Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(1): 1818-1822
Received: 14-11-2017
Accepted: 15-12-2017

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In vitro conservation of medicinal plant (*Asparagus racemosus*)

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Abstract
In the present study incubation of zygotic embryo and hypocotyl cultures in dark promoted formation of embryogenic callus, while light promoted non-embryogenic callus formation. Incubation in dark was beneficial for somatic embryo formation in *A. officinalis* (Li and Wolyn, 1995, 1996a) and other plants as *Coffea* (Giridhar *et al*. 2004) and *Echinacea* (Zobayed and Saxena, 2002). It is a very well established fact that 2,4-D is the most preferred auxin for embryogenic callus induction. Further Kn has been also used to complement the action of 2,4-D. However, for induction of somatic embryos, it is essential to remove 2,4-D from the medium. In the present study 2,4-D and Kn do induce formation of compact embryogenic callus but somatic embryo induction was observed even in the presence of 2,4-D. Prolonged exposure of 2,4-D or any other auxin even in the presence of a cytokinin promoted formation of white hairy roots and decreased the number of somatic embryos formed. Ancymidol is a known inhibitor of gibberellic acid biosynthesis and by promoting accumulation of storage protein improves somatic embryo maturation (Li and Wolyn 1996b) as has been in *A. officinalis.*

Keywords: Ancymidol, *A. officinalis. Echinacea*, embryo maturation

Introduction
Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micro propagation. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including:
The production of exact copies of plants that produce particularly good flowers, fruits or have other desirable traits. To quickly produce mature plants. The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds. The regeneration of whole plants from plant cells that have been genetically modified. The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens. The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and *Nepenthes*.
To clear particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.
Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

Techniques: Preparation of plant tissue for tissue culture is performed under aseptic conditions under HEPA filtered air provided by a laminar flow cabinet. Thereafter, the tissue is grown in sterile containers, such as petri dishes or flasks in a growth room with controlled temperature and light intensity. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so their surfaces are sterilized in chemical solutions (usually alcohol and sodium or calcium hypochlorite) [1] before suitable samples (known as explants) are taken. The sterile explants are then usually placed on the surface of a sterile solid culture medium, but are sometimes placed directly into a sterile liquid medium, particularly when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar.

Literature review

1. Bopana N *et al.* (2008) Murashige and Skoog's medium with 2, 4-D and kinetin induced callus in the shoot segments of *Asparagus racemosus*. Regeneration of shoot buds and clonal multiplication of excised shoots through proliferation of nodal buds could be achieved by the use of IAA and BAP in the medium. Rooting was achieved with half strength MS basal medium plus IBA. Complete plants with cladode, crown and root systems were developed in hormone free medium. The plants were successfully transferred to soil.
2. Gomase V.S *et al.* (2010) *Asparagus racemosus* is important medicinal plant of tropical and subtropical India, is a potent phytoestrogen, which is used extensively in the treatment of menopausal symptoms, diarrhea, dyspepsia, and neurodegenerative disorders. The multiple uses of this species have increased its commercial demand resulting in over-exploitation. Consequently, the plant is recognized as being "critically endangered" in its natural habitat. Development of an efficient Isolation, structure elucidation and biotransformation studies protocol will not only play a significant role in meeting the requirement of planting material for commercial cultivation, but also in aiding the conservation process.
3. Arti Sharma *et al.* (2013) the plant *Asparagus racemosus* is widely distributed in the Himalayan and sub-Himalayan regions of India. Based on preliminary reports, there is a lot of interest in using the roots of this plant for treating various disorders in indigenous system of medicine such as antioxidant, anti-diarrheal agent, aphrodisiac, used in the treatment of menopause and immune system modulator. The purpose of work is to study medicinally active substances present in ethanol-extract, Aqueous extract and Benzene extract obtained from roots powder of *Asparagus racemosus*. Preliminary Phytochemical screening of the extracts revealed the presence of Alkaloids, Carbohydrates, Phenolic compounds, tannins, Saponins, Steroids and Flavonoids.

Aim & objectives of work

Aim: *In vitro* conservation and phyto-chemical evaluation of different parts of medicinal plant (*Asparagus racemosus*) of Rajasthan.

Objectives

To develop efficient, reproducible and commercially viable tissue cultureprotocol for high frequency regeneration of *Asparagus racemosus.*

Development of technology for induction multiplication and regeneration of somatic embryo directly from explants and through callus culture and encapsulation of somatic embryo to produce artificial seeds.

To evaluate phytochemicals from different parts of *Asparagus racemosus* using chemoprofiling tools like HPLC, GC, MS etc.

