Bionature, 30 (2), 2010 : 139-145

MICROPROPAGATION OF MEDICINAL PLANTS II. *WEDELIA CHINENSIS* (OSBECK) MERR.

SUSANTA KUMAR MAITY* BRIJ KUMAR TIWARI AND ANADI KUMAR KUNDU

Department of Botany, Chandernagore Govt. College, Chandernagore, Hooghly, West Bengal

ABSTRACT

Methods for *in vitro* micropropagation of *Wedelia chinensis* by indirect organogenesis via callus were investigated by optimizing the concentrations and combinations of different phytohormones in MS medium. Callus was induced from the leaf explants in MS medium containing BAP and NAA. Callus proliferation with development of associated nodular structures was achieved on subculturing in MS medium fortified with BAP and L-glutamine. Caulogenesis was recorded after the subculture of the nodular light green friable calli on MS basal medium in 2 weeks time. Rhizogenesis was observed 7 days after reinoculation of the shoots in MS basal medium.

No detectable differences were recorded between the donor and *in vitro* generated plants with regard to biochemical and cytological parameters.

Key words : Medicinal plant, *Wedelia,* Micropropagation.

Introduction

Wedelia chinensis (Osbeck) Merr. is a valuable medicinal plant belonging to the family Asteraceae and distributed in several wet parts of India including Uttar Pradesh, Assam and Arunachal Pradesh. It is also found to grow in Sri Lanka, Japan and China. The plant is well recognized in traditional medicine for dyeing grey hairs and promoting their growth. *Wedelia* is reported to contain medicinal principles which can combat several diseases including carcinoma and the diseases of central nervous system. The active principle found in leaves is wedelactone, a lactone of 5:6 dihydroxybenzofuran -3- carboxylic acid that is a structural analogue of coumestrol (Chadha, 1976). Several essential oils have been obtained from the leaves of two different species of *Wedelia* (Cramer & Bridgen, 1997).

Wedelia chinensis is a procumbent herb with light camphor like odour and is propagated mainly by shoot cuttings. Seed setting is very poor. Besides this, in dry places and during summer season, the propagation of this plant is very difficult. The present investigation was therefore undertaken to optimize *in vitro* conditions for multiplication of this potential medicinal plant.

Commercial exploitation and elimination of natural habits consequent to urbanization has led to gradual extinction of several rare, endangered and threatened medicinal plants. *In vitro* propagation is an effective approach to conserve such germplasm.

* Corresponding author : Email: smaity.bot@gmail.com

In recent years, there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of medicinal plants (Ajithkumar and Seeni, 1998; Prakash *et al.* 1999). Micropropagation has proven as a potential technology for mass scale production of medicinal plant species (Martin, 2003; Faisal *et al.* 2003; Azad *et al.* 2005; Hassan and Roy, 2005). Tissue culture technique can play an important role in the clonal propagation of elite clones and germplasm conservation of this medicinal herb. There have been no reports to date on micropropagation of *Wedelia chinensis* using leaf explants. The present study was, therefore, undertaken to develop a protocol for mass clonal propagation by using leaf explants of this important medicinal herb through *in vitro* culture.

Materials and methods

Leaves of *W. chinensis* were taken as explants, these were sectioned (0.5-1 cm) and surface disinfected with 0.1% mercuric chloride solution for 8-10 minutes, followed by 6-8 washes in sterile distilled water. For induction, growth and maintenance of callus tissues, Murashige and Skoog's (1962) basal medium (MS) supplemented with different auxins (2,4-D, IAA and NAA) at 0.5-2 mg/l, cytokinin (BAP) and L-glutamine was used. The pH of medium was adjusted to 5.6 prior to adding agar (0.7% w/v, Qualigens) and autoclaved (121°C, 15 minutes). The cultures were incubated at 24±1°C under 16 hours daily illumination with fluorescent light (12000 lux). The cultures were grown either in culture tubes (25X150 mm) containing 20 ml medium or in 100 ml wide mouth conical flasks with 50 ml culture medium. As and when required, cultures were multiplied by subculturing on fresh medium every two weeks. For growth and further development of callus, calli were transferred to MS medium supplemented with different concentrations of BAP and L-glutamine. For induction of shoots, calli were transferred to MS medium lacking any growth regulators. The shoots were removed and were further subcultured in the same medium for root initiation.

