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Vol. 9(37), pp. 962-967, 3 October, 2015
DOI: 10.5897/JMPR2015.5929
Article Number: 34D43D355681
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

In vitro micropropagation of *Orthosiphon aristatus* (Blume) Miq.

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Received 17 August, 2015; Accepted 17 September, 2015

The present study deals with the mass propagation of *Orthosiphon aristatus* - a multipurpose highly valuable ethnomedicinal shrub. Stem and inflorescence explants were inoculated into the Murashige and Skoog medium (MS medium) fortified with different concentrations and combinations of auxins and cytokinins. The maximum number of shoots achieved was 25 ± 0.78 and 17 ± 0.67 at 5 mg/l benzylaminopurine (BAP) + 2.5 mg/l kinetin (kn) and 3mg/l BAP + 1.5mg/l Kn from nodal and inflorescence explants, respectively. For *in vitro* rhizogenesis, the elongated micro shoots were aseptically excised and were transferred to the half strength MS liquid medium supplemented with different concentrations of Indole-3 acetic acid and Indole butyric acid (IAA and IBA). The best rooting was achieved at 2 mg/l IBA giving maximum of 12 ± 0.67 roots per shoot. The well established plantlets were hardened by transferring them to the poly cups containing sterile soil and vermicompost in the ratio of 1:1. After 3 weeks of hardening, established plantlets were transferred to the field with 95% survivability.

Key words: Stem, inflorescence, callus, multiple shoots, acclimatisation, *O.aristatus.*

INTRODUCTION

Genetic diversity is the foundation of sustainability as it provides raw material for evolution and survival of species and individuals especially under changed environmental and disease condition (Erikson et al., 1993). India has a rich repository of medicinal plants and Ayurveda being an integral part of Indian culture, has documented the diversity of Indian medicinal plants and their uses, which is globally followed now. There is growing concern about the diminishing and depletion of plant biodiversity because of the increasing demand of herbal drugs. Traditional healers and pharmaceutical industries are indiscriminately harvesting the medicinal plants to meet out demands, which have threatened their existence in the wild. Herbal medicine is now expanding its base at a faster rate due to great inputs from ethnomedicinal practitioners which are being proved

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scientifically. *Orthosiphon aristatus* (Blume) Miq. is a multipurpose ethnomedicinal shrub which belongs to family Lamiaceae. It is widely used by tribal people for the treatment of various ailments. It has been reported to treat diseases like fever, syphilis, gonorrhoea, menstrual disorders, kidney stones and influenza (Akowuah et al., 2004).

The plant has been used as antihypertensive, anti-inflammatory, antiallergic and anticancer (Matsubara and Bohgaki, 1999). *O.aristatus* has also been reported to treat hepato-renal syndrome (Shantanova et al., 1997), renal ischaemia (Nikolaev et al., 1996) and suppressive effect on rat thoracic aorta (Ohasi et al., 2010). *O.aristatus* is variably distributed and very rare in the field and hence the conservation of this species is needed to ensure its sustainable utilisation (Rajendran et al., 2001). Plant tissue culture techniques provide an effective tool for the conservation of medicinal plants and biodiversity conservation. Hence the present study was taken up to standardise the protocol for the effective conservation and rapid multiplication of *O.aristatus* in order to meet the market demands while simultaneously minimizing the possible threat to the wild existence.

MATERIALS AND METHODS

O.aristatus plants were collected from the Nilgiri Hills of Tamil Nadu, India from October to November 2012 and are being maintained in the medicinal plant garden of the Department of Studies in Botany, University of Mysore, Mysore, Karnataka-India. Explants; stem (nodal and intermodal region) and inflorescence of *O.aristatus* were collected from the garden and were washed under running tap water for 10 to 15 min to ensure the removal of soil and dust particles followed by 1% (w/v) bavistin treatment. The explants were then washed with sterile distilled water and dipped into 70% alcohol for 45 sec. Inside laminar air flow explants were treated with 0.01% (w/v) $HgCl_2$ for 5 min followed by washing with sterile distilled water (4 to 5 times) to ensure no trace of $HgCl_2$ is left. The explants were cut into appropriate sizes and the excess water was removed by using sterile blotter discs.