Isolation, Identification and purification of secondary metabolites and aromatic compounds present in culture of the proposed plant and their comparison with naturally growing plants using molecular markers.

In vitro studies using cell suspension culture system will function as continuous and reliable source for the production of medicinally important compounds in large scale.

Analysis using combination of various biotechnological tools will an approachtowards conservation of *Asparagus racemosus*.

Plan of work

The proposed studies will be carried out in two phases:-

Development of reproducible and commercially viable tissue culture protocol for clonal mass micro-propagations of proposed plant species.

(a). Survey and collection of explants.
(b). Surface sterilization.
(c).Culture initiation, establishment & Multiplication.
(d). Callus culture.
(e). Somatic Embryogenesis.
(f). Root induction.
(g). Suspension culture.
(h). Hardening and acclimatization of *in vitro* plantlets.
(i). Field trials.

Biochemical Analysis

Extracted biochemical's will be analyzed by TLC (thin layer chromatography), GC (gas chromatography), HPLC and MS (mass spectrometry) to identity various secondary metabolites and other useful bio-chemicals. RAPD molecular marker technique will be used to find out genetic variability of *Asparagus racemosus.*

Material s & method

Survey and collection of explants

Healthy and disease free *Asparagus racemosus* plants were collected from the Department of Horticulture, SHIATS. Auxiliary buds, root segments, shoot tips and fruits were used as explants.

Washing of glassware and appliance: The glassware was washed thoroughly with a detergent solution. They were then cleaned with a jet flow of tap water, further rinsed with distilled water and oven dried. Approximately 300 seeds were immersed in a beaker with 150 ml of solution, stirred for 20 min, and rinsed three times with distilled water, each for 5 min. Seeds were germinated on Murashige and Skoog (MS) basal salts (Murashige and Skoog, 1962; Phyto Technology Laboratories Inc, USA) with 3% sucrose, rooting vitamins (Table 2) and 7% agar; the pH was adjusted to 5.9 prior to autoclaving at 21 ℃ for 20 min. In each 25 x 150 mm Borosil test tube containing 12 ml of medium, two seeds were germinated and grown for 37 days to serve as a source of explants for the experiment determining optimal hormone concentrations. Plants were grown under conditions described below.

Surface sterilization

Explants used for experiments included such as auxiliary buds, root segments, shoot tips and fruits, the explants were initially washed thoroughly with liquid detergent (Extran) for 5 min followed by continuous washing under running tap water for 1 hour until all traces of the detergent were removed. After rinsing thoroughly in tap water explants were surface sterilized with a solution of bavestin along with 8HQC and indophyl for upto 7 min. They were further surface sterilized with $HgCl_2$ (0.1% w/v) for 5-7 min in the laminar air flow chamber. The surface sterilized plant materials were then rinsed in sterile distilled water several times till all the traces of sterilants were removed.

Preparation of culture media

MS basal medium was used for all *in vitro* studies. The stock solutions for MS medium (macronutrients, micronutrients, iron and organic) were prepared as given in Table 3.1 using distilled water. Appropriate amounts of Major, Minor, Iron, and Organic were taken from the stock solutions and the volume made up to at least half that required with distilled water. 3 % (w/v) sucrose as sugar cubes (Daurala, India) was added for all experiments unless otherwise stated to the media as the carbon source. The medium was supplemented with various growth regulators and other growth adjuvants as per requirement. The the rmost able growth regulators were added to the medium before making up the total final volume with distilled water. Prior to autoclaving pH of the media was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCl with a pH meter (Lab India, India). 0.8 % agar (Hi-Media, India), used for gelling, was added to the medium and heated to melt it completely. The molten media were dispensed into 20 × 150 mm rimless culture tubes (20 ml) or 250 ml jam bottles. Non-absorbent cotton wrapped in a single layer of cheese cloth was used to plug the culture tubes and the flasks.

The medium culture tube was sterilized by autoclaving at 121 °C, 15 Psi (1.06 kg m^{-2}) for 20 min. After sterilization the tubes were taken out in culture tube racks and allowed to gel as slants. All the thermolabile growth regulators were sterilized by filtering through 0.22 µm filter membranes (Millipore, USA) fitted in a polystyrene filter assembly (Tarsons, India) inside the laminar air flow chamber prior to addition to the sterilized medium. The medium was then poured directly into pre-sterilized Petri plates or jars in the laminar air flow chamber. To prevent condensation of water droplets the media was allowed to gel before covering with lids.

