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Regeneration of *Asparagus racemosus* by shoot apex and nodal explants

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ABSTRACT

In vitro propagation of Asparagus racemosus was carried out using shoot apex and nodal explants in three stages. (a) Initiation of multiple shoots on MS -medium supplemented with 6-Benzyl Adenine (8.9 μM) + Naphthalene Acetic Acid (0.27 μM) at 25^{0}C having 16 h photoperiod (with 60 μ mol m^{-2} s^{-1} light intensity) and 8 h dark period. (b) Elongation of shoots on MS medium supplemented with 15% coconut milk + 2-iso Pentenyl Adenine (19.6 μM), the light intensity was increased to 80 μ mol m^{-2} s^{-1}. (c) Rooting of elongated shoots by giving it a pre-culture treatment with MS medium augmented with Indole Butyric Acid (7.35 μM) for 48 h and then transfer to MS medium with 15% coconut milk. The rooted healthy plantlets were best hardened in a mixture of 1:1 sand and soil in a moist saturated chamber having 60 -80 % humidity. Plants were transferred to fields, after 5 wks of hardening.

Key words: Shatavari, Micropropagation, Tissue Culture, *In vitro* Culture.

INTRODUCTION

Asparagus racemosus (L) belongs to Liliaceae family. It bears numerous fusiform, succulent tuberous roots about 30 -100 cm long and 1-2 cm thick, which are widely used in Ayurvedic medicinal preparations as refrigerant, demulcent, diuretic, aphrodisiac, antispasmodic, antidiarrhoeaic, expectorant, galactogougue tonic and for renal and reproductive system disorders[1]. Active ingredients of *Asparagus racemosus* are believed to be four saponins (Shatavarin I to IV). Shatavarin IV is a glycoside of sarasapogenin having two molecules of rhamnose and one molecule of glucose [2, 3].

Asparagus racemosus is usually propagated by planting the separated tuberous roots along with shoot apex. Since roots are the organs used for medicinal purpose there has been a practice of using seeds for plant propagation; but there are few technical problems involved in seed derived multiplication.

1. The germination % of seeds is low (Approx. 20%).
2. Seed propagated plants are slow grower,

3. Existence of heterozygosity in seed germinated plants

During our survey of yield of asparagus roots harvested from Kashele and Savle (near Mumbai) it was found that root yield varied from 200 gm/ plant to 3 kg/ plant in 9 months old plants. Hence it was decided to develop a protocol for clonal propagation of elite high root yielding plants of *Asparagus racemosus* by in *vitro* technique using shoot apex and nodal explants.

MATERIALS AND METHODS

Explant preparation: Plants of *Asparagus racemosus* yielding 3 kg roots were obtained from Academy of Developmental Sciences, Kashele, Karjat, India. Moreover, the active ingredient present in the root that is Shatavarin was also analyzed and was found to be 2% of root dry weight. Two days prior to collection of explants, the mother plant was sprayed with 1% Bavistin (a fungicide). Shoot apex and nodes were collected and washed with dilute solution of Teepol and then with tap water. They were kept in 1 % Bavistin for 1 h. Explants were sterilized with $HgCl_2$ (0.2%) for 3 min followed by 3 rinses with sterile distilled water. Shoot apex and nodes were finally trimmed to 0.5 cm under aseptic conditions.

Culture medium: Micropropagation was done in three stages - Culture initiation, shoot elongation and rooting.

For initiation of multiple shoots MS medium [4] supplemented with BA (2.2 – 4.4 µM 6-Benzyl Adenine) and Kin (2.32 – 4.6 µM Kinetin) along with NAA (0.27 – 2.69 µM Naphthalene Acetic Acid) was used.

Initially cultures were incubated in dark for 2 wk and then transferred to 60 µ mol m^{-2} s^{-1} light intensity for 16 h followed by 8 h of dark period regularly.

For elongation, the regenerated shoots were separated and transferred on to the medium containing 2iP (2.46 – 19.6µM 2-iso Pentenyl adenine) along with CM (5, 10, 15 % coconut milk). For faster shoot elongation higher light intensity (80 µ mol m^{-2} s^{-1}) was tried.

To induce root formation from the cut basal ends of the shoots, three types of experiments were carried out using 6 – 7 cm long regenerated shoots.

