH of culture media is normally between 5.0-6.0, and is also very important as it affects uptake of ions⁴¹.

Plant growth hormones

Growth hormones regulate various physiological and morphological processes in plants and are also known as plant growth regulators (PGRs) or phytohormones^{41, 47}. PGRs are synthesized by plants; therefore many plant species can grow successfully without external medium supplements⁴⁸⁻⁵⁰. Hormones can also be added into cultures to improve plant growth and to enhance metabolite synthesis^{39, 41}. As observed in *S. potatorum*, *in vitro* callusing was not achieved without adequate concentrations exogenous hormones.

Culture Browning

Explants in cultures release phenol compounds, which are oxidised by enzymes known as polyphenol oxidase, and cause the media to turn brown^{32, 51}. Browning can be minimized by adding antioxidants or phenol absorbents for e.g. ascorbic acid, glutathione, activated charcoal and polyvinylpyrrolidone³⁸ or by transferring explants into new culture media on regular intervals^{32, 52}.

Callus Induction

Callus is an undifferentiated mass of tissue which appears on explants within a few weeks of transfer onto growth medium with suitable hormones⁴¹. Callus formation occurs from reversed process of cell differentiation, known as dedifferentiation or redifferentiation³¹. Different growth hormones are used to promote callus induction and development. New plants can be successfully regenerated from callus through organogenesis⁵³. The 18 variant of basal MS media were tested to stimulate callusing (with 1/10 auxin/cytokinin ratio). Callus induced from leaf explants. Callus cultures were multiplied and maintained by subculturing onto MS medium using 0.4 mg/l KIN and 3 mg/l 2,4-D at three week intervals.

RESULTS AND DISCUSSION

In the present study, we have tried different growth media for the maximum induction of calli. It includes Murashige and Skoog, Gamborg's B5, and White's media having different hormonal combinations of auxins (2,4-D, NAA, IAA and IBA) and cytokinins, (Kinetin, 6-BAP and BAP). Murashige and Skoog's medium was found to be suitable for the induction of calli while all other media showed no response.

A simple and effective protocol was developed for the *in vitro* callusing for micropropagation of *S. potatorum*. We investigated the effect of different auxins and cytokinins



on the efficiency of callusing. Callus development from leaf explants was unsuccessful for most of the cytokinin treatments. The combination of cytokinin Kinetin (KIN), at the lower concentration (0.4 mg/L) and auxins 2,4-Dichlorophenoxyacetic acid (2,4-D), at (3 mg/L) produced the callus from leaf explants, and resulted in the maximum growth at this concentration. During the initial stage (up to 2 weeks of incubation), callus growth was initiated from the base of some explants and expansion and proliferation of cells at the cut surface were observed.

In *Cephaelis ipecacuanha* 2, 4-D and NAA along with kinetin promoted callus induction and growth⁵⁴. Finnie

and Van Staden⁵⁵ observed that MS basal medium with only 2,4-D showed the callus formation *Gloriosa* and *Sandersonia* plant. Jadhav and Hegde⁵⁶ also reported that the callus formation from *Gloriosa* occurs at 2,4-D (18.08 μ M) + Kn 23.20 μ M + CH (10 mg/l) + CW (20%). On the contrary, in the present study, we have reviewed many research articles on the *S. potatorum* but yet there is no report of callusing from any explants for micropropagation. This is the first hand report of its own kind. There is no such work type of research has been carried out.