Explants were inoculated into the MS (Murashige and Skoog, 1962) medium containing 3% sucrose, 0.08% agar and fortified with different concentrations and combinations of growth regulators (2,4-D, IAA, NAA, BAP, BAP + 2, 4-D, BAP + IAA, BAP + NAA, BAP + Kinetin) (Table 1). Prior to autoclaving at 121°C for 15 min, pH of the medium was adjusted to 5.8 using 1N NaOH and 1N HCl. The culture flasks were maintained in the incubation chamber under a 16 h photoperiod at light intensity of 25 $\mu mol/s^2/m^2$ for 4 weeks. Each treatment had 6 replicates and was repeated thrice. Means were compared by using SPSS 16 version (DMRT). *In vitro* regenerated shoots were aseptically excised and transferred to the half strength MS liquid medium supplemented with IAA (0.5, 1, 2 and 3 mg/l) and IBA (0.5, 1, 2 and 3 mg/l). The data was collected after 4 weeks, measuring root lengths and number of roots per shoot. *In vitro* rooted plantlets were removed from the culture tubes and hardened by transferring to polycups containing sterile soil and vermicompost in the ratio 1:1. After three weeks of hardening, plantlets were transferred to the field conditions with maximum survivability.

RESULTS AND DISCUSSION

Stem (node and internode) and inflorescence were used as explant source. Callusing was achieved from the cut ends in different combinations and concentrations of PGRs used (Table 1). Out of the auxins used, 2,4-D (3 and 5 mg/l) was found more effective in callusing than IAA and IBA. This is in concurrence with the earlier studies carried on *Anisochilus carnosus* (Nissar et al., 2014) and *Arnica montana* (Petrova et al., 2011). Stem segments were reported to be good source for callusing, which may be due to their physiological state which provides actively dividing cells (Chen et al., 1988). The compact and nodular callus from the cut ends may be due to wound caused during the cutting which resulted in synchronous cell division, which is in concurrence with the result of *Saccharum officinarum* (Tahir et al., 2011). This is considered as a process of de-differentiation of organised tissues and is similar to the result of various studies carried out earlier (Zing et al., 2010). Upon combination of benzylaminopurine (BAP) with different auxins, BAP + IAA was found to be effective than BAP + NAA and BAP + 2,4-D.

Results revealed that BAP + IAA stimulates callus induction and shows synergistic effect. Synergism between growth regulators have been reported earlier by several researchers; e.g Chowdhury et al. (2011) in Vitex negundo and Rani and Rani (2010), *Tylophora indica.* Multiple shoots were induced in both stem (node) and inflorescence explants (Figure 1b and c). Varied concentrations and combinations of PGRs were tested, out of which few combinations were reported to induce multiple shoots (Table 1). The best PGR combination for maximum number of multiple shoots was found to be BAP + Kn, which produced maximum of 25 ± 0.78 shoots at 5 mg/l BAP + 2.5mg/l Kn from nodal explant (Figure 1b and d). Explants developed shoots after 3 to 4 weeks of inoculation and the shoot length was more in BAP + Kn combination than other combinations tested. The culture medium with specific growth regulator combinations and concentrations influenced the organogenesis in the present study. BAP played a major distinctive role in the shoot induction in nodal explants. Multiple shoot induction by using BAP at different concentrations has been well reported in several plants; for example, *Ocimum* spp (Pattnaik and Chand, 1996), *Withania somnifera* (Manickam et al., 2000), *Phyllanthus carolinierisis* (Catapan et al., 2000) and *Piper* spp. (Bhar et al., 1995).

Inflorescence explants developed multiple shoots after 4 to 5 weeks of inoculation with maximum number of 17 ± 0.67 shoots (Figure 1c and e) at 3 mg/l BAP + 1.5 mg/l Kn. When inflorescence segments with intact floral buds were cultured, they showed a considerable enlargement between 2 to 3 weeks of culture. At the end of 4 weeks, green vegetative buds started emerging out from the

Table 1. Effect of different PGRs on callusing and shooting of *O.aristatus* stem and inflorescence.

