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"*IN- VITRO* PROPAGATION OF AN ENDANGERED MEDICINAL PLANT-*ASPARAGUS RACEMOSUS* WILLD. (SATAVARI)".

ROLLY AND CHANDER PRABHA

Tissue Culture Lab., Deptt. of Botany, Patna University, Patna 800005, (Bihar)

Correspondence to: cp_ptc_lab@yahoo.com

Asparagus racemosus Willd. is a high value, neglected and critically endangered medicinal plant. So, its propagation through *in-vitro* technique is essential to meet the growing demands of global market. In the present investigation, explants were taken from local population of Bihar with high saponin content. MS medium with BAP (3mg/l), IAA (2mg/l), CM (10%), sucrose (3%) and purified agar, served as "callus- producing medium" for aerial stem segments. Two types of regeneration patterns were observed with nodal stem segments: Indirect and Direct Organogenesis. 80% nodal explants proliferated into healthy and compact calli while 15% developed (2- 3) shoots/ node, followed by development of healthy callus from the same origin. Synergistic actions of BAP, IAA, IBA and CM in basal medium were able to induce shoot differentiation either from the explants or from the callus. Multiple shoots (95%) was achieved in ½ MS with BAP (2.5mg/l), IBA (1.25mg/l) and 5% CM. Healthy adventitious roots appeared in ½ MS basal medium with IBA (2.5mg/l) and IAA (2mg/l) with and without agar. After 60 days, 29% of the adventitious roots turned into storage roots in solid and liquid medium, 20% of the microshoots produced fibrous roots with dense root hairs from the basal region of nodes. BAP played good role in shoot and bud inductions whereas IBA is effective both at multiplication and rooting stages. 46% hardening was achieved and plants were successfully transferred to soil with 95% survival rate.

Key words: *Asparagus racemosus*, Callus, CallusProducing medium, Direct and Indirect organogenesis, Propagation.

Conservation of Biodiversity through tissue culture techniques have given fruitful results in horticulture plants e.g., African violet, Banana, *Eucalyptus*, Ferns, Orchids, *Gerbera*, *Rhododendron*

Abbreviations: MS, Murashige and Skoog's, 1962, BAP, 6-Benzyl amino purine, IAA, indole-acetic acid, CM, coconut milk, BM, basal medium.

The technology holds center stage in many of the endangered plants, for it alone holds key to the rapid bulking of elite germplasms (Purohit, 2002). like *Rauvolfia serpentina* (Roy *et al*.1995) *Podophyllum hexandrum, Rauvolfia* micrantha and *tetraphylla, Aegle marmelos, Cucurligo orchioides* (Ahuja *et al.* 2004) and *Chlorophytum borivilianum* (Kemat *et al*. 2010) The investigated herb, *Asparagus racemosus* called 'Satavari' stands in the list of 'Endangered' plants. Its bioactive constituents (Saponin, Sapogenin and Shatavarin I, II, III & IV) are phytoestrogen compounds with anti- oxytocic and anti- ADH activity. The species have been commercially exploited for the preparation of Ayurvedic drugs used as general vitalizer, female organ debility, antispasmodic, antirheumatic, antidiarrhoetic, refrigerant, etc. It has nutritive value also as it contains Folic Acid, Potassium, Vitamins (A, B, B_6 & C) and fibres. Its therapeutic power has given it a unique nomenclature, "The Female Rejuvenative Herb" in Ayurveda and its conservation has been prioritized by National Medicinal Plants Board.

Review of literature reveals that the medical utility of the plant and its endangered status was realized, so systematic efforts by various workers were under taken for its conservation by the plant tissue culture

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Table-1: Growth response of nodal explants of *A. racemosus* at establishment stages of culture

Sl.No.	Media	% Response	Colour of Callus	Callus diameter(cm) After 15 days	Callus diameter(cm) After 30 days
1	**MS**	0	-	-	-
2	**MS-A_1** **MS+ CM(10%)+ BAP(3mg/l)**	13	Green	Swelling	(0.2- 0.3)
3	**MS-A_2** **MS+CM(10%)+BAP(3mg/l)+ IAA(2mg/l)**	(i)80 Callus	Pale green	(0.58- 0.81)	(1.0- 1.5)
		(ii)15 Shoots	Green	2- 3 shoots/ node followed by pale yellow Callus (1.0- 1.5mm)	

