

***In vitro* Regeneration of *Blepharispermum subsessile* DC: An Endangered Medicinal Plant of Odisha, India using Cotyledon Explants**

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Key words: Blepharispermum subsessile, Endangered, In vitro regeneration, Cotyledon

Abstract

Multiple shoots were induced on cotyledon explants of *in vitro* grown seedlings of *Blepharispermum subsessile* DC, cultured on MS medium supplemented with various combinations and concentrations of BAP, IBA and GA₃. The highest regenerative response was observed on medium containing 2.5 mg/l BAP where shoot buds initiated after 12 days of inoculation and about 32 shoots were produced in 30 days time. Addition of GA₃ played a key role in leaf expansion and elongation of shoot buds. Addition of the auxin IBA to the induction medium resulted in more callus proliferation rather than shoot bud induction. The elongated shoots were transferred to root induction medium consisting of half strength MS supplemented with IAA, NAA and IBA. Highest rooting response (90%) was recorded in ½ MS supplemented with 1.0 mg/l IAA. Acclimatized plants were maintained in polybags with garden soil for future re-introduction program to their natural habitat.

Introduction

The genus *Blepharispermum* belongs to Asteraceae and constitutes a number of medicinally acclaimed species. Among these 'Rashna Jhadi' (*Blepharispermum subsessile*) is distributed in Odisha, Karnataka, Madhya Pradesh and Maharashtra states in India. This plant has been used in pharmacology and is a popular ethnomedicinally important plant. It is used in the treatment of common cold, rhinitis, as a wormicidal and as tonic (Gupta et al. 2004), diarrhea (Dash and Padhy 2006), eye troubles, backache and rheumatism (Prusti and Behera 2007), irregular menstruation (Shukla et al. 2008). Its rhizome has an aromatic odour

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and is used in havan samagri (Garg 1992). Four different chromenes were isolated from the aerial parts of *B. subsessile* (Kulkarni et al. 1987). In addition to the previously isolated desmethyl isoencecalin, a new chromene compound 5-hydroxy-6-acetyl-2-hydroxy methyl-2-methyl chromene was isolated from the rhizomes of this plant (Agarwal et al. 1999, 2000). These compounds were known to have anti-fertility, anti-feedant and anti-fungal activities (Kulkarni et al. 1987, Agarwal et al. 1999, 2000).

Blepharispermum subsessile is an important but endangered medicinal plant of Odisha. Local people collect rhizomes and seed materials of this plant from the wild. Seed setting of this plant is very low. Propagation through seed is hampered by a low germination rate and low viability. The collection of this plant from the wild to meet its growing demand is threatening the very existence of this plant. Still there is no report available for its commercial cultivation. There is no previous report on micropropagation of any species of *Blepharispermum*. Therefore, there is an urgent need not only to cultivate this plant but also to develop conservation strategies for it. During the last few years, *in vitro* culture techniques have been developed into a successful and rapid means of asexually propagating a large number of plant species. Clonal propagation by plant tissue culture is extremely desirable to regenerate sufficient population of plants with similar characteristics, decreasing or eliminating the possibility of anomalies arising through other methods (Bajaj et al. 1988). *In vitro* propagation will also be useful to conserve and rapidly propagate the rare, endangered and threatened (RET) medicinal plants (Surasan et al. 2006). Regeneration through micropropagation has been obtained in many medicinal plant species (Lameira et al. 1994, Johnson et al. 1997, Anand et al. 1998; Nayak et al. 2007, Kalidass et al. 2008, Kalidass and Mohan 2009 and Nayak et al. 2010). The aim of the present study is to develop an efficient protocol for *in vitro* plant regeneration from cotyledon explants of aseptically grown seedlings of *B. subsessile* for the purpose of conservation of this endangered medicinal plant.

Materials and Methods

The seeds collected from Brahmagiri, Kalahandi district, Odisha were used as the source material. The study was conducted at Taxonomy and Conservation Division, Regional Plant Resource Centre, Bhubaneswar, Odisha.

The seeds were washed in running tap water for 20 min and then dipped in a liquid detergent Tween 20 (Hi-Media, India) 1% (v/v) to remove the superficial dust particles as well as fungal and bacterial spores. Surface disinfection was made with 70% (v/v) ethanol for 2 - 3 min. After washing with double distilled water, seeds were treated with 0.2% (w/v) bavistin (Bayer, India) for 5 min.

