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Effect of Different Cytokinins and Media Types on *In vitro* Shoot Proliferation of *Asparagus racemosus* Willd.

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Abstract

The evaluation of the effects of different cytokinin concentrations and media types on *in vitro* shoot proliferation of *Asparagus racemosus* Willd. is reported. Maximum shoot number and shoot lengths were found with 0.25 mg/l BA which was statistically similar with 0.1 mg/l Kn. Maximum multiplication and growth were found in MS. The protocol could thus be helpful for *in vitro* mass propagation of *A. racemosus*.

Introduction

Asparagus racemosus Willd. is an important medicinal herb belonging to Asparagaeceae. This species is found in the tropical and subtropical regions in India. It is characterized by thorny woody stems and leaves reduced to cladodes. Storage roots are tuberous and tapering at both ends. The plant is traditionally being used for the treatment of female related disorders as it possesses phytoestrogenic properties and is extensively used in combating menopausal symptoms (Sabnis et al. 1968, Mitra et al. 1999). Steroidal saponins called Shatavarin I - X are the major phytoconstituents in *A. racemosus* which impart major properties to it as immunomodulant, galactogauge, adaptogen, antitussive, anticarcenogenic, antioxidant and antidiarrhial (Rao 1952, Joglekar et al. 1967, Gaitonde and Jetmalani 1969, Thatte et al. 1987, Rice 1988, Shao et al. 1997, Oketch Rabah 1998). Due to indiscriminate use, natural population of *A. racemosus* is shrinking. It has been included in the list of 32 prioritized medicinal plants for conservation and development by the National Medicinal Plants Board (NMPB 2002). The plant species is at the verge of being threatened and conditions would be more adverse if not properly managed.

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Micropropagation offers an alternative to overcome the high demand of most of the medicinal plant at the same time conserving the species in its natural habitat. Shoot proliferation using *in vitro* technique plays a promising role in ensuring the easy availability of identical, disease free and superior quality plant material throughout the year. The success of this technique mainly depends on the choice of nutrient medium, optimum concentrations of growth regulators and environmental conditions as it varies from species to species. Regarding the nutrient medium, MS is most widely used for plant tissue culture. However, in some reports medium other than MS has also been found to support the growth of plants (Kassim et al. 2010, Komalavalli and Rao 2000) as the optimal growth and morphogenesis were found to vary for different plants according to their nutritional requirements.

The present work emphasizes on optimizing an efficient micropropagation protocol for *A. racemosus* by standardizing the best cytokinin concentration and the media types. So far this is the first report utilizing different media types for *in vitro* propagation of *A. racemosus*.

Materials and Methods

The single nodal segments were used for establishment of cultures collected from the plants of *A. racemosus* maintained in the greenhouse of School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, India. The explants were surface sterilized by treating with 0.1% mercuric chloride for 10 minutes and washed three times with sterile distilled water. Sterilized explants were inoculated into MS with 3% sucrose and 0.75% agar supplemented with different concentrations of cytokinins from 6-benzylaminopurine, BA, (0 - 4 mg/l) alone and in combination with Kinetin, Kn (0 - 3 mg/l). The pH of medium was adjusted to 5.8 before autoclaving at 121ºC and 15 lbs pressure for 20 minutes. The vitamins, sucrose and agar used were of Himedia, India.

The *in vitro*-formed shoots after 4 weeks on initiation medium were excised from the nodal segment and cultured on semi-solid MS containing 3% sucrose and 0.75 % agar. To observe the effect of cytokinins on shoot multiplication MS was supplemented with BA (0.1 - 1 mg/l) and Kn (0.1 - 1 mg/l) alone and in combinations.

Shoot clusters from initiation medium were also cultured on four different basal media MS, NN (Nitsch and Nitsch 1969), WM (White 1963) and LS (Linsmaier and Skoog 1965) procured from HiMedia Laboratories, India. Each medium was supplemented with 0.25 mg/l BA, 3% sucrose and 0.75% agar. The entire medium was sterilized by autoclaving at 121ºC and 15 lbs pressure for 20

mins. Experiments were conducted in glass flasks (Borosil, India), containing 60 ml of medium with 3 shoot clusters each. All cultures were maintained at 25 ± 2°C, under a 16 hrs photoperiod with a light intensity of approximately 3,500 lux provided by cool, white fluorescent tubes.

