*Medicinal and Aromatic Plant Science and Biotechnology* ©2007 Global Science Books

# Rapid *in Vitro* Protocol for High Multiple Shoot Induction, Rooting and Flowering in *Asparagus racemosus* L.

**Kalpana N. Reddy*** • **A. Manoj Kumar** • **B. Abdul Rahiman**

Department of Biotechnology, Kuvempu University, Shankaraghatta - 577 451, Karnataka, India

*Corresponding author*: * **kalpana_reddyn@rediffmail.com**

## ABSTRACT

A high frequency shoot multiplication protocol for *Asparagus racemosus* L. was developed by culturing shoot tips and nodal bud explants. Murashige and Skoog (MS) medium with 3 mg $l^{-1}$ and 0.1 mg $l^{-1}$ α-naphthalene acetic acid (NAA), respectively formed green morphogenic calli which produced more than 95 multiple shoots in the same medium after 35 days of culture. The stunted shoots were transferred to shoot elongation medium with 2 mg $l^{-1}$ 6-benzylamino purine (BAP) and 0.1 mg $l^{-1}$ NAA. Rooting was obtained on half MS with 7 mg $l^{-1}$ BAP after 45 days of culture. The shoots inoculated on lower concentrations of NAA did not show any change. Some shoots produced floral buds which bloomed to produce normal flowers *in vitro* when left undisturbed on NAA medium (3 mg $l^{-1}$) for 60 days and the plants bearing flowers wilted after 3 days. The plantlets with a morphologically normal appearance produced from adventitious shoots were transferred to soil and acclimatized in the growth chamber with a 62% survival rate.

**Keywords:** *Asparagus racemosus*, BAP, NAA, *in vitro* flowering, rooting
**Abbreviations: ancymidol**, α-cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidinemethanol; **BAP**, 6-benzylamino purine; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog medium; **NAA**, α-naphthalene acetic acid, **SEM**, shoot elongation medium; **SIM**, shoot induction medium; **RIM**, root induction medium

## INTRODUCTION

The Liliaceous species *Asparagus racemosus* L., popularly known as '*shatavari*' is a plant of very high medicinal value. The plant as a whole is used in ayurvedha, sidha and homeopathy systems of medicine. *Asparagus racemosus* root paste or root juice has been used to treat various ailments and as a health tonic (Goyal *et al*. 2003). *A. racemosus* is a well known Ayurvedic Rasayana which prevents ageing, increases longevity, imparts immunity, improves mental function and vigor, and adds vitality to the body; it is also used to treat nervous disorders, dyspepsia, tumors, inflammation, hyperpiesia, neuropathy, and hepatopathy (Sharma 2001). Reports indicate that the pharmacological activities of *A. racemosus* root extract (ARRE) include antiulcer (Sairam *et al*. 2003), antioxidant (Kamat *et al*. 2000), anti-diarrhoeal (Vankatesan *et al*. 2005; Visavadiya and Narasimhacharya 2005), antidiabetic (Govindarajan *et al*. 2004) and immunomodulatory activities (Thatte and Dahanukar 1988). ARRE contains saponins, alkaloids (Sekine 1995), polysaccharides (Kamat *et al*. 2000), polyphenols, flavonoids and vitamin C. It also contains an anti-cancerous agent asparagin which is useful against leukemia. Being a highly useful medicinal plant it is very difficult to propagate because of poor seed germination. The need for an alternative propagation protocol was of the utmost importance and micropropagation was found to be the best technique and solution.

Within the genus, micropropagation has been extensively studied in *A. officinalis* using shoot apex and lateral buds. Murashige *et al*. (1972) and Yang (1977) could obtain well rooted plants with α-naphthalene acetic acid (NAA) (0.1 mg $l^{-1}$) and kinetin (1.0 mg $l^{-1}$), while Bojnath *et al*. (2003) obtained the highest number of shoots with NAA (0.015 mg $l^{-1}$) and 6-benzylaminopurine (BAP) (0.5 mg $l^{-1}$) with good roots when shoots were placed in 1.25 mg $l^{-1}$ indole-3-butyric acid (IBA). Biswajit (2000) in *A. verticallatus* reported that light intensity plays an important role in inducing callus in combination with 2,4-D, kinetin, BAP and NAA. Shoots were regenerated with BAP or 6-benzylamino-9(2-tetrahydropyranyl) adenine (BPA), L-arginine, and a low level of NAA. Roots were induced with indole-3-acetyl-L-alanine (IAA-L-alanine).