Subsequently the seeds were surface sterilized with 0.1% (w/v) HgCl₂ for 3 - 4 min and thoroughly washed three to four times with sterile double distilled water. This was done under the aseptic condition of the laminar air flow. Following sterilization all the seeds were inoculated in MS basal media without any growth regulators.

Cotyledon explants from 1 week old aseptically grown seedlings were excised and cultured in MS salts and vitamins with 3% (w/v) sucrose supplemented with different concentration of cytokinin BAP (0.5 - 3.0 mg/l) either alone or in combination with the auxin IBA (0.25, 0.75 and 1.0 mg/l) and/or GA₃ (0.5 and 1.0 mg/l) for multiple shoot induction and subsequent elongation. The pH of the media was adjusted to 5.7 - 5.8 prior to gelling with agar (0.6 % w/v) and autoclaving at 121°C and 15 psi for 15 - 20 min. All cultures were incubated in a culture room maintained at 25 ± 2°C and 55 - 60% relative humidity under cool-white florescent lights providing 2500 lux with a photoperiod of 16/8 hrs (day/light).

Healthy *in vitro* derived shootlets (2 - 3 cm) from cotyledon explants were separated and individually transferred to MS with IBA, NAA or IAA at different concentration (0.5 - 1.5 mg/l) for *in vitro* rooting. The rooted plantlets (4 - 5 cm) long were washed in sterile distilled water to remove the traces of medium and transplanted to root trainers (Nivedita traders, India) and poly pots containing autoclaved vermiculite saturated with half strength MS basal salts. The plantlets are being acclimatized inside a mist chamber maintained at 28 ± 2°C, 85 - 90% relative humidity for 4 weeks. The plants that survived were transferred to the polybags containing sand, soil and farmyard manure (1 : 1 : 1) and maintained in a shade-net house for another 2 weeks. Finally the hardened plants were transferred outdoors under full sun but maintained in polybags with garden soil for future re-introduction program to their natural habitat.

Observation on mean number of shoots per culture, mean shoot length and callus diameter were recorded at 30 days after inoculation in shoot induction medium. Similarly, data on per cent response, number of roots per shoot and root length were recorded from the *in vitro* shootlets after 30 days of culture. All experiments included 10 replicates and were conducted thrice. The number of shoots per explants, shoot length and basal proliferated tissue diameter were compared within each treatment by one way ANOVA. DMRT was applied at p ≤ 0.05 level of probability to compare individuals within treatment using SPSS 11.5; SPSS Inc., Chicago IL, USA software.

To trace the ontogenic stages of shoot bud regeneration, cotyledon explants were periodically fixed in 70% ethanol: acetic acid: formalin solution (18 : 1 : 1) at 3 days interval until the shoot buds became discernible to the naked eye. The

fixed tissue were dehydrated through a graded ethanol-butyl alcohol series and embedded in paraffin wax (Johansen 1940). Serial transverse sections (10 μm) were cut on a rotary microtome, deparaffinized in a xylene series, stained with safranin and fast green and mounted on glass slides with glycerol anhydrous. Photographs were taken with a camera (Canon, EOS 60D, Japan) attached to the microscope (Olympus SZ-PT).

Results and Discussion

On an average 60% seeds showed germination after 20 days of inoculation. The cotyledons from these aseptically grown seedlings were inoculated into shoot induction medium. Morphological changes of the cotyledon explants were noticed within 7 days of inoculation. At the initial stage the explants increased in size and became a mass of compact green or light green proliferated tissue. Tiny shoot buds arose from the compact tissue in about 1 - 2 weeks of inoculation (Fig. 1A). The differentiation of shoot buds was associated with proliferation of compact callus irrespective of the growth regulator combination and concentration. The degree of callus proliferation varied greatly with the growth regulator combination of the MS (Table 1). The highest diam of callus (4.8 cm) was seen in MS containing a combination of 2.5 mg/l BAP + 1.0 mg/l IAA + 1.0 mg/l GA₃ followed by a diameter of 4.36 cm in MS supplemented with 1.5 mg/l BAP + 0.5 mg/l GA₃.

