Research Article

Effect of Phytohormones on Leaf Explants of *Strychnos potatorum* L. – An Endegered 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 medicine[1] 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 India[2]. High demand of Medicinal plants leads over exploitation, loss of natural habitats, Environmental pollution etc. are responsible for disappearing the species from a natural habitat[3]. 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 species[4]. 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 resources[5-6].

An approach to *ex situ* conservation includes methods like seed storage in seed banks, field gene banks, botanical gardens, DNA and pollen storage[7]. *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 cotyledons[8]. 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 free[9]. As a rule, fragile tissues including meristems, immature embryos, cotyledons and hypocotyls requires less exposure to sterilizing agents than seeds or lignified organs[8].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 products[10]. 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 reintroduction[11]. 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 plants[10].

Strychnos potatorum L. (Fam: Loganiaceae) is a moderate sized tree found in southern and central parts of India, Sri Lanka and Burma [12]. 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, 2011[13]. The root cures all kind of leucoderma. The ripe fruit is emetic, diaphoretic, alexiteric, cures inflammation, anemia, jaundice[14]. 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 diseases[15]. Phytochemical studies revealed the presence of diaboline (major alkaloid) and its acetate[16], triterpenes and sterols[17], mannogalactans[18].The seeds are reported to have various activities like antidiabetic[19], antihypercholesterolemic activity[20], diuretic[21], antidiarrhoeal[22], hepatoprotective[23] and antiulcer[24].

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 report [25]. 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 asceptic 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 explants [27] because not all plant cells have the same ability to express totipotency [28-30]. 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 growth [30-31].

Sterilization

Microbial contamination of plant tissue culture is a common problem [31-33]. 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 media[36.] 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 death[30-33]. 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 fumigation [39]. A considerable decrease in bacterial contamination was seen by using ultrasonic sonicator[40].

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 MS[29] 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 ions [41].

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 supplements[48-50]. 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 polyvinylpyrrolidone [38] 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 hormones[41]. Callus formation occurs from revered process of cell differentiation, known as dedifferentiation or redifferentiation[31]. Different growth hormones are used to promote callus induction and development. New plants can be successfully regenerated from callus through organogenesis[53]. 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 growth [54]. Finnie and Van Staden[55] observed that MS basal medium with only 2,4-D showed the callus formation *Gloriosa* and *Sandersonia* plant. Jadhav and Hegde[56] also reported that the callus formation from *Gloriosa* occurs at 2,4-D (18.08µM) + Kn 23.20µM + 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.

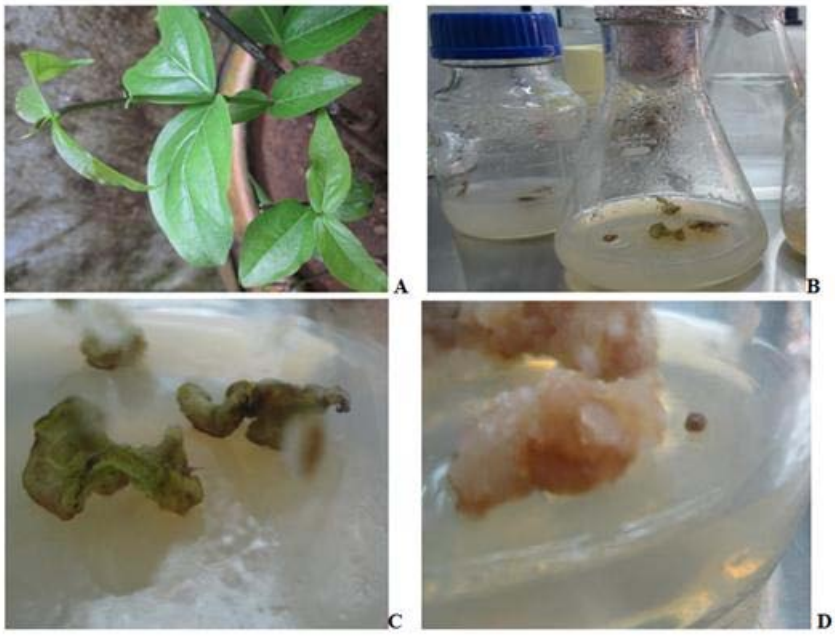

Plate-1: A- *S.potatorum* twig B-Explant, B-Callus Initiation, C- Callus

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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