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Full Length Research Paper

Shatavarin production from *in vitro* cultures of *Asparagus racemosus* Wild

Mashitha Pise, Jayshree Rudra, Sunita Bundale, Deovrat Begde, Nandita Nashikkar and Avinash Upadhyay*

Hislop School of Biotechnology, Hislop College, Temple Road, Civil Lines, Nagpur, Maharashtra – 440001, India.

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Asparagus racemosus **is one of the important medicinal plants found in India, China and other parts of the world. This plant is known to produce steroidal saponins called Shatavarins. Callus cultures of *A. racemosus* were initiated in a modified MS medium supplemented with 1.0 mg/L NAA and 2, 4-D and 0.5 mg/L BAP and compared for growth and production of saponin over a period of 60 days. Saponin production was evaluated at a regular interval of 15 days. Root calli produces more saponin compared to nodal calli and maximum accumulation was found to be 10.38 ± 0.14 mg/g of callus after 60 days of inoculation. Total saponins from the nodal calli were found to be 7.69 ± 0.136 mg/g of callus. Compared to wild type roots, *in vitro* cultures showed 20 fold increases in shatavarin levels. High performance liquid chromatography (HPLC) chromatograms of the cultures indicated that the overall saponin profile of *in vitro* and *in vivo* plant root extract is similar.**

Key words: Tissue culture, secondary metabolites, multiple growth regulators, saponins.

INTRODUCTION

Asparagus racemosus (local name Shatavari) is an important monocot medicinal plant. It is routinely used in indigenous medicines and is one of the extensively exported medicinal formulations from India for menopausal and fertility related problems. Steroidal saponins Shatavarins (Figure 1), the major bioactive component in *A. racemosus,* is particularly drawing attention because of its role as an immunomodulant, galactogauge, adaptogen, antitusive, anticarcinogen, antioxidant, antidierrhial and as a general tonic for both the sexes (Gaitonde et al., 1969; Joglekar et al., 1967; Oketch-Rabah, 1998; Rao, 1952; Rice, 1988; Shao et al., 1997; Thatte et al., 1987). Although, the entire plant is reported to contain Shatavarins, the fasciculate roots of *A. racemosus* are considered to be the richest source of Shatavarins.

The major constraint in conventional multiplication method of *A. racemosus* through seed propagation is requirement of elaborate pretreatments to break the dormancy and high mortality of seedlings. The grown-up plants can be harvested only after flowering which takes 3 to 4 years because Shatavarins are accumulated in the roots only post flowering (Rao, 1952; NMPB, 2002). The normal practice of root collection involves uprooting of the whole plant and random pretreatment methods, which usually leads to wastage of 50% of the collected material. Moreover, the growing demand for the plant has caused a serious reduction in native populations due to over-harvest and deforestation. Therefore, this is one of those several medicinal plants for which sustainable conservation methods are required on a priority basis (Rao, 1952; NMPB, 2002; Saxena et al., 2008). This plant has also been recognized as 'vulnerable' (Warner et al., 2001).

Plant tissue culture has been successfully employed for several plants facing similar problems and can be tried out for *A. racemosus* also. Very few reports are available regarding callus induction in *A. racemosus* and its *in vitro* regeneration (Kar and Sen, 1984, 1985). To the best of our knowledge, no study has been undertaken to correlate the combinations of growth regulators and their effect on callus induction, morphological aspects, the biosynthetic dynamics of the cultures with respect to

*Corresponding author. E-mail: pmmashi@gmail.com, avinash_u@yahoo.com.

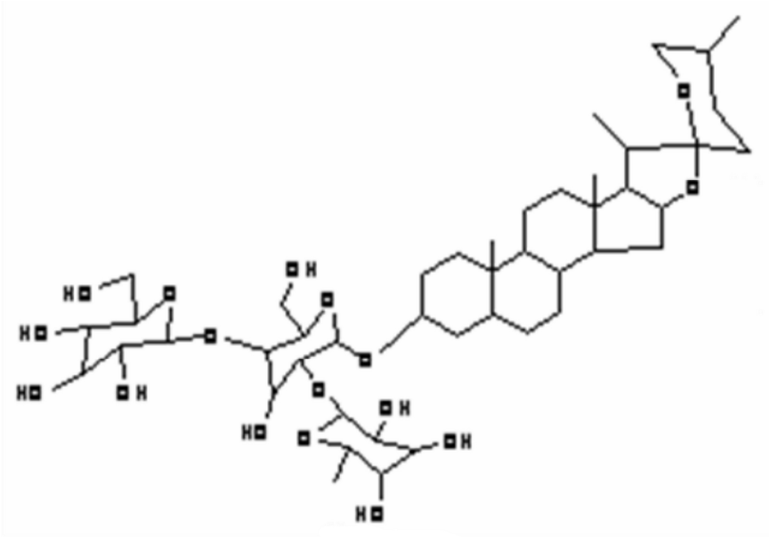

Figure 1. Structure of Shatavarin I and Shatavarin IV.