Histological investigation showed morphological changes leading to organogenesis from cotyledon explants of *B. subsessile*. It had a single layer of epidermis and multilayered cortex with vascular bundles in the centre (Fig. 2A, B). Histological investigations revealed that after 7 days of culturing, cells of subepidermal layer had undergone many divisions, which resulted in the development of shoot primordium (Fig. 2C, D). Shoot buds with well developed leaf primordia and shoot apical meristem were evident in the material fixed at 14 days of culturing (Fig. 2E, F).

The cotyledons cultured in MS basal medium devoid of growth regulators failed to show any morphological changes. Addition of cytokinin like BAP had a positive effect on shoot formation from the cotyledon explants. The highest shoot regeneration was observed at an optimum concentration of 2.5 mg/l BAP, where on an average 32 shoots per explants were produced with an average length of 4.32 cm per shoot within 30 days of inoculation (Fig. 1A, B). The frequency of shoot bud production declined when the concentration of BAP was increased to 3.0 mg/l.

Table 1. Effect of growth regulators on induction of adventitious shoots on cotyledon explants of *Blepharispermum subsessile* ^{1,2, *}

MS + growth regulators (mg/l)			Mean no. of shoots/explant	Mean shoot length (cm)	Mean callus diam (cm)
BAP	GA ₃	IBA			
-	-	-	-	-	-
0.5	-	-	8.33 ^d	3.5 ^{ef}	1.53 ^f
1.0	-	-	16 ^{cd}	4.26 ^{cde}	2.6 ^{cd}
1.5	-	-	21 ^{bc}	4.23 ^{cde}	4.5 ^a
2.0	-	-	24 ^b	3.63 ^{de}	3.5 ^{bc}
2.5	-	-	32 ^a	4.23 ^{cde}	2.53 ^{cd}
3.0	-	-	9 ^e	2.76 ^f	2.53 ^{cd}
0.5	0.5	-	11.66 ^{de}	4.43 ^{cd}	3.23 ^{cd}
1.0	0.5	-	11.66 ^{de}	4.06 ^{cde}	3.16 ^{cd}
1.5	0.5	-	21 ^{bc}	5.43 ^{ab}	4.36 ^{ab}
2.0	0.5	-	18.33 ^{bc}	4.06 ^{cde}	3.0 ^{cd}
2.5	0.5	-	14.66 ^{de}	4.7 ^{bc}	2.9 ^{cd}
1.5	0.5	0.25	10.66 ^{de}	4.8 ^{bc}	2.36 ^{de}
1.5	0.5	0.75	14.33 ^{de}	6.16 ^a	3.06 ^{cd}
2.0	1.0	0.25	11.33 ^{de}	4.53 ^{cd}	1.03 ^f
2.0	1.0	0.75	11.33 ^{de}	3.93 ^{cde}	2.9 ^{cd}
2.5	1.0	1.0	24 ^b	4.26 ^{cde}	4.8 ^a

¹Data pooled from 3 independent experiments each with 10 replicates per treatment.

²Data presented of 30 days old cultures. *Mean values within column followed by the same letter in superscript are not significantly different ($p < 0.05$; DMRT).

The combined effect of BAP and GA₃ was also evaluated for multiple shoot induction. Addition of GA₃ along with BAP to the basal media showed no significant difference in shoot induction. But the shoots produced here were with well expanded leaves and longer internodes. The shoots produced in BAP + GA₃ combination media were comparatively longer than the shoots produced in BAP containing media. This BAP + GA₃ combination media is a better choice for sub-culturing of shoot buds for the above observed character. Here maximum regenerative response was observed in MS + BAP (1.5 mg/l) + GA₃ (0.5 mg/l) where on an average 21 shoots were produced with an average length of 5.43 cm in 30 days time (Fig. 1C).

The synergistic effect of the auxin IBA was evaluated along with BAP and GA₃. Addition of IBA to the BAP + GA₃ combination media showed no significant difference in shoot induction. The longest shoots (6.16 cm) observed here were in MS + BAP (1.5 mg/l) + GA₃ (0.5 mg/l) + IBA (0.75 mg/l) media. The maximum shoot regenerated here (24 shoots/explants) was in MS + BAP (1.5

mg/l) + GA₃ (0.5 mg/l) + IBA (0.75 mg/l) media. Increasing the concentration of IBA from 0.25 to 1.0 mg/l increases the diameter of basal tissue proliferation.