Healthy rooted plantlets were taken from the rooting medium and washed several times with sterile distilled water. Plantlets were potted in sterile sand:loam: peat (in a ratio of 1:1:1) mixture, covered with a polythene bags to maintain high humidity and were kept under controlled temperature at 22-26°C and light (12000 lux) conditions in the culture room. The bags were removed periodically for gradual hardening. After 4-6 weeks when new leaves emerged from the plantlets, they were taken out of the culture room and kept in a shady place under normal temperature and light.

Isozyme analysis:

Isozyme (Peroxidase) analysis was performed by macerating 1 gm leaf material collected from mature and two months old tissue culture raised plants. The leaves were excised and kept in -70°C for some days to avoid chlorophyll interference and macerated in an ice-cold mortar and pestle with ice-cold PEB buffer (the composition of PEB buffer were 0.1 M Tris-HCl, 0.25 M Sucrose, 1 % Polyvinylpyreledon (PVP), 1 % Ascorbic acid, 0.1% Cystein HCl, 1 mM EDTA, 0.4 mM $MgCl_2$, 0.4 mM DTT, 1 % β-mercaptoethanol. All the chemicals were dissolved in double distilled water and the pH was adjusted to 6.8).

The homogenate was collected and centrifuged in a RC 5B Sorval Refrigerated centrifuge for 45 minutes at 12,000 rpm. The clear supernatant was collected and again centrifuged in a Beckman L7-55 Ultracentrifuge at 40,000 rpm for 2 hours. After centrifugation, the pellet was discarded and the supernatant was lyophilized for 10-12 hours as required. SDS-PAGE was performed using 12% polyacrylamide gels. After polymerization 150 µg of protein samples were loaded to each well with the help of a micropipette fitted with multiflex tips (MULTI, USA).

After loading the gel was run at constant current 60-80 voltage in an air-conditioned room. Ice bags and cubes were placed all around the apparatus to maintain low temperature. When the run was completed, i.e. as the tracking dye reached the anodic end, the power supply was switched off and the glass plates with the gels were removed from the apparatus. The stacking gel part was cut off and the rest was incubated in buffer and substrate solution of Isozyme. Guaicol- H_2O_2 method (Hislop and Stahmann, 1971) was followed for the visualization of peroxidase isozymes.

For cytological study

The root tips of *W. chinensis* (from donor plants and regenerated plants through callus culture) were collected and pretreated with saturated solution of PDB for 4 hours, chromosome preparation was made using aceto-orcein method.

Results

The surface sterilization protocol followed in the present investigation yielded 95% of the explant free of apparent microbial contamination. Leaf segments cultured on MS medium containing plant growth regulators enlarged and curled within a week (Fig 1). Subsequently explants showed initiation of callus after three weeks from all parts, i.e. cut margin, midrib region and from both adaxial and abaxial surfaces.The explants cultured on the MS basal medium without any plant growth regulators failed to respond and dried up within a week. Callus developed all over the surface of leaf segments within 3-4 weeks of culture initiation in MS medium containing BAP and NAA in various combinations. The callusing response varied depending on the concentrations and combinations of plant growth regulators used (Table 1).

The callus appeared light green and compact in texture (Fig 2). Leaf derived callus was subcultured on MS medium supplemented with BAP and NAA at three weeks intervals to maintain further growth and proliferation. After four subcultures (14 days interval), entire explants were seen to be covered with whitish green and friable callus (Table 1). When the explants were cultured on MS medium supplemented with different concentrations of BAP (0.5-2.5 mg/l) in combination with 2,4-D (0.5-2 mg/l) or IAA (0.5-2 mg/l), callusing rate was very poor from the beginning and light brown callus developed. Moreover, callus proliferation decreased after the first subculture and callus tissue did not survive. In presence of BAP (0.5-2.5 mg/l) and L-glutamine

Table—1 Effect of different concentrations of BAP and NAA on callus formation from leaf explants of *Wedelia* cultured on MS medium. Results are the mean of 6 replicates±SE.