secondary metabolite accumulation and its effect on overall saponin profile in cell cultures. Tissue culture methods are being successfully tried out even for several monocot plants facing similar problems. In view of the foregoing, this study was undertaken to optimize the conditions for the *in vitro* saponin production from *A. racemosus in vitro* cultures.

MATERIALS AND METHODS

Plant material

Different parts like nodal segments and roots of the field grown *A. racemosus* plant were collected after confirming the authenticity of the plant by an expert taxonomist from the Department of Botany, Hislop College, Nagpur, India. The plant materials were washed with detergent solution and distilled water for 30 min. These plant parts were surface sterilized inside a laminar hood firstly with 0.1% $HgCl_2$ for 3 min and then with 70% ethanol for 5 min followed by washings with sterile distilled water. These plant parts were then dried and cut into small pieces to be used as explants for the inoculations.

Callus induction and maintenance

An initiation medium was designed using modified MS salts supplemented with vitamins in full concentration and 3% sucrose (w/v) (Murashieg and Skoog, 1962). Three different hormones namely naphthalene acetic acid (NAA, 0 – 3.0 mg/L), 2.4-Dichlorophenoxyacetic acid (2, 4-D, 0 - 4.0 mg/L) and 6- Benzyl aminopurine (BAP, 0 - 2.0 mg/L) were used to study callus induction from nodal and root explants in various combinations. The pH of the medium was adjusted to 5.7 and 0.6% phytagel was used as the gelling agent. Medium was dispensed into tissue culture test tubes for slant preparation and autoclaved at 121°C and 15 Lb pressure for 20 min. After the inoculations of the explants, tubes were exposed to a photoperiod of 16 h of light and 8 h of darkness at 25 ± 2°C and sub cultured at regular intervals of three weeks incubation in the culture room.

Growth studies

The fresh weight of the cultured tissues was measured by carefully removing the adhered agar cells were dried in a hot air oven to a constant weight and their dry weights were recorded. In all the experiments data is expressed as mean performance of all the replicas and their standard deviations.

Extraction of saponin

Saponin was extracted from the cultures as per the previously published methods (Mathur et al., 1994). Briefly, cells were extracted separately with methanol (1:2) overnight and procedure was repeated 4 times. All the extracts were pooled and concentrated at 60°C on a rotary evaporator to dryness. The residue was redissolved in 10 ml of H_2O and further extracted with n- butanol. The n- butanol fraction was finally concentrated to dryness on a rotary evaporator under reduced pressure and redissolved in 5 ml of methanol and stored and analyzed using standard Shatavarin IV (procured from Regional Research Laboratory, Jammu, India) as the marker. These extracts were also compared with standard Sarsapogenin (Sigma chemicals), which is a precursor of Shatavarins.

For the preparation of standard solutions, Shatavarin IV was dissolved in methanol and was diluted to get a final concentration range of 100 to 500 $\mu g ml^{-1}$. The solutions were filtered through a 0.2 µm acrodisc. Evaluation of each point was repeated 3 times at 220 nm and the calibration curve was fitted by linear regression. This calibration curve was utilized for the estimation of total Shatavarin present in the methanolic extracts from the wild type plant and *in vitro* extracts. RP- HPLC analysis of the Saponin samples were carried out using a Knauer smart line manager- 5000 system (Germany) fitted with a C18 column (4 um, 150 mm x 3.9 mm I.D.), UV detector and 20µL injection loop. Acetonitrile and 30% aqueous methanol were used as the mobile phase with gradients from 8 to 100% of Acetonitrile in 60 min. The volume of sample injection was 20 µl in all the cases. The peaks of Shatavarin were