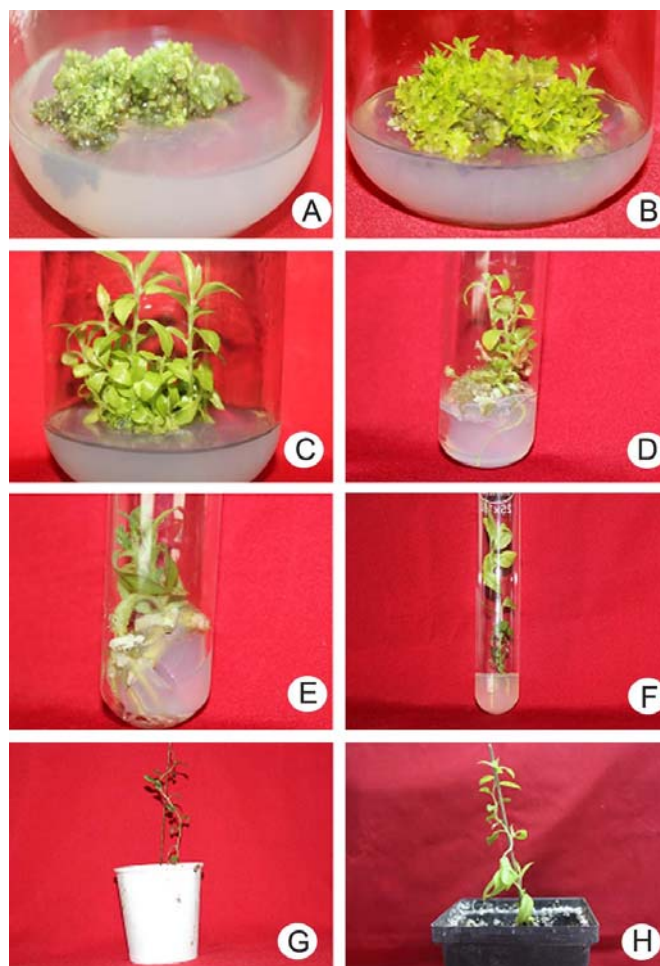


Fig. 1. *In vitro* induction, regeneration, elongation and rooting of *Blepharispermum subsessile* DC. A. Shoot bud initiation from the cotyledon explant after 2 weeks of culture on MS supplemented with 2.5 mg/l BAP. B. Differentiation of shoot buds in the same media. C. Shoot proliferation on MS supplemented with 1.5 mg/l BAP and GA₃ mg/l. D. *In vitro* shoot rooted in half strength MS + NAA (1.0 mg/l) after 2 weeks of culture. E. *In vitro* shoot rooted in half strength MS + IBA (1.0 mg/l) after 2 weeks of culture. F. *In vitro* shoot rooted in half MS + IAA (1.0 mg/l) after 2 weeks of culture. G & H. An *in vitro* regenerated plant at different stages of acclimatization.

Elongated shoots from the shoot induction medium were used for rooting experiments. Auxin had a significant influence on root induction. None of the shoots cultured in an auxin free medium (MS basal) rooted. Depending on the auxin type and concentration, root number ranging from 1 - 3 per shoot were

observed (Table 2). Root was initiated between 8 and 15 days of culture. Irrespective of the auxin type root formation was accompanied by callus formation at the junction of the root and shoot and this increases with increasing concentration of auxin. Shoots cultured on either 1.0 mg/l IBA or 1.0 mg/l NAA supplemented medium showed a maximum of 60% rooting response with an

Table 2. Effect of different concentrations of IBA, NAA and IAA on induction of roots in *in vitro* raised shoots of *Blepharispermum subsessile* ^{1,2,*}

MS + growth regulators (mg/l)			% rooting	Mean no. of roots/shoot	Mean root length (cm)
IBA	NAA	IAA			
-	-	-	-	-	-
0.5	-	-	50	1.8 ^{ab}	1.78 ^d
1.0	-	-	60	2.4 ^{ab}	1.34 ^d
1.5	-	-	60	1.6 ^{ab}	2.16 ^{cd}
-	0.5	-	70	1.8 ^{ab}	2.42 ^{bcd}
-	1.0	-	60	2.6 ^a	1.46 ^d
-	1.5	-	60	1.2 ^b	3.4 ^{abc}
-	-	0.5	70	2 ^{ab}	4.28 ^a
-	-	1.0	90	2.2 ^{ab}	4.54 ^a
-	-	1.5	70	1.2 ^b	3.86 ^{ab}