The major obstacle in asparagus micropropagation is not shoot culture and shoot multiplication but rather the difficulty in initiating roots (Natasa *et al*. 2002). Within the genus *Asparagus*, micropropagation protocols have been developed for *A. officinalis*, *A. meritimus* and *A. verticillatus* used mainly as ornamental or medicinal plants.

The present study aimed to establish a method for efficient shoot, root culture and acclimatization that can be applied for rapid large-scale *in vitro* propagation.

## MATERIALS AND METHODS

Plant material was collected from pot-grown plants in the greenhouse of Kuvempu University, Shankaraghatta, Karnataka, India. All chemicals in this study were obtained from Merck Pvt. Ltd. (UK). The shoot tips (1 cm) and nodal buds (1 cm) excised from one-year-old plants were used as explants. The explants were kept in running tap water for 30 min and then pre-treated with a solution of 0.2% carbadazime (bavistin), 0.2% cetrimide and 5-6 drops of a disinfectant, Savlon (Johnson and Johnson's, Pvt. Ltd.) for 30 min and washed with 2% (v/v) Tween-20 for 15 min then with sterile distilled water. Followed by sterilization with 0.1% $HgCl_2$ for 3 min and finally 3-4 washes in sterile distilled water were given.

The sterilized explants were transferred to 300 ml (Tarsons) culture bottles having different concentrations of BAP and NAA for callus induction, shoot bud proliferation and shoot elongation. The pH of the medium was adjusted to 5.7 prior to autoclaving. Cultures were incubated at 25 ± 1°C under a warm fluorescent light where the intensity varied from 2000-3000 lux ($20 \times 10^3$ μmol/$m^2$/s) with a 16 h photoperiod.

Callus or shoot buds from shoot induction medium (SIM)

Received: 10 August, 2007. Accepted: 13 December, 2007.

Original Research Paper

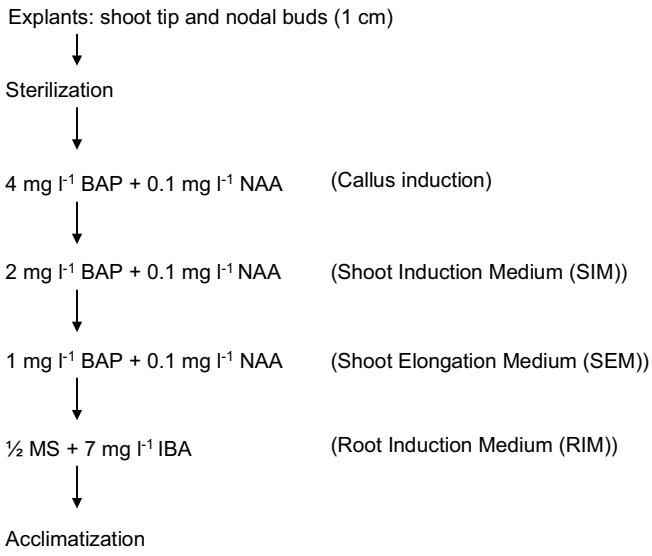


**Fig. 1 Flow diagram showing the experimental protocol.**

were transferred to the shoot elongation medium (SEM; **Fig. 1**). Both SIM and SEM were comprised of MS with 1.0, 2.0, 3.0 or 4.0 mg $l^{-1}$ BAP with 0.1 mg $l^{-1}$ NAA. Data was recorded every week. Elongated and 2-3 cm–long shoots were transferred to root induction medium (RIM), which consisted of ½ MS with 0.0, 1.0, 5.0, 7.0 or 8.0 mg $l^{-1}$ NAA and IBA. The effect of ancymidol (Sigma Aldrich, St. Louis, Missouri) on rooting was determined by transferring nodal sections of asparagus to medium with 2, 5 or 10 µM of ancymidol. Each treatment was applied to 100 shoots consisting of 20 cultures replicated five times in a randomized complete block design. Ancymidol is considered to be an efficient $GA_3$ inhibitor of root induction (Natasa *et al*. 2002).