Table 1. Growth response of nodal explants of *A. racemosus.*

Growth hormone (mg/L) 2,4-D	+NAA	+BAP	No. of tubes inoculated	No. of tubes contaminated	Growth response in nodal explants			
					% induction	Colour	Average callus wt (g) after 30 days	Average callua wt (g) after 60 days
0.5	0.0	0.5	30	0	20	Brown	0.24 ± 0.08	0.396± 0.08
0.5	1.0	0.5	30	0	40	Greenish yellow	0.34 ± 0.05	0.583± 0.13
0.5	2.0	0.5	30	3	30	Yellow	0.31 ± 0.12	0.554± 0.06
0.5	3.0	0.5	30	0	30	Brown	0.36 ± 0.03	0.583± 0.08
1.0	0.0	0.5	35	2	40	Green	0.29 ± 0.13	0.41± 0.04
1.0	1.0	0.5	35	1	100	Green	0.47 ± 0.07	0.830± 0.06
1.0	2.0	0.5	35	1	70	Greenish yellow	0.24 ± 0.09	0.425± 0.11
1.0	3.0	0.5	35	0	40	Brownish yellow	0.27 ± 0.06	0.373± 0.07
2.0	0.0	0.5	30	0	30	Green	0.28 ± 0.05	0.501± 0.14
2.0	1.0	0.5	30	3	30	Green	0.31 ± 0.03	0.583± 0.08
2.0	2.0	0.5	30	0	20	Brown	0.36 ± 0.07	0.487± 0.07
2.0	3.0	0.5	30	0	10	Brown	0.31 ± 0.08	0.431± 0.12

identified by comparing the retention time of the peaks with those of the reference compounds eluted under same conditions.

RESULTS

Standardization of media for callus induction

Based on the preliminary results, a media composition was standardized for *A. racemosu*s. The details of the combinations used are presented in Table 1. The best combination for callus induction from nodal explants was found to be 1.0 mg/L NAA + 1.0 mg/L 2,4-D + 0.5 mg/L BAP (Figure 2). Preliminary results showed that compared to single auxin, a combination of two auxins is more suitable for the callus induction.

A high auxin to cytokinin ratio was found to be better suited for induction and proliferation of callus in case of nodal explants. In all the tubes fast growing green compact callus appeared within 25 to 30 days of inoculation.

Presence of single auxin 2,4-D alone at high concentrations resulted in a brownish yellow callus (Figure 2b) and a high concentration of NAA alone, resulted in fresh green colored callus with low proliferative capacity. These negative effects were totally absent when these two hormones were used in combination at 1.0 mg/L along with 0.5 mg/L BAP.

However a slight increase in the 2,4-D level (1.0 to 1.2 mg/L) in the induction media gives higher proliferative efficiency to the induced callus. Therefore after 2-3 subcultures in the induction medium, calli were maintained in MS media supplemented with 1.0 mg/L NAA + 1.2 mg/L 2,4-D and 0.5 mg/L BAP. Under the above conditions the time required for the induction was about 25 to 30 days.

Studies on induction time

With a view to reduce the induction time required for the callus, the above medium was altered slightly with the addition of extra KH_2PO_4 (170.0–500.0 mg/L), keeping the hormone combination and levels same. *A. racemosus* explants were inoculated in the above media. It was noted that induction time decreased to 10 to 15 days when the induction media was supplemented with 250 to 300 mg/L KH_2PO4 in case of nodal explants (Table 2). Nodal explants started callusing in around 10 to 15 days, which was nearly half of the previous induction time. This effect on induction time was not seen when another salt of sodium and potassium (K_2HPO_4) was used. In presence of K_2HPO_4 explants did not show callus induction either.

Analysis of shatavarin

Callus cultures derived from the nodal and root explants were screened for the presence and accumulation of saponins at various growth phases. The results have been graphically represented in Figure 3. It was found that a callus culture derived from the root explants produces more saponin compared to nodal callus cultures and maximum accumulation was found to be 10.38 ± 0.14 mg/g after 60 days of inoculation. Total saponins from the nodal calli were found to be 7.69 ± 0.136 mg/g of callus. In the wild type roots Shatavarin IV is generally found to be 0.05 to 0.08% where as in our cultures it was found to be 1.1% which indicates that there is approximately 20 fold increase in the saponin content in the *in vitro* cultures. The high performance liquid chromatography (HPLC) chromatogram of the

Figure 2A. Callus in MS media with 1.0 mg/L NAA + 1.0 mg/L 2,4-D and 0.5 mg/L BAP.