(a)*Incorporation of auxins into the MS medium*. The auxin studied were IAA (2.85 – 57.1 µM Indole Acetic Acid), IBA (2.45 – 4.9 µM Indole Butyric Acid), NAA (2.67 – 5..7 µM Naphthalene Acetic Acid) and NOA (2.7 – 54 µM Naphthoxy Acetic acid).
(b) *Dip (pulse) treatment* - in 57.1 – 2855 µM IAA, 49 – 2450 µM IBA, 53.7 – 2685 µM NAA or 54 – 2700 µM NOA. Auxin solutions were prepared in autoclaved distilled water under aseptic conditions. Cut ends of regenerated shoots were dipped for 30, 60, 120 and 180 min in auxin solution and then transferred onto auxin free MS basal medium.
(c)*Pre-culture of regenerated shoots on high auxin containing MS medium*: 6 –7 cm long regenerated shoots were pre-cultured on various concentrations of IAA, IBA, NAA or NOA (concentrations are mentioned in table 6) for 16, 24, 48, 72 and 96 h followed by sub-culture in MS basal medium with 15% CM. Both solid as well as liquid MS medium having paper rafts to support the shoots, were used.

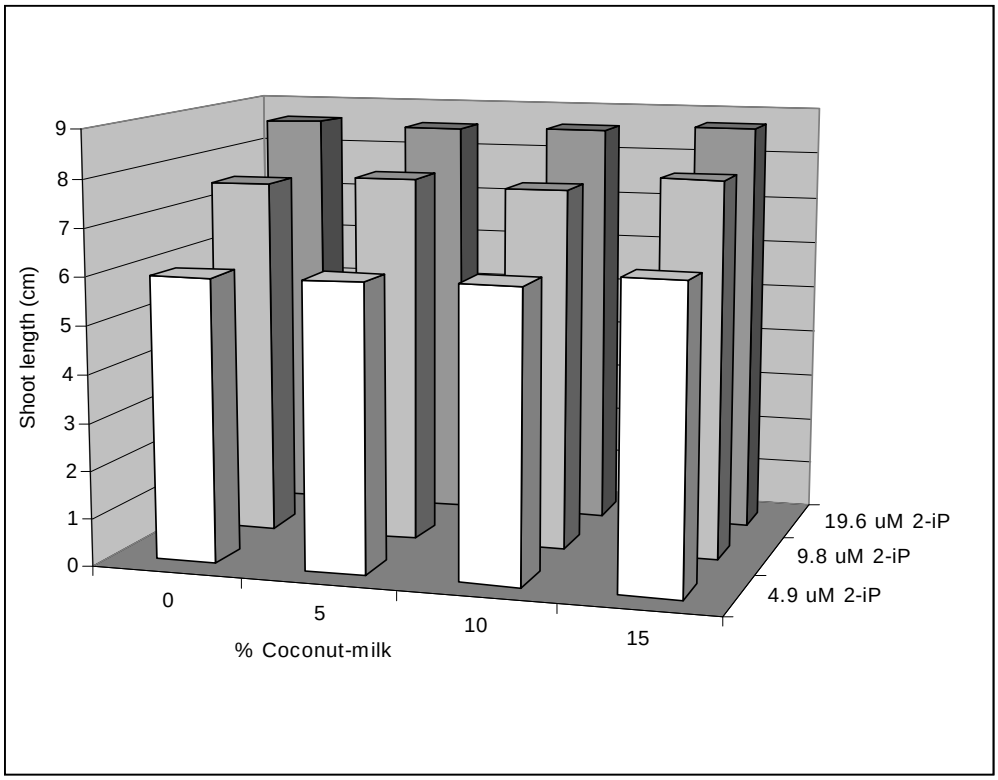
A regular subculture was maintained at an interval of 5 weeks at all the stages of culture.

Hardening - Seven wks after the root initiation, the plants were hardened in 3 x 3 cm protrays filled with different substrates i.e. cocopeat, soil, sand or 1:1 soil and sand; kept in a moist saturated chamber having 60 -80 % humidity. The plants were irrigated daily After 2 wk the established plants were transplanted to 15 x 8 cm long pots with potting mixture containing 4:1 soil and sand for further growth under nursery conditions.

RESULTS AND DISCUSSION

Initiation of multiple shoot clumps: Table 1 shows the shoot forming response to concentration and combination of growth regulators. Ineffective concentrations are not shown in the table. Within 2- 4 wk of inoculation, initiation of multiple shoots was observed in cultures grown on all the combinations of cytokinins and NAA and also on medium having only cytokinins (Table 1). However maximum growth was observed on MS medium supplemented with 8.9 µM BA + 0.27 or 0.54 µM NAA. Although both BA 17.6 µM and kinetin 18.4 µM caused maximum shoot formation the shoots developed on kinetin containing medium were very feeble and tended to dry out whereas shoot grown on medium with BA were healthy. Propagation of *Asparagus racemosus* from callus culture on medium containing IAA and BA, and IBA for rooting has been reported [5], but it was not found to be very effective in the present work. Shoot tip culture of *Asparagus officinalis* on medium containing kinetin and NAA has been reported [6]; but in the present work kinetin along with NAA was not found to be very effective for shoot growth of *Asparagus racemosus.*