¹Data pooled from 3 independent experiments each with 10 replicates per treatment. ²Data presented of 30 days old cultures. *Mean values within column followed by the same letter in superscript are not significantly different ($p < 0.05$; DMRT).

average of 2.4 and 2.6 roots per shoot, respectively. As the concentration increased there was a decline in per cent response and root number. The media fortified with 1.0 mg/l IAA showed the highest rooting response where about 90% shoots responded for rooting producing an average of 2.2 roots per shoot with average length of 4.54 cm (Fig. 1F). Although the number of roots produced per shoot was higher in the NAA supplemented medium, the shoots cultured in NAA showed poor root growth (Fig. 1D). IAA proved best with the highest per cent rooting and very less callusing at the base followed by NAA and IBA. Some difficulties were faced in acclimatizing these *in vitro* regenerated plants. Only 30 - 40% plants survived at the end of acclimatization period. Further studies are going on regarding acclimatization and hardening of *in vitro* regenerated plants of this species. The few survived and hardened plants were transferred outdoors under full sun but maintained in polybags with garden soil for future re-introduction program to their natural habitat.

The *in vitro* propagation of *Blepharispermum subsessile*, an under explored and endangered ethnomedicinal plant, is reported here for the first time. *In vitro* propagation through organogenesis from cotyledons was described here for this plant. Among Asteraceae members, direct and indirect organogenesis has been

achieved with *Stevia rebaudiana* using thin cell layers of hypocotyls (Marco and Lourdes 2015), *Artimisia vulgaris* using cotyledonary nodes (Kumar and Kumari 2010), *Cinnamomum tamala* using nodal segment, shoot tip, internode and leaf (Sharma and Nautiyal 2009), *Eclipta alba* using leaf, stem and root (Zafar and Sagar 1999), *Launaea sarmentosa* using leaves (Mahesh et al. 2012),

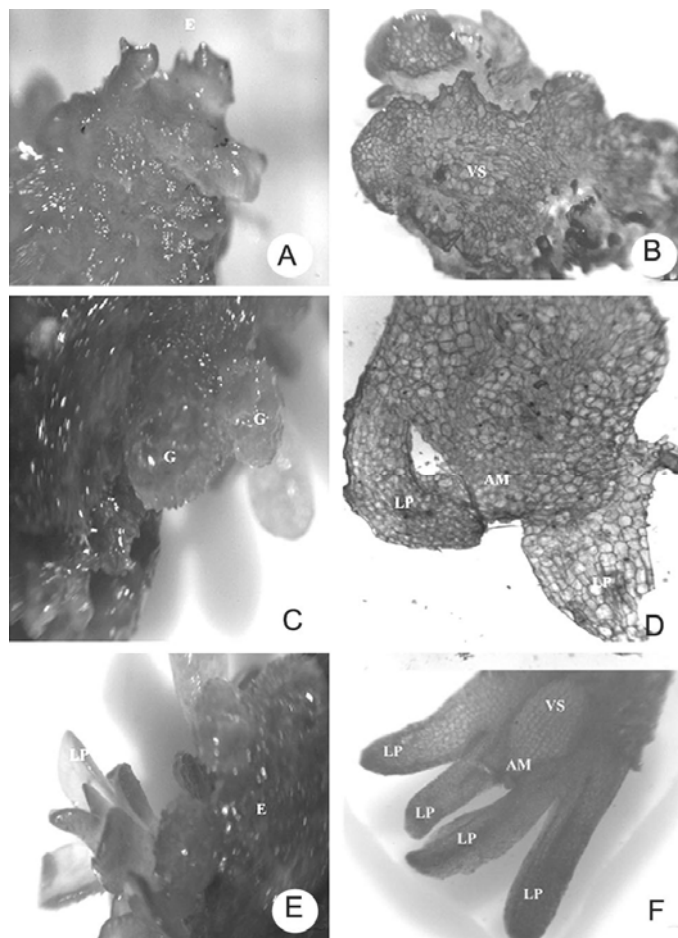


Fig. 2. Histology of adventitious shoot regeneration from cotyledon explants. A. Development of shoot buds after inoculation. B. Histology of a shoot bud showing apical meristem. C & D. Formation of apical meristems with leafy primordia. E & H. Histology of secondary leafy primordia developmental structure.