Square root transformation was used to analyze the root number and root length. The data was expressed in centimeters. The resultant data was subjected to analysis of variance (ANOVA) using SAS (SAS Institute, Inc., 1999). The treatment means were compared using Critical Difference (CD) at p=0.05.

**Table 1** Effects of BAP and NAA on multiple shoot induction in *Asparagus racemosus*.

| BAP + NAA (mg/l) | Shoot (№) | Shoot length (cm) | Callus intensity |
|---|---|---|---|
| 1.0 + 0.1 | 20 ± 1 d | 4.26 ± 0.01 a | + |
| 2.0 + 0.1 | 95 ± 1 a | 1.50 ± 0.21 b | + |
| 3.0 + 0.1 | 60 ± 1.22 b | 1.20 ± 0.37 c | ++ |
| 4.0 + 0.1 | 55 ± 1 c | 1.00 ± 0.15 d | ++++ |
| F. test | * | * | - |
| SEm± | 0.5099 | 0.0531 | - |
| C.D (p=0.05) | 1.57 | 0.16 | - |

Note: Different letters within a column are significantly different (P=0.05).

## RESULTS AND DISCUSSION

Establishment of *in vitro* cultures of *A. racemosus* was found to be slow and difficult. Fungal contamination was the major obstacle with more than 60% of primary cultures lost in the process. Shoots developed from the pre-existing axillary buds from both sides of the nodal explants. Resultant shoots grew vigorously and developed without any visible deformities. Callus formation at the base of explants increased with an increase in BAP concentration. Multiple shoot induction mainly depended on the concentration of BAP (**Table 1**). Irrespective of the explant, callus production was always observed at the base of the explants after 15-20 days. Media containing 1 mg $l^{-1}$ BAP and 0.1 mg $l^{-1}$ NAA produced dull green calli and in all other combinations from 2-4 mg $l^{-1}$ BAP with 0.1 mg $l^{-1}$ NAA produced dark green and compact calli. But dark green callus that formed at higher concentrations of BAP i.e., 2-4 mg $l^{-1}$ BAP with 0.1 mg $l^{-1}$ NAA could successfully regenerate both leaf-like structures and shoot initials. BAP at 4 mg $l^{-1}$ and 0.1 mg $l^{-1}$ NAA produced the highest amount of callus, which weighed approximately 0.5 g, more than the 0.1 to 0.35 g obtained in other media combinations.

When the explants were inoculated onto the 1 mg $l^{-1}$ BAP and 0.1 mg $l^{-1}$ NAA medium only 20 shoots were produced along with a small quantity of basal callus. With an increase in BAP concentration to 2 mg $l^{-1}$ plus 0.1 mg $l^{-1}$ NAA the number of multiple shoots drastically increased to 95 (**Fig. 2A, Table 1**) along with callus formation and was suitably considered as the SIM. An earlier report by Dipak and Sumitra (1985) on *A. racemosus* produced a maximum