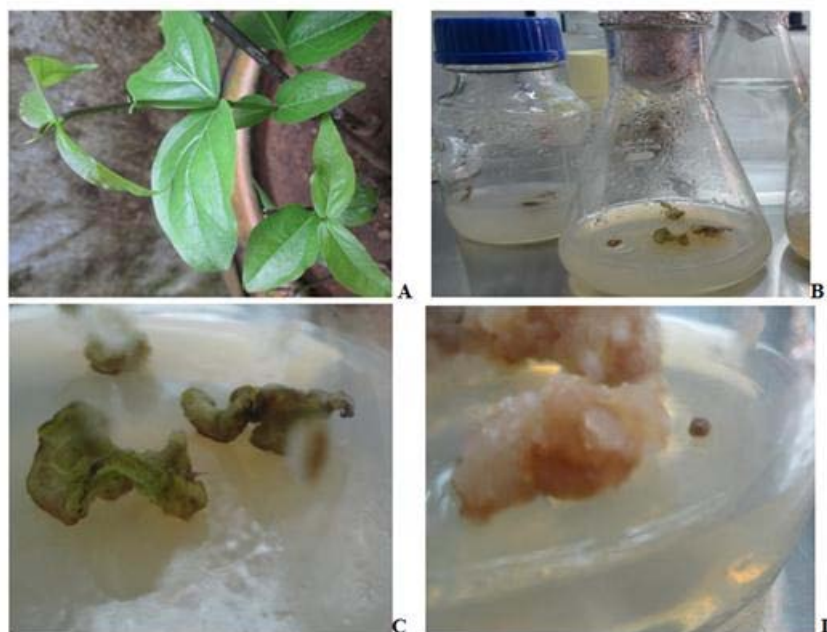


Plate-1: A- *S. potatorum* twig B-Explant, B-Callus Initiation, C- Callus

CONCLUSION

This is the first report describing callus induction protocol for micro-propagating *S. potatorum* using leaf explants. MS medium supplemented with 0.4 mg l⁻¹ KIN and 3.0 mg l⁻¹ 2,4-D is the most effective medium for callus induction. This protocol could be utilized for conservation and clonal propagation as well as chemical analysis of this medicinally important endangered plant. This protocol can be exploited for conservation and commercial propagation of this plant in the Indian subcontinent and may be useful for genetic improvement programs. The prime importance of *in vitro* propagation of rare, endangered and vulnerable plants would be to generate a large number of planting materials from a single explant without destroying the mother plant and subsequently their restoration in the natural habitat, thus conserving the biodiversity.

No any reports were found in literature regarding the *in vitro* callus production from *S. potatorum* plant. Although the plant is under threatened and endangered category, it must require a standard method for conservation and

high yield of similar secondary metabolites as in the natural plant. Above all in view and consideration the current work was undertaken to produce a standard procedure for the *in vitro* induction of callus. Advances in plant tissue culture will enable rapid multiplication and sustainable use of medicinal plants for future generations.

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REFERENCES

1. Kala, C.P., Indigenous uses, population density and conservation of threatened medicinal plants in protected areas of the Indian Himalayas. *Conservation Biology* 19, 2005, 368-378.
2. Myers N. Threatened biotas: 'hotspots' in tropical forests. *Environmentalist* 8, 1988, 187-208.
3. Yadav SR, Mayur Y. K., Threatened Ceropogias of the Western Ghats and Strategies for their Conservation. In:

- Rawat GS (Ed.), Special Habitats and Threatened Plants of India. ENVIS Bulletin: Wildlife and Protected Areas, Wildlife Institute of India, Dehradun, India 11(1) 2008, 239.
4. Constabel, F, Medicinal plant biotechnology. *Planta Med.* 56, 1990, 421–425.
 5. Ramanatha Rao V, Riley R, The use of biotechnology for conservation and utilization of plant genetic resources. *Plant Genet Resour Newsl* 97, 1994, 3-20.
 6. Withers L. A , Collecting in vitro for genetic resources conservation. In: Guarino L, Ramanatha Rao V, Reid R (eds) *Collecting plant germplasm diversity*, Technical Guideline, CABI, Wallingford, UK, 1995, 51-526.
 7. Rao N.K, Plant genetic resources: Advancing conservation and use through biotechnology. *African J Biotech* 3 (2), 2004, 136-145.
 8. Paunesca A., Biotechnology for endangered plant conservation: A critical overview. *Romanian Biotech Letters* 14 (1), 2009, 4095-4104.
 9. Fay, M.F., Conservation of rare and endangered plants using in vitro methods, *In Vitro Cellular and Developmental Biology – Plant*, 28, 1992, 1–4.
 10. Bapat, V.A., Yadav, S.R., Dixit, G.B., Rescue of endangered plants through biotechnological applications. *Natl. Acad. Sci. Lett.* 31, 2008, 201-210.
 11. Bramwell, D, The role of in vitro cultivation in the conservation of endangered species. In: Hernández, B.J.E., Clemente, M., Heywood, V. (Eds.) *Proc. Int. Congress of Conserv. Techniques in Botanic Gardens*, Koeltz Scientific Books. 1990, 3-15.
 12. Kirtikar KR, Basu BD, Indian medicinal plants. In Allahabad Edited by: Basu LM, 3, 1933.
 13. Kagithoju S., Godishala V. , Kairamkonda M., Kurra H., and Nanna R. S., Recent Advances in Elucidating the Biological and Chemical Properties of *Strychnos potatorum* L.- A Review *Int J Pharm Bio Sci*, 3(4):(B), 2012, 291 – 303
 14. Kirtikar KR, Basu BD, Illustrated Indian Medicinal Plants. Edited by: Mhaskar KS, Blatter, 2000.
 15. Asima C, Satyesh CP, The Treatise on Indian Medicinal Plants. Publications and Information Directorate, CSIR, 4, 2001, 85-87.
 16. Harkishan Singh, Kapoor KVijay, Phillipson JD, Bisset NG, Diaboline from *Strychnos potatorum*. *Phytochem*, 14,1975, 587-588.
 17. Harkishan Singh, Kapoor K. Vijay, Investigation of *Strychnos* Spp.III Study of triterpenes and sterols of *Strychnos potatorum* seeds. *Planta Med*, 28, 1975, 392-396.
 18. Adinolfi M, Corsaro MM, Lanzetta R, Parilli M, Folkard G, Grant W, Sutherland J, Composition of the coagulant polysaccharide fraction from *Strychnos potatorum*, *Carbohydrate Res*, 263,1994, 103-110.
 19. Mathuram LN, Samanna HC, Ramasamy VM, Natarajan R, Studies on the hypoglycemic effects of *Strychnos potatorum* and *Acacia arabica* on alloxan diabetes in rabbits. *Cheiron*, 10, 1981,1-5.
 20. Venkata Rao E, Ramana KS, Venkateswarao M, Revised structure and antihypercholesterolemic activity of a mannogalactan from *Strychnos potatorum*, *Ind J Pharm Sci*, 53, 1991, 53-57.
 21. Biswas S, Murugesan T, Maiti K, Ghosh L, Study on the diuretic activity of *Strychnos potatorum* Linn, seed extract in albino rats. *Phytomed*, 8, 2001, 469-471.
 22. Swathi B, Murugesan T, Sanghamitra Sinha, Antidiarrhoeal activity of *Strychnos potatorum* seed extract in rats. *Fitoterapia*, 73, 2002, 43-47.
 23. Sanmuga priya E, Venkataraman S, Studies on hepatoprotective and antioxidant actions of *Strychnos potatorum* Linn seeds on CCl4 induced acute hepatic injury in experimental rats. *J Ethnopharmacol*, 05, 2006, 154-160.
 24. Sanmuga priya E, Venkataraman S, Antiulcerogenic potential of *Strychnos potatorum* Linn seeds in aspirin+pylorus ligation induced ulcers in experimental rats. *Phytomed*, 14, 2007, 360-365.
 25. Rosli N, Maziah M, Chan KL,Sreeramanan S, Factors affecting the accumulation of 9-methoxycanthin-6-one in callus cultures of *Eurycoma longifolia*, *Journal of Forestry Research* 20, 2009, 54-58.
 26. Murashige, T., Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15,1962, 472- 497.
 27. Gamborg, O.L., T. Murashige, T.A. Thorpe and I.K. Vasil, Plant-tissue culture media. *Journal of the Tissue Culture Association*, 12, 1976, 473-478.
 28. Sasikumar, S., S. Raveendar, A. Premkumar, S. Ignacimuthu, P. Agastian, Micropropagation of *Baliospermum montanum* (Willd.) Muell. Ara.-A threatened medicinal plant. *Indian Journal of Biotechnology*, 8, 2009, 223-226.
 29. Murashige, T. and F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, 15, 1962, 473-497.
 30. Staba, E.J. and J.E.A. Seabrook, *Laboratory Culture. Plant Tissue Culture As a Source of Biochemicals*, CRC Press, Boca Raton, 1980, 1-20.
 31. Fowler, M.R., F.W. Rayns and C.F. Hunter (Editor), *The language and aims of plant cell and tissue culture*. In *Vitro Cultivation of Plant Cells*, Butterworth-Heinemann Ltd, Oxford, 1993, 1-18.
 32. Rout G.R., S. Samantaray, and P. Das, In vitro manipulation and propagation of medicinal plants. *Biotechnology Advances*, 18, 2000, 91-120.
 33. Leifert, C. and W.M. Waites, Bacterial growth in Plant tissue culture media. *Journal of Applied Bacteriology*, 72, 1992, 460-466.
 34. Leifert, C., W.M. Waites, and J.R. Nicholas, Bacterial contaminants of micropropagated plant cultures. *Journal of Applied Microbiology*, 67, 1989, 353-361.
 35. Leifert, C., J.Y. Ritchie, and W.M. Waites, Contaminants of Plant tissue and Cell cultures. *World Journal of Microbiology & Biotechnology*, 7, 1991,452-469.
 36. Somkuwar S.R., Kalkar S. A., Bodele S. K., and Chaudhary R. R, Microbial contamination in *Andrographis paniculata* and *Strychnos potatorum* leaf cultures. *Multilogic in Science* 2, (4), 2013.