Spilanthes acmella using leaf discs (Singh and Chaturvedi 2012) etc. In the present study, the effect of BAP either alone or in combination with IBA or GA₃ was evaluated on multiple shoot induction on cotyledons of *B. subsessile*. All these growth regulator combinations were found to induce multiple shoots. But, it was

observed that MS with 2.5 mg/l BAP alone was the most efficient treatment for multiple shoot induction on cotyledons than the combinations. On the contrary positive synergistic effect of cytokinins like BAP or Kn or TDZ or 2iP or zeatin in combination with an auxin like IAA, or NAA on *in vitro* shoot regeneration through organogenic differentiation has been reported by several workers (Shen et al. 2007, Unda et al. 2007, Erisen et al. 2010, Ghimire et al. 2010, Lin et al. 2011, Wadl et al. 2011, Mandal and Laxminarayana 2012, Perez-Jimenez et al. 2012 and Bhagya et al. 2013). Superior effect of BAP for shoot multiplication has been reported among various medicinal plants of Asteraceae, like *Tanacetum cinerariifolium* (Hedayat et al. 2009), *Spilanthes acmella* (Saritha and Naidu 2008), *Launaea sarmentosa* (Mahesh et al. 2012). As in the present study, the reduction of shoot regeneration potential by the combined effect of BAP and GA₃ compared to a medium with cytokinin alone was also demonstrated in *Guizotia abyssinica* (Baghel and Bansal 2014). On the contrary, a combination of BAP and GA₃ had shown better response in *Artemisia vulgaris* (Borzabad et al. 2010). The shoots developed by BAP over its optimal level (2.5 mg/l) showed stunted growth. A similar phenomenon has also been reported in *Tanacetum cinerariifolium* (Hedayat et al. 2009).

In the present study it was observed that the differentiation of shoot buds was associated with proliferation of compact callus irrespective of the growth regulator combination and concentration. This may be due to the cotyledon's varying endogenous auxin level or variation in their responsivity to growth regulators in the nutrient media, which played a role in the callus induction ability. For root induction, MS basal medium with 1.0 mg/l IAA was found superior. Higher efficacy of IAA over other auxins for root induction has been demonstrated in many medicinal plants of Asteraceae family, like *Stevia rebaudiana* (Ahmed et al. 2007), *Inula verbascifolia* (Perica et al. 2008), *Artemisia petrosa* (Pace et al. 2004).

The results presented here suggest an efficient, reproducible and easy-to-handle protocol for *in vitro* regeneration of *B. subsessile* using cotyledons as explants for large scale propagation. This study can be used for commercial cultivation and domestication of this valuable medicinal plant after development of a proper hardening procedure. This will not only help in *ex situ* conservation, but will also contribute to the rehabilitation of this endangered plant in nature.

Acknowledgement

The authors are grateful to the Chief Executive, Regional Plant Resource Centre, Bhubaneswar for the encouragement and facilities. They are also

thankful to Forest & Environment Department, Government of Odisha, Bhubaneswar for financially supporting this project.