PGR	Concentration (mg/l)	Stem		Inflorescence	
		Callus %	Shooting	Callus %	Shooting
2,4-D	0.5	55	-	20	-
	1	70	-	25	-
	3	98	-	55	-
	5	95	-	60	-
IAA	0.5	40	-	10	-
	1	60	-	23	-
	3	75	-	42	-
	5	78	-	49	-
NAA	0.5	45	-	34	-
	1	55	-	42	-
	3	70	-	52	-
	5	65	-	50	-
BAP	0.5	35	-	30	-
	1	50	-	44	
	3	70	5 ± 0.45^{a}	60	6 ± 0.66^{ef}
	5	70	5 ± 0.62^{a}	63	5 ± 0.67^{b}
BAP + 2,4-D	1 + 0.5	80	-	42	-
	3 + 1.5	100	-	62	-
	5 + 2.5	95	-	78	-
BAP + IAA	1 + 0.5	55	-	42	-
	3 + 1.5	80	8 ± 0.45^{ef}	52	7 ± 0.67^{cd}
	5 + 2.5	75	6 ± 0.43^{cd}	67	6 ± 0.65^{ef}
BAP + NAA	1 + 0.5	50	-	40	-
	3 + 1.5	65	6 ± 0.67^{cd}	52	6 ± 0.67^{ef}
	5 + 2.5	60	5 ± 0.63^{a}	62	7 ± 0.62^{cd}
BAP + Kn	1 + 0.5	50	5 ± 0.58^{a}	48	7 ± 0.63^{cd}
	3 + 1.5	75	16 ± 0.65^{e}	56	13 ± 0.74^{e}
	5 + 2.5	76	25 ± 0.78^{f}	67	17 ± 0.67^{f}

*All treatments with 6 replicates and were repeated thrice. Each value represents Mean ± S.D. Statistical analysis using DMRT ($P \leq 0.5$). Means followed by same letters within column are not significantly different.

inflorescence axis which later developed into shoots, covering the whole inflorescence axis.

Our results concur with the studies carried out earlier in *Gynandropsis pentaphylla* (Nagarathnamma et al., 2013), *Zingiber officinale* (Kirmal et al., 1992) and *Curcuma longa* (Neeta et al., 2000). Primarily, cytokininis have major role on plant development, such as regulation of shoot formation, multiplication and the promotion of cell division and expansion (Mok and Mok, 2001). Our study revealed that the effect of BAP and Kn combination on multiple shoot induction is more compared to BAP or Kn when used separately. This is in confirmation with the earlier studies in *Bambusa glaucescens* (Shirin and Rana, 2007) and *Lagenaria siceraria* (Saha et al., 2007). Srivastava and Joshi (2009) reported that BAP tested individually was more effective than Kn alone on shoot multiplication of *Portulaca grandiflora* but their combination was most advisable for shoot multiplication. They elaborated that Kn stimulated BAP-dependent shoot growth.

After 7 to 8 weeks, shoots attained the length of 3 to 5 cm and were aseptically excised and transferred to half strength MS liquid medium fortified with different concentrations of IBA and IAA (0.5,1, 2 mg/l) for rhizogenesis (Table 2). In the medium containing IBA at 2 mg/l, best rooting with maximum of 12 ± 0.67 roots per shoot was achieved (Figure 1g). The results were recorded after four weeks with average number of roots