Figure 2B. Yellow coloured callus developed in MS media containing only 1.0 mg/L 2, 4-D and 0.5 mg/L BAP.

in vitro culture and the natural plant root extract were compared and it was found that all the major peaks were present in the *in vitro* extract and the overall saponin profile was similar to the natural root extract (Figures 4A and B).

DISCUSSION

The results of the experiments show that *A. racemosus* can be cultured rather easily in synthetic media, which is generally not the case for most of the monocots. Previous

Table 2. Effect of KH_2PO_4 on induction time and growth of callus.

Sr. No.	Additional KH_2PO_4 (mg/L)	No. of tubes inoculated	Induction time (days)	Average wt. of callus after 30 days	Average wt. of callus after 60 days
1.	170.0	20	30	0.098 ± 0.3	0.409± 0.6
2.	200.0	20	25-30*	0.191 ± 0.2	0.598± 0.6
3.	250.0	20	15- 20	0.212 ± 0.6	0.734± 0.3
4.	300.0	20	10-20	0.153 ± 0.4	0.830± 0.7
5.	350.0	20	20-25	0.103± 0.7	0.397 ± 0.5
6.	400.0	20	30 -40	0.106 ± 0.3	0.343 ± 0.2
7.	450.0	20	40	Only swelling	----

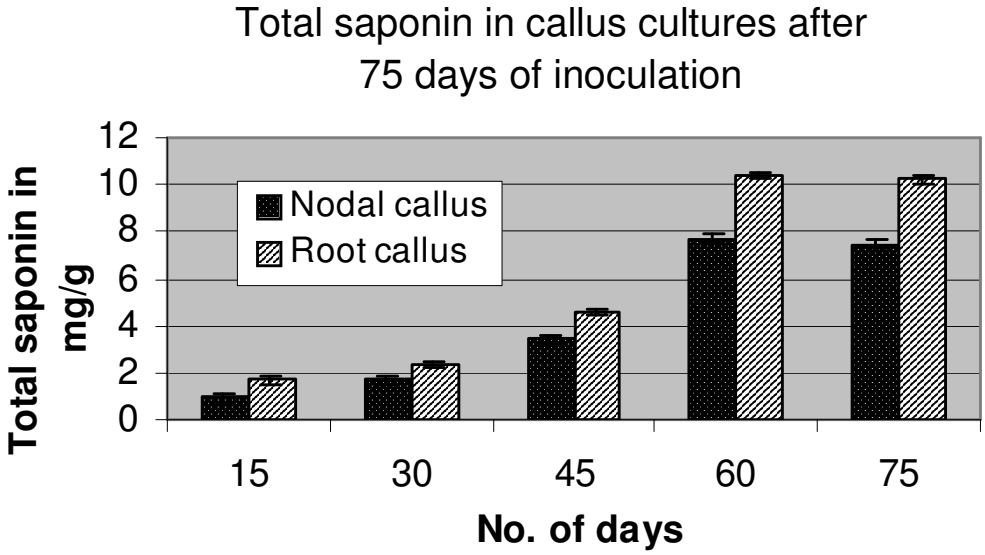

Figure 3. Total saponin content in callus cultures of *A. racemosus.*

workers have observed callus induction to occur when equal levels of auxin and cytokinin are provided in the medium but we found that a high auxin to cytokinin ratio gives better results for callus induction and morphology for all the explant types. Compared to single auxin in high concentration a combination of auxins is better suited for the induction and growth of callus. The auxin NAA seems to support the development of chloroplast as in presence of NAA, callus acquires green colour. For root explants, a medium supplemented with high levels of auxin is good for induction, but for growth, calli must be transferred to a high cytokinin medium. Many plants are known to behave differently on a high phosphorus medium. Phosphorus in general is believed to support good growth and differentiation and the same is true for potassium too (Tsutomu et al., 1987; Gyulai et al., 1992). Some of the earlier reports have even suggested that presence of exogenous additional phosphorus and potassium in terms of KH_2PO_4 enhances secondary metabolite production, especially hecogenin which is also a steroidal saponin (Gyulai et al., 1992). In *A. racemosus*, callus induction time was reduced to half when the explants were grown on a medium with high auxin, low cytokinin and high KH_2PO_4. Therefore this can be considered as a good variation if fast induction in the explants is the objective and may also serve to enhance saponin production.

