IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 2 Issue 8, August 2015.
www.ijiset.com
ISSN 2348 – 7968

Micropropagation studies in *Cocculus hirsutus* (L) Diel.

P.V. Shelar[1], U. R. Wayase[2,*], S. S. Deokule[1]

[1] Department of Botany, Savitribai Phule Pune University, Ganeshkhind, Pune- 411007, Maharashtra Pune, India

[2] Post Graduate Research Center, Department of Botany, Modern College of Arts, Science & Commerce, Shivajinagar, Pune -411005, Maharashtra, India

* Corresponding author, e-mail: (botanica5050@gmail.com)

Abstract

The present investigation was aimed to develop a reproducible and proficient regeneration protocol for organogenesis in *Cocculus hirsustus* (L) Diel. Plant material for the present work was collected from various spots from Pune district including Katraj hill, Savitribai Phule Pune University campus and Mulshi dam area. They were grown and maintained in green house of Savitribai Phule Pune University botanical garden. Nodal sectors of these seedlings were excised and used as explants. The surface sterilized explants were inoculated on MS basal medium supplemented with BAP (0.00-11.05 µM) and kinetin (0.00-11.60 µM) alone and also in combinations. The cultures were incubated at 25 ± 2°C under white fluorescent light (65 µE/m^2/ s) and with 16 h photoperiod. The shoots were visible after 10-15 days after inoculation. After 21 days the *in vitro* grown shoots were transferred for induction of roots on MS basal medium supplemented with IAA (0.00-14.24 µM). The early initiation of shoots were observed 8.3 ± 1.63 days after inoculation from nodal sector explant on MS + 2.21 µM BAP but maximum percentage (84.1±1.97%) of shoot initiation was observed in MS + 4.42 µM BAP. MS basal supplemented with 2.32 µM Kinetin showed early shoot formation i.e. 8.3 ±1.29 days after inoculation and 4.64 µM Kinetin showed maximum percentage (63.3±1.96%) of shoot initiation. MS basal supplemented with 2.21µM BAP in combination with 2.23 µM kinetin showed early initiation 11.33 ± 1.06 days after inoculation and 4.42 µM BAP + 4.64 µM Kinetin showed maximum percentage (73.6±1.14%) of shoot initiation. *In vitro* grown shoots were inoculated on MS basal medium supplemented with IAA (0.00-11.05 µM). Auxin containing medium resulted in maximum number of roots (3.33 ± 0.88) and maximum percentage of cultures (83.5 ±1.51 %) showed root initiation in *in vitro* grown shoots in MS + 2.85µM IAA. The outcomes presented describe an effective and reproducible tissue culture regeneration protocol.

Keywords: *Cocculus hirsutus* (L) Diels., *in vitro.*

1. Introduction

Indiscriminate use of synthetic drugs and antibiotics has resulted in to serious symptoms all over the world, the demand of plant based raw material for pharmaceuticals have increased enormously. The world health organization has emphasized the utilization of indigenous systems of medicines based on locally available raw plant material. *Cocculus* shows one of the greatest diversities in alkaloid types in the family Menispermaceae. About 135 alkaloids of 13 different classes have been isolated. Due to different phytochemicals the genus *Cocculus* is used in the treatment of various diseases. *Cocculus hirsutus* (L) Diel reported to contain essential oil, β-sitosterol, ginnol (Merchant *et al.*, 1962), glycoside, sterol and alkaloids (Das *et al.*, 1964). *Cocculus hirsutus* (L) is dioecious plant therefore sexual reproduction is difficult process. Because of its medicinal properties the demand for Cocculus hirsutus (L) Diels has increased by many folds which has lead to indiscriminate harvesting of *Cocculus hirsutus* (L) Diels. Roots of this plant serve as a source of many phytochemicals but harvesting roots means destroying the entire plant. Urbanization has also decreased the number of the plant further decreasing the chance of sexual reproduction. Therefore, it becomes essential to develop a useable protocol for micro-propagation and organ culture for *Cocculus hirsutus* (L) Diels. Multiple shoots induction through axillary buds of *Cocculus hirsutus* (L) Diel has been successfully achieved (Meena and Singh, 2012). The literature available on tissue culture of *Cocculus hirsutus* (L) Diels is meagre and there is no useable protocol available for the multiplication.