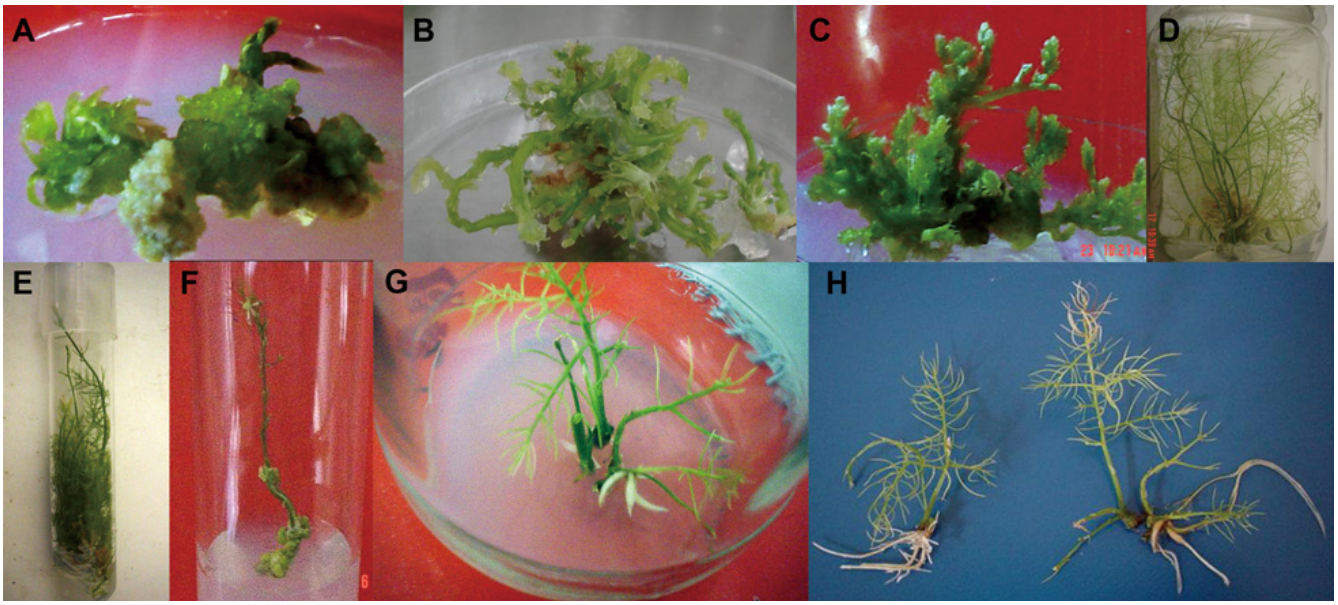


**Fig. 2 Micropropagation of *Asparagus racemosus* from nodal explants.** (**A**) Induction of calli on shoot tips and nodal bud explants on MS medium supplemented with 4 mg $l^{-1}$ BAP and 0.1 mg $l^{-1}$ NAA. (**B**) Adventitious shoot formation from surface of calli on MS with 2 mg $l^{-1}$ and 0.1 mg $l^{-1}$ BAP (SIM). (**C**) Regenerated shoots on SEM with 1 mg $l^{-1}$ BAP and 0.1 mg $l^{-1}$ NAA. (**D, E**) Well-established shoots after 4 weeks in culture. (**F**) Plantlet showing *in vitro* flowering. (**G**) Rooted microshoots on RIM with ½ MS + 7 mg $l^{-1}$ IBA. (**H**) Rooted *in vitro* plants ready for acclimatization.

**Table 2** Effects of auxins and cytokinins on callus induction and regeneration in *Asparagus racemosus*.

| Treatments (mg/l) | Description of callus | Regeneration | Intensity |
|---|---|---|---|
| BAP +NAA | | | |
| 0.0 + 0.0 | None | - | - |
| 1.0 + 0.1 | Dull Green | S | + |
| 2.0 + 0.1 | Dark Green | S, LS | + |
| 3.0 + 0.1 | Dark Green | S, LS | ++ |
| 4.0 + 0.1 | Dark Green | S, LS | ++++ |

LS: leaf-like structures; S: shoot regeneration.

**Table 3** Effects of IBA on rooting in *Asparagus racemosus*.

| ½ MS + IBA (mg/l) | Root (No.) | Root length (cm) |
|---|---|---|
| 0.0 | 0.0 ± 0 (0.707) c | 0.0 ± 0 (0.707) c |
| 1.0 | 0.0 ± 0 (0.707) c | 0.0 ± 0 (0.707) c |
| 5.0 | 0.0 ± 0 (0.707) c | 0.0 ± 0 (0.707) c |
| 7.0 | 5.6 ± 0.14 (2.46) a | 2.88 ± 0.13 (1.84) a |
| 8.0 | 1.4 ± 0.2 (1.36) b | 0.7 ± 0.12 (1.08) b |
| F. test | * | * |
| S Em± | 0.0458 | 0.0261 |
| C.D (*P=0.05*) | 0.13 | 0.07 |

Figures in parenthesis indicate transformed values.
Different alphabets in the column exhibit significant differences.