Fig. 1.Effect of various concentrations of 2-iP and coconut milk supplemented to MS medium on *in vitro* elongation of regenerated shoots of *Asparagus racemosus*. Results are mean of 30 readings



Since 8.9, 13.2 and 17.6 µM BA produced more shoots; these three concentrations were tried along with three different concentrations of NAA (Table 1), to further stimulate the initiation of

multiple shoots. It was found that 8.9 µM BA was more effective with lower concentrations of NAA (0.27 and 0.54 µM) and induced shoot initiation in as much as 90% cultures.

Shoot apices showed slightly more (90%) initiation of multiples than nodal (88%) (Table – 1) explants. The number of shoot buds regenerated from one shoot apex and a nodal explant in forty days was 66 and 57 respectively. The shoot apex was found to have significantly more regenerative capacity than nodes. Further separation of each shoot bud on the same medium again produced more than 66 shoots in forty days time. This multiplication factor/ subculture have continued for last 10 subcultures.

Table 1. Effect of various growth regulators supplemented to MS medium on *in vitro* shoot growth from shoot apices and nodal explants of *Asparagus racemosus*. Results are mean ± S.E. of 100 nodal and 20 shoot apex explants.

Growth regulators (µM)	From Shoot Apex		From Nodes	
	% explants showing initiation on 15th d	No. of shoot buds regenerated on 40th d	% explants showing initiation by 15th d	No. of shoot buds regenerated on 40th d
BA				
2.2	5	2.8 ± 1.3	5	2.4 ± 1.1
4.4	10	6.2 ± 0.8	11	5.6 ± 0.9
8.9	45	21.8 ± 1.9	42	20.6 ± 1.8
17.6	85	30.2 ± 1.3	77	29.8 ± 0.8
26.4	20	3.8 ± 0.8	15	3.6 ± 1.1
Kin				
2.32	3	3.6 ± 0.9	2	3.2 ± 1.1
4.6	5	6.6 ± 1.5	7	7.0 ± 1.5
9.3	45	24.0 ± 1.6	48	21.8 ± 2.3
18.4	82	32.6 ± 2.5	80	30.6 ± 1.5
27.8	10	2.6 ± 0.4	12	2.8 ± 0.8
BA + NAA				
8.9 + 0.27	**90**	**66.4 ± 1.8**	**88**	**57.0 ± 1.2**
8.9 + 0.54	**90**	48.8 ± 2.8	86	46.8 ± 1.3
8.9 + 1.07	45	28.8 ± 1.3	50	30.2 ± 1.3
13.2 + 0.27	75	44.4 ± 1,1	73	44.8 ± 1.3
13.2 + 0.54	65	27.8 ± 2.2	60	25.2 ± 2.5
13.2 + 1.07	35	21.2 ± 2.0	30	21.2 ± 1.1
17.6 + 0.20	60	25.8 ± 2.3	63	21.6 ± 2.1
17.6 + 0.54	55	26.4 ± 1.5	50	21.4 ± 1.1
17.6 + 1.07	30	15.6 ± 1.1	25	9.6 ± 1.1
Kin + NAA				
9.3 + 0.27	28	1.1 ± 2.0	25	9.6 ± 1.1
9.3 + 0.54	15	5.2 ± 1.3	20	5.0 ± 1.2
9.3 + 1.07	10	4.0 ± 1.4	10	4.0 ± 1.6
13.8 + 0.27	24	11.2 ± 1.3	26	10.6 ± 1.1
13.8 + 0.54	10	5.0 ± 1.0	15	4.8 ± 0.8
13.8 +1.07	5	3.2 ± o.4	5	3.2 ± 0.8
18.4 + 0.27	50	10.8 ± 1.9	48	10.0 ± 1.6
18.4 +0.54	35	8.0 ± 1.9	30	7.4 ± 1.7
18.4 + 1.07	5	3.2 ± 0.8	4	3.0 ± 1.0

Shoot Elongation: As it can be seen from figure 1 and figure 2b, 19.6 µM 2-iP along with 15% coconut milk was found to be best supplement for shoot elongation. Increase in 2-iP concentration in presence of coconut milk stimulated the number of lateral branches.