2. Material and Methods:

Plant material and *in vitro* culture:

Plant material for the current work was collected from various spots from Pune district including Katraj hill, Savitribai Phule Pune University campus and Mulshi dam area. Seeds of the plant were collected and seedlings from these seeds were grown and maintained in green house of Savitribai Phule Pune University botanical garden. Using nodal sectors explants of a young and healthy branches, culture were initiated. Explants were washed under

ISSN 2348 – 7968

running tap water for 20-30 min. with 2-3 drops of Tween 20. Then the explants were washed by bavistin (0.02% w/v) and rinsed with sterile distilled water, followed by surface sterilization with 0.075% of $HgCl_2$ for 2 min. and finally the explant were washed with sterile distilled water for 5 times to remove any traces of $HgCl_2$. Explants were inoculated on MS basal medium supplemented with BAP (0.00-11.05 µM) and kinetin (0.00 – 11.60 µM) alone and in combinations.

The cultures were incubated at 25 ± 2°C under white fluorescent light (65 µE/m^2/ s) and with 16 h photoperiod. The shoots were visible after 10-15 days after inoculation. After 21 days the *in vitro* grown shoots were transferred for induction of roots on MS basal medium supplemented with IAA (0.00-14.24 µM).

For regeneration and rooting studies *in Cocculus hirsustus* (L) Diel**.** MS media with 3% (w/v) sucrose and 0.8% (w/v) agar (Himedia, India) was used. The pH of the media was adjusted to 5.8 with 0.1N NaOH before autoclaving at 120°C for 15 min.

In vitro rooted plantlets from culture vessels were removed and washed with sterile distilled water to remove the traces of culture media. The plantlets were transferred to plastic containers containing garden soil added with vermiculite and sand (1:1:1). The plastic bags were used to cover plantlets to maintain high (70 to 80%) humidity levels. Cups were incubated in the growth room illuminated with 650-lux light intensity and 25 ± 2ºC temperature. Cups were maintained in the shade with 650-lux light intensity and at temperature 25 ± 2°C and with 60-65% relative humidity. $^1/_2$ strength MS basal medium was added to pots at the interval of 4 days for nourishment of the plantlets. Above mentioned conditions were maintained for first 1 week. After the 1 week polythene bags were removed. The cups were maintained in the shade for one week. Plantlets were exposed progressively to full sunlight, and then plantlets were taken to the field condition. All the above mentioned experiments were repeated three times and standard errors of the means were calculated. Data were analyzed by using analysis of variance procedure (ANOVA) in MS Excel program.

2. Results and Discussion

Review of literature reveals that there is no usable tissue culture protocol available for *Cocculus hirsutus* (L) Diels. Therefore present study, it was proposed to establish a simple protocol for rapid *in vitro* multiplication of *Cocculus hirsutus* (L) Diels. Effect of increasing concentrations of kinetin (0.00-11.60 µM) on the nodal sector explants suggests that 4.64 µM kinetin was the most optimum concentration for induction of shoot from nodal sector explant 63.3±1.96 % cultures showed shoot initiation and 2.32 µM Kinetin showed early (8.3±1.29 days after inoculation) shoot initiation, with maximum shoot length attended after 3 weeks was 2.1±0.24cm.

Similar observations were recorded by Sobhakumari and Lalithakumari (2003), Sevimay *et al.* (2005), Girija *et al.* (2006), Rao et al. (2006), Sharma et al. (2007), Akbas *et al.* (2009), Negi and Saxena (2011) and Meena *et al.* (2012) in different plants.

Effect of increasing concentration of BAP (2.21 – 11.05 µM) on nodal sector explant indicated that the 4.42 µM concentration of BAP used in the experiment induced maximum percentage of shoot initiation (84.1 ±1.97 %) in *Cocculus hirsutus* (L) Diels. nodal sector explant and 2.21 µM BAP was the most optimum concentration for early shoot initiation (8.3 ± 1.63 days after inoculation) from nodal sector explant, where single shoot was produced from the nodal sector with maximum height of 1.8 ± 0.23 cm .

The results are in line with researches indicating the efficiency of BAP for shoot culture initiation and multiplication (Uranbey *et al.*, 2003; Khawar *et al.*, 2005; Tiwari *et al*., 2007; Meena and Patni, 2007; Jain *et al.*, 2009; Akbas *et al*., 2011; Meena *et al.*, 2012).