of 12 multiple shoots with BAP at 1.0 mg $l^{-1}$ and IAA at 0.1 mg $l^{-1}$. Later when this callus was transferred to 1 mg $l^{-1}$ BAP and 0.1 mg $l^{-1}$ NAA considered as shoot elongation medium (SEM), shoots increased in length (**Fig. 2B**). Subculture of microshoots onto SEM helped the shoots attain a very good height of 4.26 cm (**Fig. 2C**); 2 mg $l^{-1}$ BAP and 0.1 mg $l^{-1}$ NAA gave very stunted shoots which failed to grow beyond 1.5 cm. BAP concentrations at 3 mg $l^{-1}$ and 5 mg $l^{-1}$ together with 0.1 mg $l^{-1}$ NAA gave very compact calli which was highly morphogenic similar to 2 mg $l^{-1}$ plus 0.1 mg $l^{-1}$ NAA. The shoots on these BAP concentrations were very short ranging from 1.0 to 1.2 cm. However, as the concentration of BAP increased, the shoot length decreased, and the shoot number and amount of callus increased (**Table 2**). Shoots failed to elongate with optimum concentrations of growth regulators, therefore they were transferred to media containing lower levels of growth regulators (Bhat *et al*. 1992).

The observations in the present study with ancymidol were in agreement with those found by Natasa *et al*. (2002) when working with *A. meritimus* in which the lowest concentration of ancymidol (0.39 µM) promoted the highest shoot multiplication rate (11.9 shoots/crown), while medium without ancymidol promoted higher shoot multiplication (9.4 shoots/crown) than media supplemented with higher concentrations of ancymidol (1.95 and 2.73 µM (7.1 and 7.9 shoots/crown, respectively)). The results could be due to ancymidol's property of disrupting apical dominance and altering carbohydrate metabolism as reported by Desjardin *et al*. (1987) who obtained more shoots (19.4) in ancymidol (5 µM)-containing medium than medium without ancymidol (14) in *A. officinalis*.

Rooting was attempted with different hormones at different concentrations with both full and half strength MS. Usage of ancymidol, a potential $GA_3$ inhibitor did not improve rooting. NAA, which was used at 0.1 mg $l^{-1}$ to 3.0 mg $l^{-1}$ on full and half strength MS did not result in any roots. As is evident from **Table 3**, IBA at 1.0 and 5 mg $l^{-1}$ also did not show any rooting while shoots rooted in medium with IBA at 7 and 8 mg $l^{-1}$ after 45 days and served as RIM following transplant (**Fig. 2G**). The roots were well established and a good root system was formed by 60 days (**Fig. 2H**). The roots were healthier and longer (2.88 cm) in 7 mg $l^{-1}$ IBA but were only 0.7 cm in 8 mg $l^{-1}$ IBA media. Desjardins *et al*. (1987) reported that increasing the concentration of sucrose from 3 to 7% in media containing ancymidol increased rooting.

*In vitro* flowering is a unique phenomenon which has intrigued plant tissue culturists. In the present study, flowering was observed only when the shoots were placed on MS medium with 3 mg $l^{-1}$ NAA for a period of 55 days (**Fig. 2G**). Flowering in *in vitro* conditions has been reported in numerous plant species, reviewed in Taylor and van Staden (2006) and Sudhakaran *et al*. (2006). Shoots bearing flowers turned yellow and dried off slowly, which may have been due to the production of ethylene by the withering flower parts (Dhanaraj 1992).

This report provides a comprehensive outlook on the possibilities of multiplying a very important medicinal plant, '*shatavari*', which is highly recalcitrant to regeneration. In order to meet the demand to production ratio the method standardized in the present investigation could be suitably used to mass produce this plant. The study outlines a rapid and a successful technique to overcome the major problem of rooting in asparagus species, especially in *in vitro* studies.

## Acclimatization

Rooted plantlets were washed in running tap water followed by a dip in 1% IBA before transferring to thumb-sized pots containing soilrite. The plants were maintained in a mist chamber for 30 days were later transplanted to pots having sand: soil: FYM (1:1:1) with 70% survival (**Fig. 2H**).

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge Head of the Department, Biotechnology Department, Kuvempu University for his co-operation to carry out research work.