Rooting: *Asparagus racemosus is* a tuberous-root containing plant and it was hard to initiate tuberous-roots from the regenerated shoots. However, when the auxin was incorporated into the

medium some rooting was observed, but the results were not very encouraging (Table 2), not more than 54 roots could be initiated from the cut ends of regenerated shoots. Hence, a pulse treatment or dipping of cut ends of the regenerated shoots into rather high concentration of various auxins was tried but this method also did not yield more than 10% rooting in regenerated shoots i.e. when 1470 µM IBA was given for 180 min (Table 3).

Table 2.Rooting response by regenerated shoots of *Asparagus racemosus,* cultured on MS medium supplemented with various auxins, as recorded on 60th day after subculture. Values are mean of 30 readings.

IAA		**IBA**		**NAA**		**NOA**	
Conc (µM)	% Rooted Shoots	Conc (µM)	% Rooted Shoots	Conc (µM)	% Rooted Shoots	Conc (µM)	% Rooted Shoots
2.85	0	2.45	0	2.67	0	2.7	0
5.71	0	4.90	0	5.37	0	5.4	0
5.72	0	9.80	0	10.74	0	10.8	0
11.42	0	14.70	0	16.11	0	16.2	0
17.13	0	24.50	5	26.85	0	27.0	0
28.55	5	49.00	5	57.70	0	**54.0**	**0**
57.1	5						

Table 3. Effect of pulse (dip) treatment in various auxins given to regenerated shoots of *Asparagus racemosus,* and then cultured on auxin free MS medium; on the rooting. Values are mean of 50 readings.

Auxin Conc.(µM)	% rooting response when pulse treatment was given for	
	120 min	180 min
IAA		
57.1	0	0
285.0	0	0
856.0	0	0
1713.0	0	0
285.0	0	0
IBA		
49.0	0	0
245.0	0	0
735.0	5	10
1470.0	5	5
2450.0	0	0
NAA		
53.7	0	5
268.0	0	5
805.0	0	0
1611.0	0	0
2685.0	0	0
NOA		
54.0	0	0
270.0	0	0
810.0	10	10
1620.0	0	0
2700.0	0	0

Since, it is reported [7] that rooting is promoted in *Asparagus officinalis* when transplanted to a medium containing no growth regulators after pre-culture on a medium containing auxins with high concentrations, and the results of experiments [8] suggested the same possibilities, an experiment was carried out on the same lines to improve the rooting i.e. after 5 weeks of growth on elongation medium, the shoots were precultured on NOA, NAA, IBA, IAA and then transferred to MS basal medium plus 15 % coconut milk to induce rooting (Table 4). IAA was completely ineffective in rooting the shoots. IBA was the most effective auxin. At all the tested

concentrations it showed some rooting, however, best rooting was observed when shoots were pretreated with 735 µM of IBA. It showed as much as 66% rooting (Table 4).

Figure - 2. *In vitro* plant regeneration of *Asparagus racemosus*

(a) Various stages of initiation and proliferation of shoot buds from apex
(b) Elongated multiple shoots, (c) Rooted shoots (d)In vitro cultured plant growing in 1:1 soil and sand mixture