37. Prakash, S. and J. Van Staden, Micropropagation of *Hoslundia opposita* Vahl--a valuable medicinal plant. South African Journal of Botany, 73, 2007, 60-63.
38. Matkowski, A, Plant in vitro culture for the production of antioxidants - A review. Biotechnology Advances, 26, 2000, 548-560.
39. Rayns, F.W., M.R. Fowler and C.F. Hunter, Media design and use. In Vitro Cultivation of Plant Cells, Butterworth-Heinemann Ltd. Oxford, 1993, 43-64.
40. Garro-Monge, G., A.M. Gatica-Arias, and Valdez-Melara, Somatic embryogenesis, Plant regeneration and Acemannan detection in Aloe (*Aloe barbadensis* MILL.). Agronomia Costarricense, 32, 2008, 41-52.
41. Bhojwani, S.S. and M.K. Razdan, Plant tissue culture: Theory and Practice: Developments in crop science Elsevier, Amsterdam 5, 1996.
42. Huang, L. and T. Murashige, Plant tissue culture media: major constituents; their preparation and some applications. Tissue Culture Assoc, 3, 1977, 539-548.
43. Linsmaier, E.M. and F. Skoog, Organic growth factor requirement of tobacco tissue cultures. Physiol. Plant, 18, 1965, 100-127.
44. Schenk, R.V. and A.C. Hilderbrandt, Medium and techniques for induction and growth of monocotyledonous dicotyledonous plant cell cultures. Canadian Journal of Botany. 50, 1972, 199-204.
45. Lloyd, G.B. and B.H. McCown, Commercially feasible micropropagation of mountain laurel (*Kalamia latifolia*) by use of shoot tip culture. Proc. Int. Plant Propagators Soc. 30, 1980, 421-437.
46. Nitsch, J.P. and C. Nitsch, Haploid plants from pollen grains. Science, 163, 1969, 85-87.
47. Srivastava, L.M., Plant Growth and Development: Hormones and Environment Academic Press, New York, 2002, 140-143.
48. Bhavisha, B.W. and Y.T. Jasrai, Micropropagation of an Endangered Medicinal Plant: *Curculigo orchoides* Gaertn. Plant tissue Culture, 13, 2003, 13-19.
49. Hussey G, In vitro propagation of monocotyledonous bulbs and corms. Plant Cell, Tissue and Organ Culture, 19, 1982, 677-680.
50. Baksha, R., M.A.A. Jahan, R. Khatum and J.L. Munshi, Micropropagation of *Aloe barbadensis* Mill. through In vitro Culture of Shoot tip Explants. Plant tissue Culture and Biotechnology. 15, 2005, 121-126.
51. Bhat, S.R. and K.P.S. Chandel, A novel technique to overcome browning in tissue culture. Plant Cell Reports, 10, 1991, 358-361.
52. Krishnan, P.N. and S. Seeni, Rapid Micropropagation of *Woodfordia fruticosa* (L) Kurz (Lythraceae), A rare medicinal plant. Plant Cell Reports, 14, 1994, 55-58.
53. Sears, R.G. and E.L. Deckard, Tissue culture variability in wheat- callus induction and plant regeneration. Crop Science, 22, 1982, 546-550.
54. Rout G.R, Samantaray S., and Das P., In vitro somatic embryogenesis from callus cultures of *Cephaelis ipecacuanha* A. Richard. Scientia Horticulturae, 86, 2000, 71-79.
55. Finnie JF and Van Staden J, Micropropagation of *Gloriosa* and *Sandersonia*. J Plant Cell Tiss Organ Cult 19, 1994 151-158.
56. Jadhav SY, Hegde BA, Somatic embryogenesis and plant regeneration in *Gloriosa* L. Indian J Exp Biol, 9, 2001, 943-946.

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