Results on effect of MS basal media supplemented with BAP in combination with kinetin indicate that all the combinations of kinetin and BAP used in the experiment, induced a single shoot per explant from the nodal sector explant of *Cocculus hirsutus* (L) Diels. 2.21 µM BAP + 2.32µM Kin was the most optimum combination of BAP and kinetin for early shoot induction, (13.33 ± 1.43 days after inoculation) compared to other concentration. The percentage of shoot initiation was maximum (73.6 ± 1.14 %) in MS basal + 4.42 µM BAP + 4.64µM Kinetin combination. The shoot length of the shoots produced by nodal sectors MS basal + 4.42µM BAP + 4.64µM Kinetin was also found to be maximum shoot length (10.6 ± 1.23 cm).

At lower concentrations shoot initiation was observed. Similar effects of lower concentrations of cytokinin on shoot initiation and elongation have been reported also by Kaur *et al.* (1998), Dave and Purohit (2002), Parmaksiz and Khawar (2006), Arya *et al.* (2008), Basalma *et al.* (2008a), Basalma *et al.* (2008b), Gayathri *et al.* (2009), Uranbey et al. (2010) and Meena et al. (2012).

Results of effect of increasing concentrations of IAA (0.00-14.24 µM) on shoot explants of *Cocculus hirsutus* (L) Diels showed highest percentage of root initiation (83.5 ±1.51 %) on 2.85 µM IAA concentration of IAA, and the percentage of root formation linearly decreases with the increase in the concentration of IAA. 2.85 µM IAA concentration showed maximum root initiation (3.33 ± 0.88 roots / shoot). The 2.85µM concentration of IAA

ISSN 2348 – 7968

was the optimum concentration and showed maximum root initiation.

The supplementation of auxin either individually or in combination for rooting was also testified in many plant species (Gopi *et al*. 2006; Baksha *et al*., 2007; Kalidass *et al*., 2008; Aasim *et al*., 2008a; Kalidass and Mohan, 2009; Aasim *et al*., 2009). The addition of IBA also favored rooting in some medicinal plants (Chandra *et al*., 2006; Ozel *et al*., 2006, 2008; Sivanesan, 2007; Meena *et al*., 2010; Chordia *et al*., 2010; Nagesh and Shanthamma, 2011 and Meena et al., 2012).

3. Tables and Figures

Table No. 1 Effect of Kinetin on nodal sector explant of *Cocculus hirsutus* (L) Diels.

Kinetin (µM)	Shoot initiation days after inoculation	Percent of cultures producing shoots	Shoot length (cm)
MS + 0.00	00	00	00
MS + 2.32	8 .3 ±1.29	13.3 ±1.2	1.6 ± 0.3
MS + 4.64	10±0.83	63.3 ±1.96	2.1 ± 0.24
MS + 6.96	11.6 ± 0.95	26.3 ± 1.89	1.5 ± 0.20
MS + 9.28	12.3 ± 1.56	38.6 ±1.60	1.2 ± 0.37
MS +11.60	13.6± 1.37	7.6 ±1.56	0.9 ± 0.20

Results are mean of three replicate (30X3) ± S.D.

Table No. 2 Effect of BAP on nodal sector explant of *Cocculus hirsutus* (L) Diels.

Results are mean of three replicate (30X3) ± S.D.

BAP (µM)	Shoot initiation days after inoculation	Percent of cultures producing shoots	Shoot length (cm)
MS + 0.00	-	00	00
MS + 2.21	8.3 ± 1.63	37.5 ±1.45	0.8 ± 0.17
MS + 4.42	10.6 ±1.58	84.1 ±1.97	1.8 ± 0.23
MS + 6.63	11.3± 1.26	33.5 ±1.14	1.4 ± 0.24
MS + 8.84	13.6 ±1.42	28.3 ±1.32	1.2 ± 0.33
MS +11.05	16.0 ± 1.76	24.6 ±1.19	0.9 ± 0.06

Table No. 3 Effect of Kinetin and BAP on nodal sector explant of *Cocculus hirsutus* (L) Diels.

BAP and Kinetin (µM)	Shoot initiation days after inoculation	Percent of cultures producing shoots	Shoot length (cm)
MS	00	00	00
BAP 2.21 + Kin 2.32	11.33 ± 1.06	42.5 ± 1.52	6.8 ± 1.63
BAP 4.42+ Kin 4.64	13.33 ± 1.43	73.6 ± 1.14	10.6 ± 1.23
BAP 6.63 + Kin 6.96	12.00 ± 1.51	32.6 ± 1.53	3.9 ± 0.89
BAP 8.84 + Kin 9.28	14.33 ± 1.23	29.5 ± 1.65	2.8 ± 0.96
BAP 11.05 + Kin 11.60	16 .33 ± 1.86	14.5 ± 1.19	1.9 ± 0.63

Results are mean of three replicate (30X3) ± S.D.