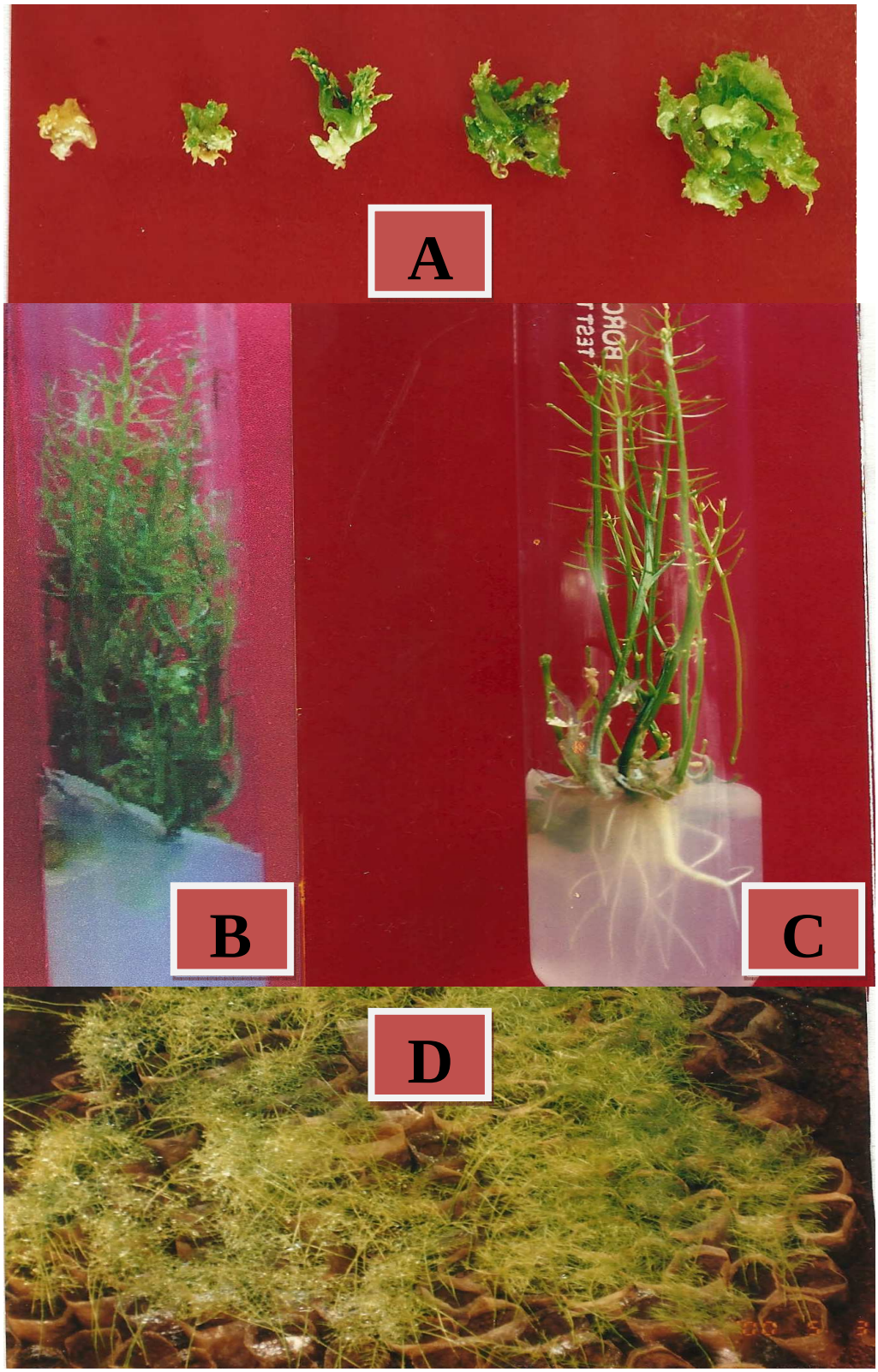


Table 4.Effect of pre-culture of regenerated shoots of *asparagus racemosus* on high concentrations of various auxins for limited period (16 – 96 h), and then sub-cultured on MS medium supplemented with 15% coconut milk. Rooting response was measured on 30th day after sub-culture. Results are mean of 50 readings.

Auxin (µM)	% Rooted shoots after following hours of pre-culture				
	16 h	24 h	48 h	72 h	96 h
IBA					
24.5	0	0	0	4	4
49.0	0	0	0	16	20
245.0	8	10	20	44	16
490.0	50	0	30	54	6
735.0	0	0	66	10	0
NOA					
27.0	0	0	0	0	4
54.0	0	0	0	0	60
270.0	0	0	0	0	0
540.0	0	0	46	40	0
810.0	26	20	20	25	50
NAA					
5.37	0	0	0	0	0
53.71	0	0	0	0	24
107.4	0	0	20	0	0
268.5	0	0	42	0	0
540.0	0	0	0	0	50

A pre-culture time of 48 hours with 735 µM IBA showed vigorous and healthy growth of roots (Fig. 2c). The requirement of pre-culture time, the type of auxins and concentration was different in case of *Asparagus officinalis*[7].

Hardening: Trials indicated that 1:1 mixture of soil and sand was the best substrate, as 70 % plants could survive after transfer to *in vivo*. Excessive flooding during hardening was disastrous for the growth of the plant. The plants, which were transferred to the shade house without any humidity control, started drying from the top of the spear and only 15% could survive. Plants were successfully hardened for 5 weeks in shade houses with humidity control, which was slowly decreased from 80% initial RH to ambient RH of 50%; and then were transferred to the field (Figure 2d).

CONCLUSION

Results mentioned above revealed shoot apex and nodal segments have the potential to induce multiple shoots when cultured on the medium containing BA, kinetin, BA + NAA; but for the elongation the shoots, incorporation of coconut milk along with 2 iP is needed into the medium. For rooting of the regenerated shoots a pre-culture treatment with high concentration of IBA followed by transfer on MS medium supplemented with coconut milk was needed. Since multiplication factor of the both the explants are very high, this protocol can very successfully be used for viable commercial micropropagation of *Asparagus racemosus*. From inoculation to first batch of rooted plant, it takes only six months.

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