techniques. Chin (1982) used ancymidol to promote formation of shoots and roots under *in vitro* conditions. Kar and Sen (1985 & 1993) worked on *in vitro* propagation aspect and they obtained complete plant of *A.racemosus* with cladodes, crown and roots in hormone free MS medium. Desjardins *et. al* (1987) used nodes as explant and investigated the effects of sucrose and ancymidol on rooting, where as Khunachak *et.al* (1987) reported promotion of shoot and root growth by growth retardants. Mehta and Subramanian (2005) used nodal stem segments as explants and obtained multiple shoots in *A.adscendens*. They conducted phytochemical fingerprinting studies by HPTLC and found no phytochemical variation among *in vivo* and *in vitro* grown rhizomes. Saxena and Bopana (2009) recognized the investigated herb as "critically endangered" in its natural habitat, so they emphasized to develop efficient micropropagation protocol for its conservation. They cultured single node segments in MS medium, obtained multiple shoots by axillary branching and also assessed the clonal fidelity of regenerates by using inter simple sequence repeat marker. Multiple shoots were obtained by Pant and Joshi (2009) when they inoculated shoot tips, nodes and internodes in MS medium. Callus was induced from nodal and root explants by Pise *et. al* (2011) to compare saponin production over a period of 60 days. They reported more production of saponin in root calli than nodal calli and 20 fold increases in Shatavarin level in cultured roots.

Its utility in formulations of several herbal drugs as well as its endangered status gave us an impetus to propagate it through defined steps of a typical micro propagation system. The present report is concerned with selection of best suitable explants, media, growth regulators along with their optimum concentration and mode of regeneration to develop a reproducible protocol. The findings have added interest for manipulating and culminating the *in- vitro* stages for reducing the cost of Tissue Cultured plants.

MATERIAL AND METHODS

Seeds of *Asparagus racemosus* were obtained from the local population of Bihar. Authentication of plant species was done by Botanical Survey of India (BSI), Sibpur, Howrah, West Bengal. Saponin content in the storage roots was determined which amounts to 45%. The seeds were sown in the nursery of the Glasshouse. When the seedlings were 2.5- 3.5" tall, they were replanted on raised beds in the Polyhouse, Deptt.of Botany, P.U. The composition of the soil used for raising nursery and replantation composed of Sand: Soil: Farm Yard Manure (FYM):: 1:1:1. Nodal segments of aerial stem from 5- 6 months old climbers were used as explants. The selected stem pieces were washed in running tap water for ½ hr.,

rinsed in 1% solution of cetramide, followed by rewashing. Later, they were surface sterilized by immersing for 3 min. in 70% alcohol, 3-5 min. in a freshly prepared filtered solution of 3% calcium hypochlorite and submerged in $HgCl_2$ solution (0.05%) for 3 min. Subsequently, they were washed thrice in sterile distilled water to remove traces of Mercury and Chlorine. Under sterile conditions the nodal stem segments (0.5- 1.0 cm. long) were aseptically placed in each culture tube containing slants of MS, MS-A_1 and MS-A_2 media (Table- 1). The cultures were incubated in diffused artificial light [10-20ft (1-ft-c=10.76391 Lux)] at $25^0 \pm 2$ºC. For each treatment, 72 cultures were raised in three replicates of 24 each. The culture tubes were marked for details of media, ex- plant, date of inoculation, etc., daily observed for signs of growth responses along with elimination of contaminated tubes.

Multiplication was induced in MS- A_2 and MS- A_3 and for rooting MS- A_4 and MS- A_5 (Table- 2) media were used and regularly observed to record the frequency and nature of shoots and roots developed. Hardening was carried in small earthen pots with sterile soil mixture by gradual weaning process (Phillips 1988).

RESULTS

Culture Establishment: The response of MS basal medium was not at all encouraging. 13% of explants responded in MS- A_1 in which 10% CM and BAP (3mg/l) were added. The explants swelled but organized callus was not formed.