Table 1: Composition of Murashige and Skoog (1962) medium and preparation of its stock solution

Stock	Components	Amount	(Stock solution)	Stock Solution
		(mg L^{-1})	(mg L^{-1})	concentration
	NH_4NO_3	1650	33000	
MS Major	KNO_3	1900	38000	20X
	$CaC1_2.2H_2O$	440	8800	
	$MgSO_4.7H_2O$	370	7400	
	KH_2PO_4	170	3400	
	KI	0.83	166	
	H_3BO_3	6.2	1240	
MS Minor	$MnSO_4.4H_2O$	22.3	4460	200X
	$ZnSO4.7H_2O$	8.6	1720	
	$Na_2MoO_4.2H_2O$	0.25	50	
	$CuSO4.5H_2O$	0.025	5	
	$CoC12.6H_2O$	0.025	5	
MS Iron	$FeSO_4.7H_2O$	27.8	5560	200X
	$Na_2EDTA.2H_2O$	37.3	7460	
	Myo-Inositol	100	20000	
MS Organic	Nicotinic Acid	0.5	100	200X
	Pyridoxine HC1	0.5	100	
	Thiamine HC1	0.1	20	
	Glycine	2	400	

Preparation of Plant Material: The explants were surface sterilized before placing themon the culture medium. The adopted sterilization protocol for different explants is given below.

Seeds: The de-husked seeds of *A. Racemosus* were dipped in Savlon (Johnson & Johnson, UK) for 5 to 6 min in a conical flask covered with a single layer of cheese cloth followed by through washing under running tap water for 10 min. Seeds were surface sterilized by a quick rinse with 70 % ethanol followed by treatment with 0.1 % w/v mercuric chloride for 5 min with intermittent stirring, Traces of $HgCl_2$ seeds were removed by washing four times with sterile distilled water and the seeds were soaked overnight, in sterile distilled water, at room temperature. The soaked seeds were put for germination in three different concentrations of MS basal medium (1/4MS; 1/2MS; MS), in a medium supplemented only with agar and water (without MS salts), or in pre-sterilized jars with cotton beds wetted with tap water.

Hypocotyl from seedings: The germinated seedlings were taken out in a sterilized Petri-plate using (Sterilized) forceps and the seedlings were cut into uniform 0.5 cm segments with a scalpel and inoculated in callus induction medium.

Zygotic Embryo: The berries of *A. Racemosus* were collected from the Botanic Garden of the Institute and were first sterilized by a quick rinse for 15 sec with 70 % ethanol followed by flaming of each berry. The berries were then dissected under a stereo-microscope, using sterile forceps and a scalpel blade (Blade no. 11) and embryos were squeezed out by application of gentle pressure. Zygotic embryo (1 mm) was gently removed from the berry with the smooth side of the blade followed by inoculation on a callus induction medium.

Nodes: The young nodal segments were excised from mother plant growing in the field and soaked in 1% Savlon for about 5 min followed by a 10 min wash under running tap water to remove any attached dust particles. The explants were surface sterilized by a quick rinse with 70% ethanol followed by treatment with a 0.1% mercuric chloride solution for 8 min in a laminar air hood. The explants were thoroughly washed with sterilized distilled water for six times to remove traces of $HgCl_2$. The forceps and scalpel were periodically dipped in 70

% ethanol followed by flaming in between inoculations to avoid possibility of contamination.

Culture Media
Culture media varied depending on the type of experiment.

Somatic Embryo formation
Callus Induction Medium (CIM): Young embryos and internodes from germinated seedling were cultured on MS medium supplemented with various concentrations of 2,4-D (1.1, 1.54, or 2.2 mg L^{-1}) alone or in combination with 0.43 mg L^{-1} kinetin. Cultures were incubated at 25 ± 2 °C either in light or in dark. Cool white fluorescent tubes with 30 µE m^{-2} s^{-1} irradiance were used as the light source at 16 h photoperiod. Experiments zygotic embryos in CPM were incubated in dark. The callus was maintained by periodic subculture after every four weeks. Callus incubated in dark only produced somatic embryos. Therefore, in all subsequent experiments zygotic embryos in CIM were incubated in dark. The callus was maintained by periodic subculture after every four weeks.

Result & discussion

(Plant of *Asparagus racemosus)*

Micropropagation
Two approaches were adopted for developing micro propagation protocol for *Asparagus racemosus* in the present investigations. In one approach inter node segments from in-vitro germinated seedlings and immature young embryos isolated from fruits were used for establishing callus for somatic embryo induction. The second approach involved using nodal explants from field grown plants is establish axemic cultures for shoot multiplication through axillary bud break. The details of the experiments and their results are discussed in the following paragraphs.