The above results prove that *in vitro* cultures can be used as an alternative source for Shatavarins. However, further work need to be done on establishment of suspension cultures, secretion of saponins into liquid media and evaluation of bioactivities to validate the protocol for scale up studies.

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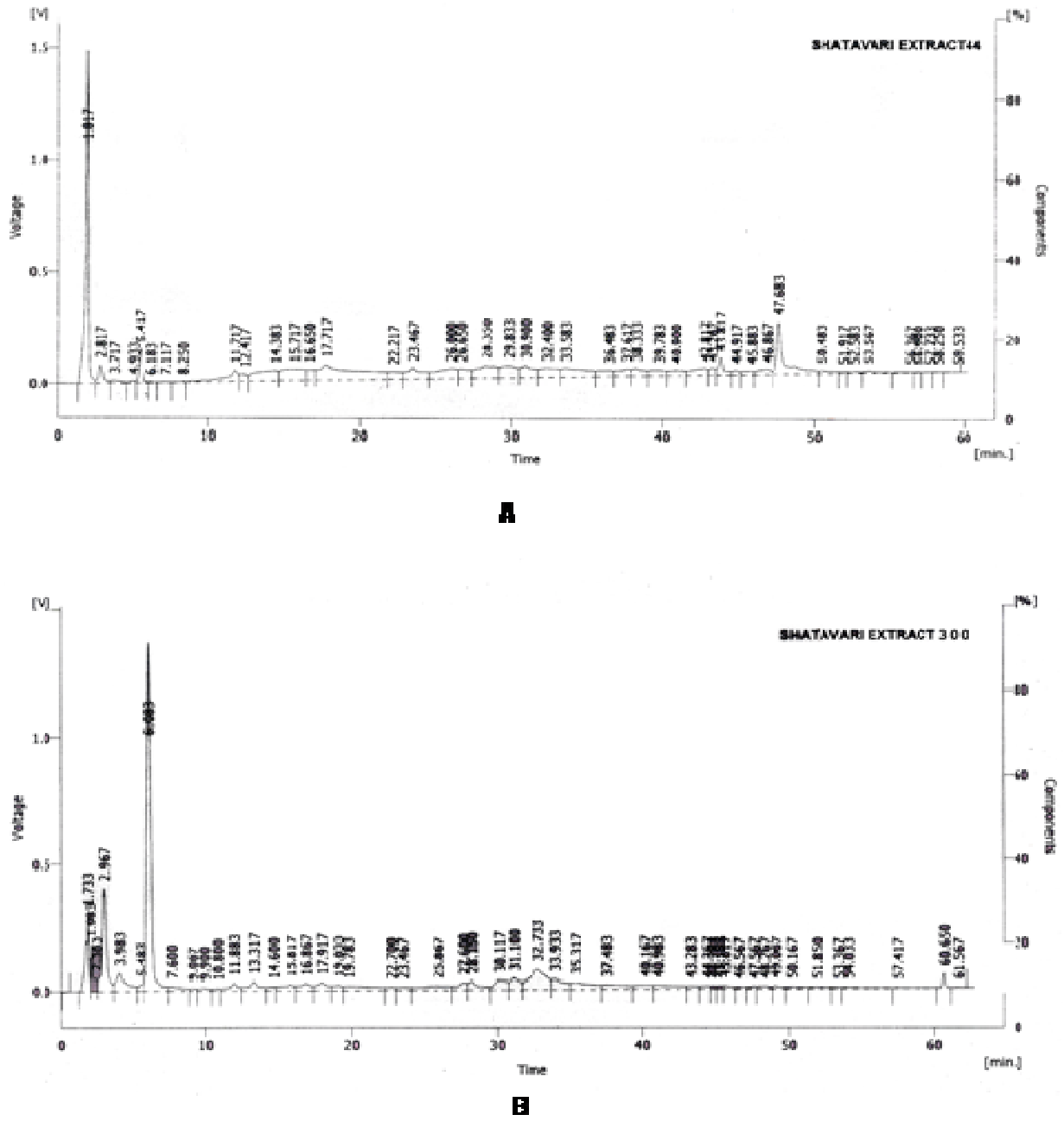

Figure 4. HPLC profiles of: (A) Natural root extract (B) Nodal callus extract.

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