## REFERENCES

**Biswajit G** (2000) Plant regeneration in *Asparagus verticillatus* L. *Journal of Herbs, Spices and Medicinal Plants* **4**, 41-43

**Bojnath G, Puchooal S, Bahorun T** (2003) *In vitro* regeneration of *Asparagus officinalis*: Preliminary results. AMAS. Food and Agricultural Research Council, Reduit, Mauritius, pp 7-15

**Desjardins Y, Tiessen H, Harney PM** (1987) The effect of sucrose and ancymidol on the *in vitro* rooting of nodal sections of asparagus. *HortScience* **22**, 131-133

**Dhanaraj A** (1999) *In vitro* flowering in *Jasminum scambac* var. "aryaneanum" (Udupi Mallige). *Indian Perfumery* **43**, 88-92

**Dipak KK, Sumitra S** (1985) Propagation of *Asparagus racemosus* through tissue culture. *Plant Cell, Tissue and Organ Culture* **5**, 89-95

**Govindarajan R, Vijayakumar M, Rao CV, Kumar V, Rawat AKS, Pushpangadan P** (2004) Action of *Asparagus racemosus* against streptozotcin-induced oxidative stress. *Natural Product Sciences* **10**, 177-181

**Goyal RK, Singh J, Lal H** (2003) *Asparagus racemosus* - An update. *Indian Journal of Medical Sciences* **57**, 408-414

**Kamat JP, Boloor KK, Devasagayam TP, Venkatachalam SR** (2000) Antioxidant properties of *Asparagus racemosus* against damaged induced by gamma radiation on rat liver mitochondria. *Journal of Ethnopharmacology* **71**, 425-435

**Murashige T, Shabde MN, Hasegawa PM, Takatori FH, Jones JB** (1972) Propagation of asparagus through shoot apex culture. *Journal of the American Society of Horticultural Science* **97**, 158-161

**Murashige T, Skoog F** (1962) A revised medium or rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

**Natasa S, Borut B, Marijana J** (2002) *In vitro* propagation of *Asparagus maritimus* - A rare Mediterranean salt-resistant species. *Plant Cell, Tissue and Organ Culture* **70**, 269-274

**Sairam K, Priyambada S, Aryya NC, Goel RK** (2003) Gastroduodenal ulcer protective activity of *Asparagus racemosus*: An experimental, biochemical and histological study. *Journal of Ethnopharmacology* **86**, 1-10

**SAS** (1996) SAS/STAT User's Guide, Version 6.12. SAS Institute Inc., Cary NC, USA

**Sekine T** (1995) Structure and relative stereochemistry of a new polycyclic alkaloid, sparagamine a, showing anit-oxytocin activity, isolated from *Asparagus racemosus*. *Journal of Chemical Society, Perkin Transactions* **1**, 391-393

**Sharma PV** (2001) Cikittsastana. In: *Ccaraka Samhita* (Vol 2) Chaukhambha Orientalis, Varanasi, pp 7-14

**Sudhakaran S, Teixeira da Silva JA, Sreeramanan S** (2006) Test tube bouquets – *in vitro* flowering. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol II), Global Science Books, Isleworth, UK, pp 336-346

**Taylor NJ, van Staden J** (2006) Towards an understanding of the manipulation

of *in vitro* flowering. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol IV), Global Science Books, Isleworth, UK, pp 1-22

**Thatte UM, Dahanukar SA** (1988) Comparative study of immunomodulating activity of Indian medicinal plants, lithium carbonate and glucan. *Methods in Experimental and Clinical Pharmacology* **10**, 639-644

**Venkatesan N, Thiyagarajan V, Narayanan S, Arul A, Raja S, Vijayakumar S** (2005) Anti-diarrhoeal potential of *Asparagus racemosus* willd root extracts in laboratory animals. *Journal of Pharma and Pharmaceutical Science* **8**, 39-45

**Visavadiya N, Narasimhacharya RL** (2005) Hypolipidemic and antioxidant activities of *Asparagus racemosus* in hypercholesteremic rats. *Indian Journal of Pharmacology* **37,** 376-380

**Yang HY** (1977) Tissue culture technique developed for asparagus propagation. *HortScience* **12**, 140-141