Shoot clusters containing 3 - 4 well developed shoots were inoculated into half strength MS containing 0.1 mg/l NAA, 3% sucrose and 0.75% agar. Rooted plants after 40 days in rooting medium were hardened by keeping it into pots containing cocopeat and finally potted in soil.

The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means and the treatment means were compared using DMRT at a 5% probability level using software SPSS 16.0.

Results and Discussion

Micropropagation through axillary branching is the most efficient procedure to obtain clonally propagated plants. Direct organogenesis through nodal explants is a promising method for obtaining true to type plantlets (Altman and Lobert 1998). Thus, in the present study nodal explants have been utilized for establishment of *in vitro* cultures. The percentage shoot initiation obtained in the present work was 99 by following the mentioned protocol for surface sterilization. Bud break was achieved within 3 - 4 days of inoculation. The effect of different concentrations of cytokinins alone and in combinations on shoot multiplication were observed as summarized in Table 1. MS without any hormone it was even possible to induce bud break but the number of shoots obtained were less. BA was effective at lower concentrations in both initiation and multiplication phase. Increase in concentrations of BAP enhanced the rate of proliferation of shoots during initiation of cultures as reported by Sharan et al. (2011) but simultaneously induced callus formation. This observation confirms the report of Choudhary (2012). The combinations of 0.5 mg/l BA and 0.5 mg/l Kn proved to be most effective for culture initiation with healthy shoots and no sign of abnormal shooting. For multiplication of shoots, MS with 0.25 mg/l BA was found to facilitate statistically significant multiplication rate with considerably good shoot length as well (Fig. 1A). However, the 0.1 mg/l Kn gave statistically similar results for shoot multiplication but the shoots obtained tends to dry out earlier than on medium containing BA.

The earliest attempt to propagate *A. racemosus in vitro* was made by Kar and Sen (1985) in which the adventitious shoots were regenerated by callus. Bopana and Saxena (2008) worked through axillary branching of *A. racemosus* by using nodal explants in which they obtained a multiplication rate of 4.57 shoots/node

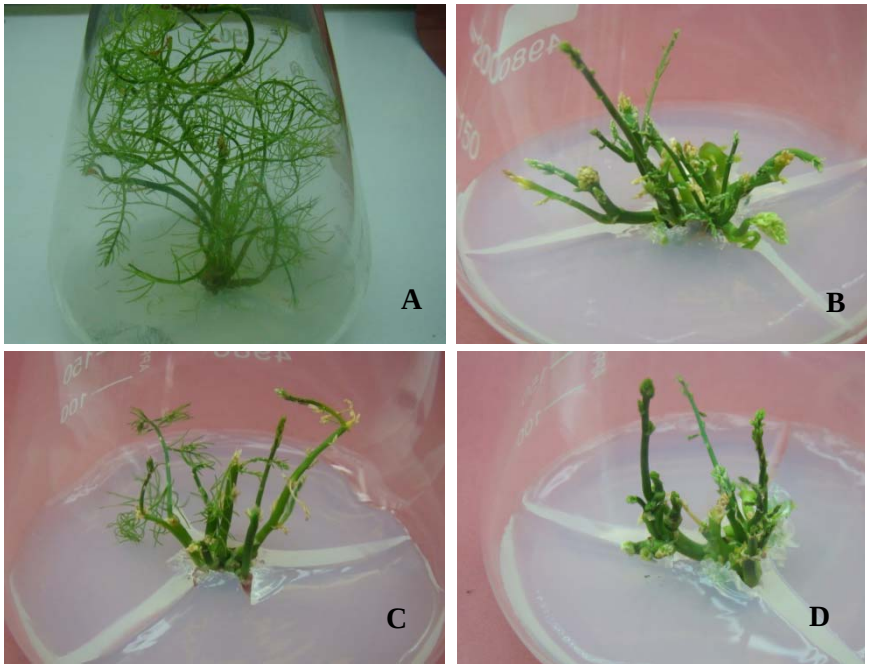

Fig. 1. Effect of media types on shoot proliferation of *A. racemosus* containing 0.25 mg/l BA. A - MS, B - N&N, C -WM, D - LS medium.