References

- Agarwal SK, Verma S, Singh SS, Kumar S and Keshri G** (1999) Antifertility chromene from *Blepharispermum subsessile*. *Fitoterapia* **70**(4): 435 - 437.
- Agarwal SK, Verma S, Singh SS, Tripathy AK, Khan ZK and Kumar S** (2000) Antifeedant and antifungal activity of chromene compounds isolated from *Blepharispermum subsessile*. *J. Ethnopharmacol.* **71**(1-2): 231-234.
- Ahmed MB, Salahin M, Karim R, Razvy MA, Hannan MM, Sultana R, Hossain M and Islam R** (2007) An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni.) in Bangladesh. *Am-Eurasian J. Sci. Res.* **2**: 121-125.
- Anad A, Rao CS and Latha R** (1998) Micropropagation of *Uraria picta*, a medicinal plant, through axillary bud culture and callus regeneration. *In Vitro Cell Dev Biol Plant* **34**: 136-140.
- Baghel S and Bansal YK** (2014) Synergistic effect of BAP and GA₃ on *in vitro* flowering of *Guizotia abyssinica* Cass. - A multipurpose oil crop. *Physiol. Mol. Biol. Plants* **20**(2): 241-247.
- Bajaj YPS, Furmanowa M and Olszowska O** (1988). Biotechnology of the micropropagation of medicinal and aromatic plants. *In: Bajaj YPS, ed., Biotechnology in agriculture and forestry. vol. 4: Medicinal and aromatic plants I*, Springer, Berlin, Germany. 60-103.
- Bhagya N, Chandrashekar KR, Karun A and Bhavyashree U** (2013) Plantlet regeneration through indirect shoot organogenesis and somatic embryogenesis in *Justicia gendarussa* Burm. f., a medicinal plant. *J. Plant Biochem. Biotechnol.* **22**(4): 474-482.
- Borzabad RK, Sudarshana MS and Niranjana MH** (2010) *In vitro* plant regeneration from leaf explants of *Artemisia vulgaris* L. - A medicinal herb. *Modern Appl. Sci.* **4**: 9.
- Dash SK and Padhy S** (2006). Reviews on ethnomedicines for diarrhoea diseases from Orissa prevalence versus culture. *J. Hum. Ecol.* **20**: 59-64.
- Erisen S, Yorgancilar M, Atalay E and Babaoglu M** (2010). Prolific shoot regeneration of *Astragalus cariensis* Boiss. *Plant Cell Tiss. Org. Cult.* **100**: 229-233.
- Garg SN** (1992) Chemical examination of the essential oil of *Blepharispermum subsessile* rhizomes - A good source carvicrol. *Proceeding of exploration indigenous raw materials for essential oil industry*, Bharat Jyoti, Lucknow, India. 107-111.
- Ghimire BK, Seong ES, Goh EJ, Kim NY, Kang WH, Kim EH, Yu CY and Chung IM** (2010) High-frequency direct shoot regeneration from *Drymaria cordata* Willd. leaves. *Plant Cell Tiss. Org. Cult.* **100**: 209-217.
- Gupta AK, Sharma M and Tandon N** (2004). Reviews on Indian medicinal plants, Indian Council of Medical Research, New Delhi, 2004. pp. 250-251.