In MS- A_2 medium, both indirect (via callus) and direct organogenesis was observed. 80% explants developed healthy compact callus (1.0- 1.5cm in diameter) after 30 days of inoculation (Fig. A) and 2- 3 shoots appeared on the nodes in 15% of cultures established from the nodal stem segments after 15- 30 days of inoculation in MS-A_2 medium. Initially, the nodes exhibited swelling, followed by green coloured small friable calli (1.0- 1.5 mm diameter) which bursted to form shoots from single node. After the growth of shoots, healthy

Table-2: Growth Response of nodal explants of *A. racemosus* at Multiplication and Rooting stages of culture

Sl.No	Media	% Response	Mean length of shoot (cm)(Mean± SE) after 45 days	Mean length of root (cm)(Mean± SE) after 45 days
	Multiplication			
1	MS -A_2 MS+ CM(10.0%)+ sucrose(3.0%)+ BAP(3mg/l)+ IAA(2mg/l)	80	2.10± 0.21	
2	MS-A_3 1/2MS+ CM(5.0%)+ sucrose(1.5%)+ BAP(2.5mg/l)+ IBA(1.25mg/l)	95	3.92± 0.24	
	Rooting			
3	MS-A4 1/2MS+ Agar(0.8%)+CM(5.0%)+ sucrose(1.5%)+ IBA(2.5mg/l)+	80		3.14± 0.33
4	IAA(2.0mg/l)	29		(adventitious roots)
	MS -A5 1/2MS+ CM(1.50%)+ sucrose(1.5%)+ IBA(2.5mg/l)+ IAA(2mg/l)	20		Tuberous roots Fibrous roots with dense root hairs
5	**Rejuvenation**	80	Healthy, Green coloured	
6	**Hardening**	46		
7	**Survival**	95		