Table No.4 Effect of IAA on in vitro grown shoot explants of *Cocculus hirsutus* (L) Diels.

IAA (µM)	Percent of cultures producing Roots	Average number of roots produced
MS	00	00
MS + 2.85	83.5 ±1.51	3.33 ± 0.88
MS + 5.70	56.3 ± 1.11	2.66± 0.54
MS + 8.55	43.6 ± 1.26	2.33 ± 0.95
MS + 11.40	38.5 ±1.09	1.66 ± 0.72
MS + 14.24	21.1 ± 1.55	1.33± 0.09

Results were recorded 3 weeks after inoculation Results are mean of three replicate (30x3) ± S.D.

4. Conclusions

Low concentrations of Kinetin (2.23 µM) and BAP (2.21 µM) singly as well as in combination showed early shoot initiation, shoot thus produced where single. BAP 4.42+Kin 4.64 combination showed maximum percentage of shoot initiation. Optimum rooting induction on shoot explant was observed on MS Supplemented with 2.85 µM IAA. Furthers different concentrations of growth hormones or even different hormones can be tasted for their effect on *Cocculus hirsutus* (L) Diels. This study can serve as a base for further studies.

Acknowledgment:

This research work was monetarily supported by BCUD, Pune University, a research project sanctioned to PV.Shelar. The laboratory facilities provided by authorities of MCASC, PUNE -5 are gratefully acknowledged




ISSN 2348 – 7968

A) B) C) D)

E)  F) 

Plate I: Organogenesis in *Cocculus hirsutus* (L) Diels A) shoot regeneration on 4.64 µM Kin. B) shoot regeneration in 4.42 µM BAP C) Shoot regeneration on 4.64 µM Kin.+ 4.42 µM BAP D) Root regeneration on 2.85 µM IAA E& F) Hardening process.

References

[1] Akbas F, Isikalan C, Namli S, Karakus P and Basaran D (2011). Direct plant regeneration from *in vitro*-derived leaf explants of *Hypericum spectabile*, a medicinal plant. *J. Med. Plant Res.* 5(11): 2175-2181.

ISSN 2348 – 7968

[2] Arya D, Patni V. and Kant U. (2008). *In vitro* propagation and quercetin quantification in callus cultures of Rasna (*Pluchea lanceolata* Oliver & Hiern.). *Indian J. Biotechnol*. 7: 383-387.

[3] Badole S, Patel N, Bodhankar S, Jain B and Bharadwaj S. (2006). Antihyperglycemic activity of aqueous extract of leaves of *Cocculus hirsutus* (L.) Diels in alloxan induced diabetic mice. *Ind. J. Pharmacol*. 38(1): 49-53.

[4] Baksha R, Jahan MAA, Khatun R, Munshi J L (2007). *In vitro* rapid clonal propagation of *Rauvolfia serpentina* (Linn.) Benth. Bangladesh J. Sci. Ind. Res. 42(1): 37-44. Basalma D, Uranbey S, Gurlek D, Ozcan S (2008a). TDZ-induced plant regeneration in *Astragalus cicer* L. *African. Journal of Biotechnol*. 7: 955-959.

[5] Chandra B, Palni LMS and Nandi S K (2006). Propagation and conservation of *Picrorhia kurrooa* Royle ex Benth.: an endangered Himalayan medicinal herb of high commercial value. *Biodivers. Conserv*. 15, 2325-2338.

[6] Chordia A M, Kumari R S, Kannan RR (2010). *In vitro* regeneration of plantlets from internodal callus culture of *Vitex leucoxylon* L. – A rare medicinal plant. J. Med. Plant Res. 4(22): 2399-2403.

[7] Gopi C, Nataraja S Y, Ponmurugan P. (2006). *In vitro* multiplication of *Ocimum gratissimum* L. through direct regeneration. *Afr. J. Biotechnol.* 5(9): 723-726.

[8] Das P K, Nath V, Gode K D, Sanyal A K, (1964). Preliminary phytochemical and pharmacological studies of *Cocculus hirsutus* Linn, *Ind. J Med Res*,; 52: 300-307.

[9] Dave A; Purohit S D (2002). Factors influencing *in vitro* culture of 'Safed Musli' (*Chlorophytum borivilianum*). *Oikoassay*, 15: 19-27.