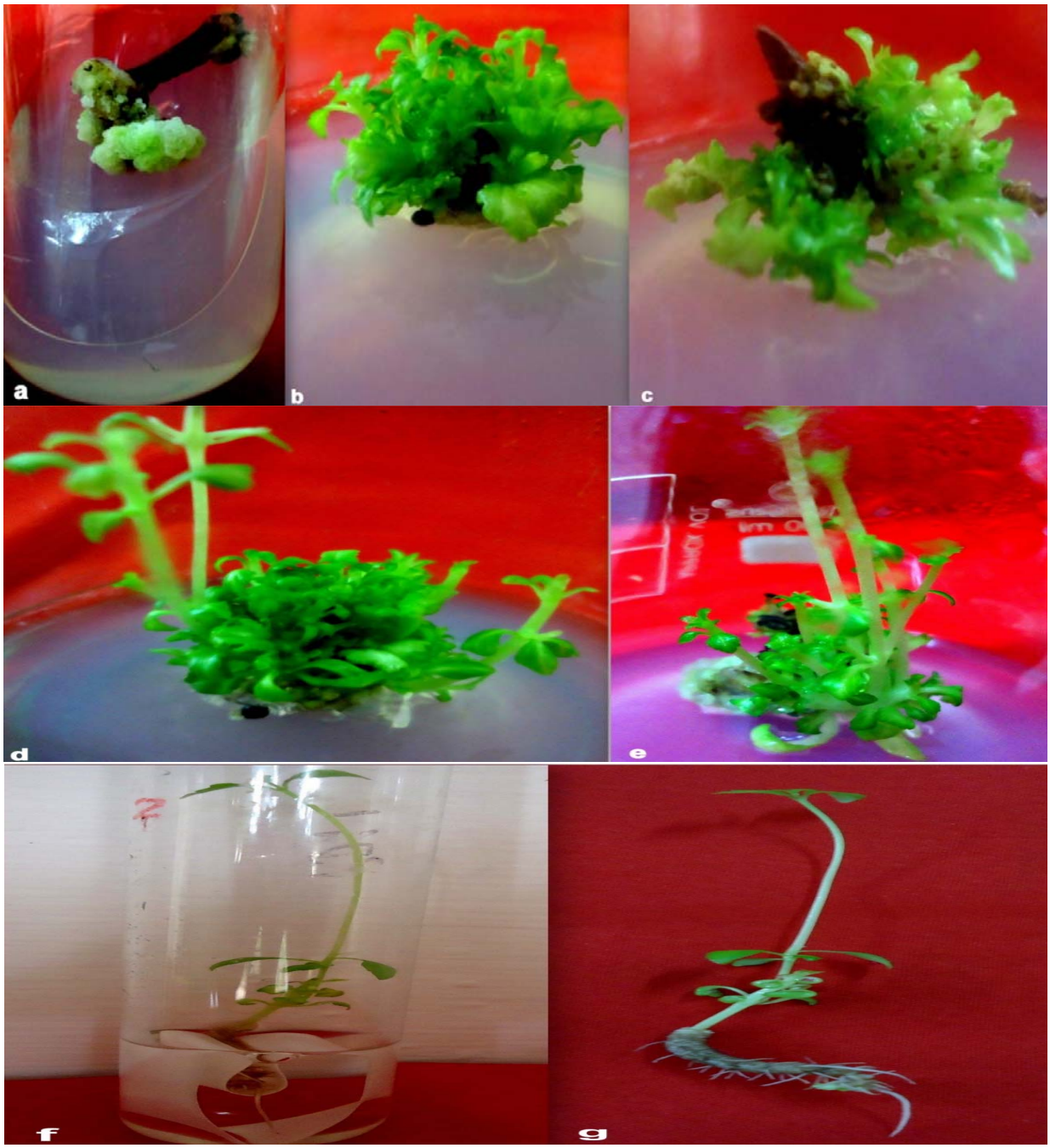

Figure 1. (a) Callusing of internodal segment; (b). Multiple shoot emergence from nodal explants; (c). Multiple shoot emergence from inflorescence explants (d). Shoot proliferation in nodal explants; (e). Shoot proliferation in inflorescence explants; (f). *In vitro* rhizogenesis; (g). Rooted plantlet.

and root length per shoot. There are several earlier studies which have confirmed IBA as a best rooting growth regulator; for example, *Thapsia garganica* (Mukunga et al., 2006) and *Psudarthria viscid* (Vinothkumar et al., 2010). The well rooted *in vitro* regenerated plantlets were hardened for three weeks in polycups containing sterile soil and vermicompost in the ratio 1:1 inside growth chamber (Figure 2a, 2b). After 21

Table 2. Effect of IBA and IAA on *in vitro* rhizogenesis.