- Hedayat M, Abdi Gh and Khosh-Khui M** (2009) Regeneration via direct organogenesis from leaf and petiole segments of pyrethrum [*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.]. *Am-Eurasian J. Agric. Environ. Sci.* **6**(1): 81-87.
- Johansen DA** (1940) *Plant microtechnique*, McGraw-Hill, New York. pp. 126-154.
- Johnson TS, Narayan SB and Narayana DBA** (1997) Rapid *in vitro* propagation of *Saussurea lappa*, an endangered medicinal plant through multiple shoot cultures. *In Vitro Cell Dev. Biol. Plant* **33**: 128-130.
- Kalidass C and Mohan VR** (2009). *In vitro* rapid clonal propagation of *Phyllanthus urinaria* (Euphorbiaceae) - A medicinal plant. *Researcher* **1**: 56 - 61.
- Kalidass C, Manickam VS and Glory M** (2008). *In vitro* studies on *Leptadenia reticulata* (Retz.) Wight. & Arn. (Asclepidaceae). *Ind. J. Multi. Res.* **4**: 221-225.
- Kulkarni MM, Nagasampagi BA, Deshpande SG and Sharma RN** (1987) Five chromenes from *Blepharispermum subsessile*. *Phytochem.* **26**: 2969-2971.
- Lameira OA, Costa MP and Pinto JEBP** (1994). The efficiency of shoot and plantlet formation of *Cephaelis ipecacuanha* after three subcultures *in vitro*. *Ciencia Rural* **24**: 523-526.
- Lin GZ, Zhao XM, Hong SK and Lian YJ** (2011) Somatic embryogenesis and shoot organogenesis in the medicinal plant *Pulsatilla koreana* Nakai. *Plant Cell Tiss. Org. Cult.* **106**: 93-103.
- Mahesh A, Thangadurai D and Melchias G** (2012). Rapid *in vitro* plant regeneration from leaf explants of *Launaea sarmentosa* (Wild.) Sch. Bip. ex Kuntze. *Biol. Res.* **45**: 131-136.
- Mandal J and Laxminarayana U** (2012). *In vitro* organogenesis of *Quisqualis indica* Linn.- An ornamental creeper. *Amer. J. Plant Sci.* **3**(9): 1272-1282.
- Marco A Ramírez-Mosqueda and Lourdes G Iglesias-Andreu** (2015) Direct organogenesis of *Stevia rebaudiana* Bertoni using thin cell layer (TCL) method. *Sugar Tech.* **17**(55): 1-5.
- Nayak P, Behera P R and Thirunavoukkarasu M** (2007). High frequency plantlet regeneration from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *In vitro Cell Dev. Biol. Plant* **43**(3): 231-236.
- Nayak P, Behera P R, Thirunavoukkarasu M and Chand P K** (2010). High frequency plant regeneration through adventitious multiple shoot organogenesis in epicotyl explants of Indian gooseberry (*Emblica officinalis* Gaertn.). *Sci. Hort.* **123**(4): 473-478.
- Pace L, Pacioni G and Spano L** (2004). *In vitro* propagation of *Artemisia petrosa* ssp. *eriantha*: potential for the preservation of an endangered species. *Plant Biosyst.* **138**: 291-294.
- Perez-Jimenez M, Carrillo-Navarro A and Cos-Terrer J** (2012) Regeneration of peach (*Prunus persica* L. Batsch) cultivars and *Prunus persica* × *Prunus dulcis* rootstocks via organogenesis. *Plant Cell Tiss. Org. Cult.* **108**: 55-62.
- Perica M C, Vrsek I and Mitic B** (2008) *In vitro* propagation of *Inula verbascifolia* (Willd.) Hausskn. sub species *verbascifolia*. *Plant Biosyst* **142**: 1-4.
- Pradeep Kumar S and Ranjitha Kumari BD** (2010) Effect of aminoacids and growth regulators on indirect organogenesis in *Artimisia vulgaris* L. *Asian J. Biotechnol.* **2**(1): 37-45.

- Prusti AB** and **Behera KK** (2007) Ethnomedicobotanical study of Sundargarh district, Orissa, India. *Ethnobotanical Leaflet*. **11**: 148-163.
- Sarasan V**, **Cripps R**, **Ramsay MM**, **Atherton C**, **McMichen M**, **Prendergast G** and **Rowntree JK** (2006). Conservation *in vitro* of threatened plants - Progress in the past decade. *In Vitro Cell Dev. Biol. Plant* **42**: 206-214.
- Saritha KV** and **Naidu CV** (2008) Direct shoot regeneration from leaf explants of *Spilanthes acmella*. *Biol. Plant* **52**: 334-338.
- Sharma G** and **Nautiyal AR** (2009) Influence of explants type and plant growth regulators on *in vitro* multiple shoots regeneration of a laurel from Himalaya. *Nature and Science* **7**(9): 1-7.
- Shen X**, **Chen J** and **Kane ME** (2007) Indirect shoot organogenesis from leaves of *Dieffenbachia* cv. Camouflage. *Plant Cell Tiss. Org. Cult.* **89**: 83-90.
- Shukla R**, **Moyna C** and **Gautam MP** (2008) Indigenous medicines used for treatment of gynecological disorders of tribals of Chattisgarh. *Ind. J. Med. Plants Res.* **2**: 356-360.
- Singh M** and **Chaturvedi R** (2012) Screening and quantification of an antiseptic alkylamide, spilanthol from *in vitro* cell and tissue cultures of *Spilanthes acmella* Murr. *Ind. Crop Prod.* **36**: 321-328.
- Unda F**, **Kalynyak P** and **Riseman A** (2007) Organogenesis plant regeneration from leaf explants of *Exacum* Styer group. *Plant Cell Tiss. Org. Cult.* **89**: 105-111.
- Wadl PA**, **Dattilo AJ**, **Vito LM** and **Trigiano RN** (2011) Shoot organogenesis and plant regeneration in *Pityopsis ruthii*. *Plant Cell Tiss. Org. Cult.* **106**: 513-516.
- Zafar R** and **Sagar BPS** (1999) *In vitro* plant regeneration of *Eclipta alba* and increased production of coumestans. *Fitoterapia* **70**: 348-356.