Table 1. Effect of cytokinin concentrations on *in vitro* multiplication of *A. racemosus* on MS and 3% sucrose after 4 weeks.

Treatments	Cytokinins (mg/l)	Shoot number	Length of shoots (cm)
MS	Nil	12.60 ± 0.97abc	1.99 ± 0.12d
BA	0.1	12.80 ± 0.85abc	2.11 ± 0.09d
	0.25	**15.00 ± 0.94a**	**3.35 ± 0.23c**
	0.50	10.60 ± 0.81cde	1.62 ± 0.18de
	0.75	12.20 ± 0.99bcd	1.83 ± 0.26de
	1.00	09.00 ± 0.59e	1.22 ± 0.09e
Kn	**0.10**	**14.00 ± 0.94ab**	**4.68 ± 0.27a**
	0.25	9.70 ± 0.96de	4.19 ± 0.23ab
	0.50	10.2 ± 0.91cde	4.64 ± 0.40a
	1.00	8.80 ± 0.64e	3.64 ± 0.13bc
BA + Kn	0.10 + 0.10	8.70 ± 0.47e	3.94 ± 0.13bc
	0.10 + 0.25	10.60 ± 0.27cde	4.42 ± 0.19a
	0.25 + 0.10	5.50 ± 0.31f	3.79 ± 0.23bc
	0.25 + 0.25	6.30 ± 0.62f	2.94 ± 0.30bc

*All the values are mean ± SE, means followed by different letters differ significantly at 5% as analysed by DMRT using SPSS 16.0. Each treatment contains 10 replicates. (Shoot number) df = 13; $F = 12.806$; $p < 0.001$. (Shoot length) df =13; $F = 28.60$; $p < 0.001$.

and a shoot length of 3.39 cms when 2ip was incorporated into the medium. Moreover, study conducted by Choudhary (2012) promises a rate of 15.87 shoots per cluster with a length of 2.93 cm on MS with 0.08 mg/l BA. Through supplementing the medium with 0.25 mg/l BA we obtained a similar proliferation of 15 shoots per cluster with much better shoot length of 3.35 cm. BA has been widely used to enhance shoot proliferation in many species like *Andrographis paniculata* (Purkayastha et al. 2008), *Chlorophytum borivillianum* (Dave et al. 2003) as it is the most effective cytokinin known to induce multiplication *in vitro*.

Table 2. Effect of different types of media on *in vitro* multiplication of *A. racemosus* with 0.25 mg/l BA and 3% sucrose after 4 weeks.

Media type	Shoot number	Length of shoots (cms)
MS	**15.00 ± 0.94a**	**3.35 ± 0.23a**
White's	6.00 ± 0.36c	1.53 ± 0.16c
Nitsch & Nitsch	8.67 ± 0.42b	1.52 ± 0.97c
Linsmeir & Skoog	6.67 ± 0.55bc	2.43 ± 0.45b

*All the values are mean ± SE, means followed by different letters differ significantly at 5% as analysed by DMRT using SPSS 16.0.

Different types of medium formulations have been utilized for *in vitro* propagation of plant species, but MS is the most popular basal media used (Huang and Murashige 1977, Rout et al. 2000). In some species, other devised medium formulations were also reported to enhance the shoot proliferation rate. In present study, MS was found to support maximum growth of shoots with significantly good shoot number (15 per cluster) and shoot length (3.35 cms) and the findings are supported by reports of many workers (Joshi and Jadhav 2013, Bhaskaran and Jayabalan 2005). Following MS, NN medium also facilitated the growth of *in vitro* cultures of plant with 8.67 ± 0.42 shoots per cluster but with reduced shoot length of 1.52 ± 0.97 cm. Minimum growth was observed in White's medium (Table 2, Fig. 1). The positive effect of MS could be attributed to relatively higher supply of nitrate-nitrogen within the medium that influences the pH of the medium and in turn determines the absorption of other nutrients (Ramage and Williams 2002). Well developed plantlets which were rooted and successively hardened in coco-peat and potted in soil.

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