PGR	Concentration (mg/l)	Mean root no./shoot	Mean root length/shoot
IBA	0.5	-	-
	1	-	-
	2	12 ± 0.67	3 ± 0.62
	3	4 ± 0.66	2 ± 1.00
IAA	0.5	-	-
	1	-	-
	2	-	-
	3	-	-

*All treatments are with 6 replicates and were repeated thrice. Each value represents Mean ± S.D. Statistical analysis using DMRT (P ≤ 0.5).

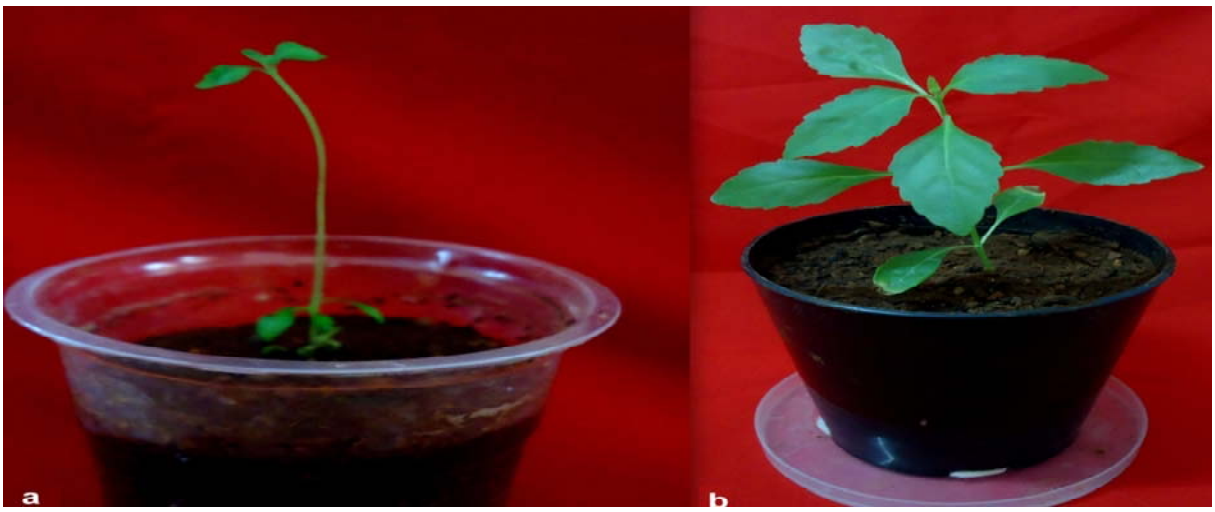

Figure 2. (a) Acclimatisation of plantlet; (b). Acclimatised plant after 4 weeks.

days of hardening, established plantlets were transferred to the field with maximum survivability.

CONCLUSION

Our study describes the standardised protocol for the callus induction and mass propagation of *O. aristatus* using nodal and inflorescence explants. The lack of proper cultivation practices and indiscriminate way in which the plant is collected by pharmacists and traditional healers from its natural habitat, poses a serious threat to its existence in the wild. So propagation through tissue culture technique offers a viable alternative for the conservation of such highly valuable medicinal plant while simultaneously meeting out the pharmaceutical and commercial demands.

ACKNOWLEDGEMENT

Authors are grateful to the University of Mysore for providing all the necessary facilities to carry out the present study.

Conflicts of interest

Authors declare that they have no conflict of interest.

Abbreviations

PGR, Plant growth regulator; **Kn,** Kinetin; **BAP,** Benzylaminopurine; **IAA,** Indole-3 acetic acid; **IBA,**

Indole butyric acid; **2,4-D,** 2,4 dichlorophenoxyacetic acid.

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