[10] Gayathri G; Arya K and Bastian D (2009). Rapid multiplication of Fire Flamed Bush (*Woodfordia fruticosa* (L.) Kurz) through *in vitro* techniques. *Current Biotica*. 2(4): 453-460.

[11] Girija S; Kavitha S, and Deepavathi S. (2006). Direct multiple shoot regeneration from shoot tip and nodal explants of *Ocimum sanctum* L. (Tulsi): A medicinal herb. *Plant Cell Biotechnology*. *Mol. Biol.* 7(1&2): 23-28.

[12] Iqbal S. (1993) Extended studies on the chemical constituents of *Cocculus hirsutus* [dissertation]. Karachi, University of Karachi

[13] Jain R, Singh N, and Patni V (2009). *In vitro* plantlet regeneration and isolation of quercetin from elite species of *Acacia catechu* (L.F.) Willd- A katha yielding plant. *Plant Cell Biotechnol. Mol. Biol.* 10(3& 4): 125-130.

[14] Jagannadha Rao K V, Ramachandra R L, (1961). Chemical examination of *Cocculus hirsutus* (Linn) Diels. *J Sci Ind Res,*; 20(B): 125-126.

[15] Kalidass C, Mohan V R (2009). *In vitro* rapid clonal propagation of *Phyllanthus urinaria* Linn. (Euphorbiaceae)- A medicinal plant. *Researcher*, 1(4): 56-61.

[16] Kaur K, Verma, B, Kant U (1998). Plant obtained from the Khair tree (*Acacia catechu* Willd.) using mature nodal segments. *Plant Cell Reproduction.* 17: 427-429.

[17] Merchant J R., R. M. Naik and S. N. Hirwe, (1962). Chemical investigation of *Cocculus hirsutus* (L.) Diels. *J.Ind.Chem.Soc.*39:411-416.

[18] Meena, M. K. Singh N. and Patni V, (2012). *In vitro* multiple shoot induction through axillary bud of *Cocculus hirsutus* (L.) Diels: A threatened medicinal plant. *Afric. J. of Biotechnol..*;.11(12):2952-2956.

[19] Murashige T and Skoog F 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15: 473-497.

[20] Nagesh K S and Shanthamma C (2011). Micropropagation and antioxidant activity of *Mollugo nudicaulis* Lam. *J. Med. Plant Res.* 5(6): 895-902.

[21] Negi D and Saxena S (2011). *In vitro* propagation of *Bambusa nutans* Wall. ex Munro through axillary shoot proliferation. *Plant Biotechnol. Rep*. 5: 35-43.

[22] Parmaksiz. I and Khawar K M (2006). Plant regeneration by somatic embryogenesis from immature seeds of *Sternbergia candida* Mathew Et T. Baytop, an endangenred endemic plant of Turkey. *Propagat. Ornament. Plants*, 6(3): 128-133.

[23] Rastogi B, Tiwari U, and Dubey A., (2008). Immunostimulant activity of *Cocculus hirsutus* on immunosuppressed rat, *Pharmacology* online; 3:38-57.

[24] Sevimay C S; Khawar K M, Parmaksiz I, Cocu S, Sancak C, Sarihan E O and Ozcan S (2005). Prolific *in vitro* bulblet formation from bulb scales of Meadow Lily (*Lilium candidum* L.). *Periodicum Biologurum*, 107(1): 107-111.

ISSN 2348 – 7968

[25] Sivanesan I (2007). Direct regeneration from apical bud explants of *Withania somnifera* Dunal*. Indian J. Biotechnol*. 16: 125-127.

[26] Sharma A; Meena M C, Patni V and Arora D K (2007). *In vitro* multilplication of *Acalypha wilkesiana* dwarf through nodal segment culture. *J. Plant Sci. Res.* 23(1- 2): 79-82.

[27] Sobhakumari V P and Lalithakumari D (2003). Direct plant regeneration from shoot tip cultures of *Capsicum annuum* L. cv. PLR-1. *Phytomorphology*, 53(3&4): 235-242.

[28] Tevari M N, Ramavat K G, Bhardwaj L, (1992). Antitumour activity of *Cocculus hirsutus*, *Bionature*,; 12:33-41.

[29] Uranbey S: Cocu S, Sancak C, Parmaksız I, Khawar K M, Mirici S , and Ozcan S (2003). Efficient adventitious shoot regeneration in *Cicer milkvetch. Biotechnol. Equipment*, 17 (1): 33-37.