Somatic Embryogenesis
Somatic embryogenesis in *Asparagus racemosus* was attempted through two approaches. Inter node segments from *in vitro* germinated seedling were used to initiate callus and subsequently somatic embryos. In the second approach zygotic embryos were used to initiate callus and somatic embryos. The two approaches are discussed below.

Hypocotyl segments: Hypocotyl segments from *in vitro* germinated seedlings were used to establish callus culture and subsequent somatic embryo development.

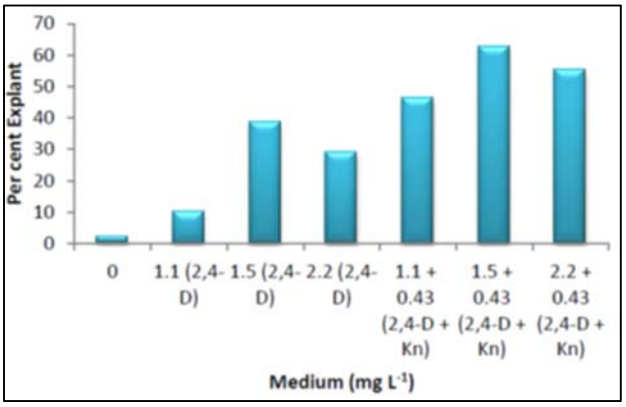
***In Vitro* seed germination:** Highest per cent of germination was obtained in seeds those were incubated in dark with wet cotton beds. Major salt concentration variations did not induce seed germination. Ten days old seedlings were used to raise callus.

Establishment of explants and callus induction: 1 cm long hypocotyl segments were cultured on MS basal medium supplemented with various concentrations of 2, 4-D (0, 1.1, 1.54, 2.2 mg L^{-1}) either alone or in combination with 0.43 mg L^{-1} Kn. Callus was formed only in the presence of the auxin. In 2,4-D the hypocotyls swell and expanded during the first two weeks and by the sixth weeks the epidermis burst open to expose creamish callus (Plate 1A,B). The degree of callusing was influenced by the concentration of 2, 4-D with increase in 2,4-D concentration. The number of hypocotyls that callused also increased with 38% explants callusing at 1.54 mg L^{-1} 2, 4-D compared to 10 % in 1.1 mg L^{-1} 2,4-D. With further increase in 2,4-D to 2.2 mg L^{-} the percent explants callusing decreased to 29 % (Table 5.1; Fig. 5.1) Presence of Kn significantly increased the number of hypocotyls that callused. The maximum per cent of hypocotyls (62.7 %) that showed callusing was in MS supplemented with 1.54 mg L^{-1} 2,4-D+ 0.43 mg L^{-1} Kn.

Effect of 2,4-D and kinetin concentrations on callus cultures of *Asparagus racemosus* induction in hypocotyl

S. No.	Growth Regulators (mg L^{-1})		% of hypocotyl from seedling* producing CEC
	2,4-D	Kn	
1	0	0	0
2	1.1	0	10
3	1.5	0	38.4
4	2.2	0	29
5	1.1	0.43	46.2
6	1.5	0.43	62.7
7	2.2	.0.43	55

*Only cultures incubated in dark produced compact embryogenic callus (CEC)



Per cent explants producing compact embryogenic callus in hypocotyl cultures of *A. racemosus*
Incubation in light or dark had a significant influence on degree of callusing and the type of callus formed. Light appeared to be inhibitory to callus formation as explants incubated in dark only showed callus formation. Culture incubated in dark profusely producing two type of calli; (i) creamish, hard, nodular, compact and slow growing (compact embryogenic callus) and (ii) Creamish, soft, friable and growing callus (Nonembryogenic callus).

Discussion

In the present study development of somatic embryos in the *in vitro* cultures of zygotic embryos and hypocotyl seedlings of *Asparagus racemosus* has been demonstrated, which is a first report for this species. The simple sterilization procedure resulted in recovering 100% sterile cultures and was adopted from an earlier report on garlic (Bhojwani 1980). Somatic embryo formation has been extensively studied in *A. officinalis* (Kunitake and Mii 1998). In *A. officinalis* the most favoured explant for somatic embryogenesis has been stems and cladodes (Li and Wolyn 1995, 1997; Limenton-Grevet and Julien 2000; Delbriel *et al*. 1994) though hypocotyls (Willmar *et al*. 1968), stems (Reuther 1977), buds (Levi and Sink 1990), shoot apices (Dupire *et al*. 1999) and cell suspension cultures (Levi and Sink 1992) have also been used.

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