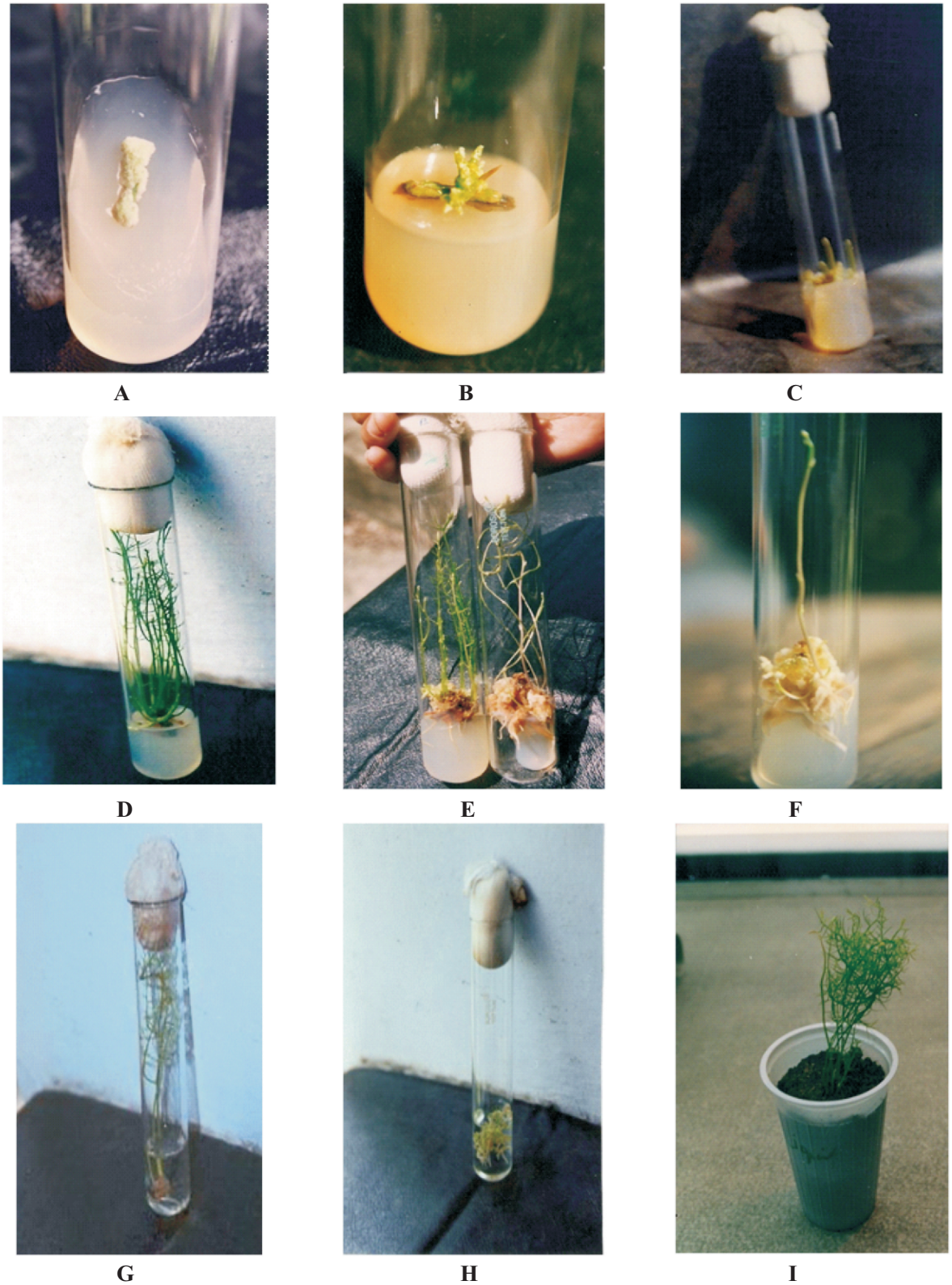

Figure1. *In- vitro* microcloning of *A. racemosus*. **A**. Callus formation; **B**. Cladophylls from nodal region; **C**. Multiple shoot initiation from callus; **D**. Vigrous shooting; **E**. Adventitious roots growing in solid media; **F**. Tuberous roots in solid media; **G**. Adventitious roots growing in liquid media; **H**. Root arising from nodal region; **I.** Hardening.

callus also developed from the same origin (Fig.B).

Multiplication: The shoots were subcultured on (MS-A_2) medium and 80% multiple shoots (2.12 ± 0.21 cm long) appeared after 45 days. 95% calli when subcultured in MS- A_3 medium (Table- 2, Figs. C& D), developed healthy and vigorous shoots (3.9± 0.24). The callus demonstrated continued capacity for differentiation during successive subculturing. From a single callus, 10- 12 cuttings were taken for 4 times. The callus proliferating on "callus- producing medium" required a period of 40- 45 days subculturing on ½ BM for differentiation. The calli were maintained on MS- A_2 medium for 4-5 months by successive subculturing. Frequent subculturing is necessary, as the tissue appears brown due to secretion of polyphenol which is oxidized to a dark brown growth inhibiting substance.

Rooting: For induction of roots, ½ MS medium was supplemented with 2.5 mg/l IBA and 2 mg/l IAA with and without agar and coded as MS- A_4 and MS- A_5 respectively (Table- 2). Three entirely different kinds of rooting trends were spotted: (i) 80% adventitious roots appeared in both solid and liquid media. 29% of the adventitious roots turned into fasciculated storage roots in MS- A_4(Figs. E& F) (ii) 80% proper healthy adventitious roots (Fig.G) in MS- A_5 and (iii) 20% cultures developed fibrous roots with dense root hairs from the basal portion of nodes in MS- A_5 after 45 days (Fig.H).

Hardening and Survival: Initially the rooted shoots of *Asparagus* exhibited symptoms of rejuvenation but with the passage of time, the survival declined to 46%. (Table-2 & Fig.H). 95% plants survived in field under in- vivo conditions.

DISCUSSION

Perusal of the results points to the fact that although BM +CM (MS- A_1 medium) was sufficient to trigger division, the formation of callus took place only when BM was supplemented with BAP and IAA. Hence, the above mentioned medium i.e., MS- A_2 will be referred to as 'callus producing medium'.

In the present investigation, direct organogenesis was noticed as shoots developed from nodal explants (Fig.B), which on sub culturing produced plantlets. The appearance of cladophylls was due to growth of axillary/ lateral buds and the phenomenon is known as 'Proliferation of Axillary Buds'. The axillary buds under the influence of growth regulators broke their dormancy to develop shoot branches which are further used as propagules for future propagation. Conservation of rare and endangered Cacti spp. is being done by axillary bud proliferation to overcome the typical propagation problem with least variation. This phenomenon has been commercially exploited in ornamentals and woody plants (Mantell *et al*., 1985, Pierik, 1987 and Chu, 1992) due to economic considerations and market demand of plants propagated by tissue culture. In the present scenario, growth of the axillary buds which are pre-formed meristems have been triggered by exogenous cytokines (BAP 3mg/l) and the best part is (i) appearance of small friable calli which developed shoots, can be used as propagules for micropropagation, (ii) growth of healthy calli which can be further multiplied in multiplication media and (iii) by manipulating proper ratio of cytokinin and auxin, entire plants with uniform genetic makeup can be developed from single stage of culture, reducing the cost of tissue cultured plantlets.

Whole plant regeneration from cultured cells occurs through (i) shoot- bud differentiation or (ii) somatic embryogenesis which is distinguished on the basis of recognizable morphological differences between the two. Cytokinins promote bud

formation but requirement of exogenous auxin and cytokinin varies with the tissue system, depending on the endogenous level of the two hormones in the tissue (Bhojwani and Rajdan 1983). MS- A_3 media contained BAP (2.5 mg/l), which supported good shoot multiplication (Figs.C & D). For shoot proliferation in hybrid willow and white clover (Bhojwani 1980 and 1981) BAP was effective, but the fact is, that the endogenous levels of auxin and cytokinin are effective in organogenesis.

The calli originating on 'callus-producing medium' required an intervening period of 6- 7 weeks before showing induction on "differentiation medium". Similar observations were made in anther culture of *Solanum surattense* (Sinha *et al*. 1979). They have discussed this in the light that such callus contained some substance (s) which was (were) inhibitory for development of shoot buds, and gradual depletion of such substance (s) during its growth on MS- A_3 medium for 40- 45 days was essential before it was able to differentiate vigorously on a suitable medium.

Our investigations indicated that, interaction of Kinetin and CM is essential for direct organogenesis or via callus. For commercial utility, this finding is very important for cost reducing of micropropagated plantlets by condensing the establishment and multiplication stages.

In- vitro studies have proved that static liquid medium should be used for root cultures (Bhagyalakshmi and Sinha 1995). Rooting is influenced by mineral salts, auxin, sugar, temp. and light (Gauthere 1969). In culture of carrot cells, it has been found that root primodium arises in association with the protoxylem strands and when this is transferred to an agar medium, it forms a complete plant.

In this report, the development of 29% tubers after 60 days in the agar medium indicates that complete *Asparagus racemosus* plants have been fully developed (Fig. F) (morphologically the plantlet looks like young seedlings of *Asparagus*) and the roots have started their function of storage due to their active metabolic cellular state. Present findings are similar to observations made in *Daucus carota* (Kato 1966 and Than & Trinh 1978) where complete plant is formed in solid medium, but such roots do not survive in hardening stage, so static liquid medium should be used for inducing rooting. The use of liquid media has been corroborated by present findings i.e., healthy adventitious roots (Fig.G), are induced by exogenous auxins IAA and IBA. IAA is easily destroyed by light and enzyme, so it is added in high concentration to the culture medium (Gauthert loc. cit.). For rooting, the cultures were incubated in dark, besides IAA (2 mg/l) was added both in 'callus producing medium' and 'multiplication medium', so it was added in moderate concentration in "rooting medium" (Table -2). Commercially exploitable observations were growth of fibrous roots from the lower surface of nodes. *Asparagus* has two modes of propagation- (i) through seeds and (ii) young plantlets developed from underground condensed stem. The cultured plantlets looked similar to runners of *Cynodon dactylon*, which bears roots and shoots from lower and upper regions of nodes respectively (Fig. H). Auxins promote adventitious root development on stem by forming root primodia (Haissig 1974), which remain dormant for some time unless stimulated by auxin. Adventitious root formation on stem cutting is the basis of vegetative reproduction. The site of adventitious root formation on stems in most species is the physiological basal portion, away (distal) from the stem apex. If the stem is placed in a horizontal position and kept moist, adventitious root appears on the lower stem surface confirming the basepetal direction of auxin transport (Haissig loc. cit. and Goldsmith 1977). Due to geotropism, higher auxin level develops in the zone of root emergence prior to

root development. In nature, this phenomenon is useful to develop additional root system. Synthetic auxins, NAA and IBA are more effective than IAA, as they are not destroyed by IAA oxidase, so they persist longer. Exogenous auxins often causes emergence of many adventitious roots in the lower internal stem region. Our investigation indicated that there must be accumulation of endogenous and exogenous IBA and IAA at the lower surface of nodes because the explants were horizontally placed on the nutrient media, triggering the root primordia to develop into adventitious roots. Transition from culture to compost is critical because the success of any micro propagation protocol is evaluated by (i) percentage of plants survived under field conditions and (ii) their field performance in terms of quantity and quality. In the present investigation, 80% plantlets exhibited symptoms of rejuvenation but only 46% were hardened and 95% survived under *in-vitro* condition.

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