BAP (mg/l)	NAA (mg/l)	Fresh wt. of callus after 60 days (gm)
0.5	0.5	4.83±0.2
1.0	0.5	6.90±0.8
1.5	0.5	6.20±0.8
2.0	0.5	5.50±0.3
2.5	0.5	4.33±0.1
0.5	1.0	4.80±0.1
1.0	1.0	5.66±0.3
1.5	1.0	3.83±0.4
2.0	1.0	2.67±0.4

(50-100 mg/l), white light brown calli were developed and could be maintained on the same media by transferring the callus repeatedly during subculturing. But such callus could not be maintained after three passages because callus induced on such medium stopped proliferation and did not develop any further. Extensive callus growth was observed in MS medium containing 1mg/l BAP and 0.5 mg/l NAA with the development of whitish green and friable callus (Fig 2).

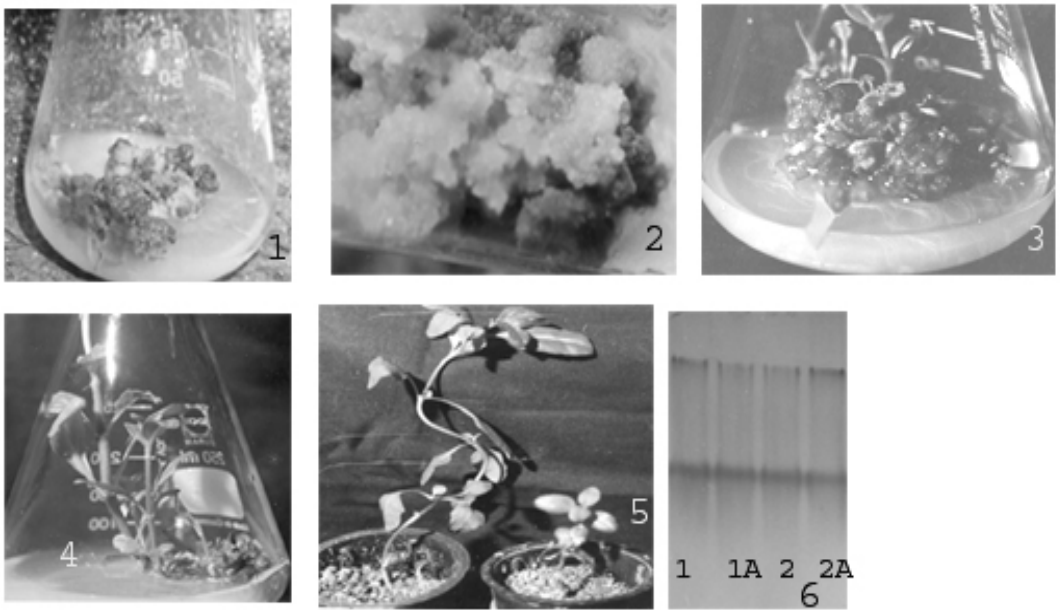

Figure captions:

In vitro* regeneration of plantlets through callus of *Wedelia chinensis

Fig.1. Induction of callus from leaf segments on MS+BAP (1.0 mg/l)+NAA (0.5 mg/l).
Fig.2. Proliferative callus on MS+BAP (1.0 mg/l)+NAA (0.5 mg/l) after six weeks of culture.
Fig.3. Regeneration of multiple shoots from callus on growth regulator free MS medium after 15 weeks of culture.
Fig.4. Rhizogenesis from callus on growth regulator free MS medium after 18 weeks of culture.
Fig.5. Potted *in vitro* raised plantlets.
Fig.6. Peroxidase isozyme pattern.
1&1A=donor plants.
2&2A= *in vitro* raised plants.

Of the two concentrations of NAA (0.5 and 1 mg/l) tested in combination with BAP, 0.5 mg/l NAA was found to be more effective than 1 mg/l in inducing callus formation and their further development. More morphologically distinct callus were developed from the leaf explants in a medium containing BAP (1 mg/l) in combination with NAA (0.5 mg/l) while those initiated from other concentrations of BAP and NAA, grew slower (Table 1). The highest amount (6.90 gm per explant) of fresh weight callus was produced on the medium containing BAP (1.0 mg/l) and NAA (0.5 mg/l) after 60 days of culture.

To further assess the morphogenic potential the callus pieces (0.5 to 1 cm in diameter) were subcultured on MS medium augmented with BAP (0.5-1.5 mg/l) and L-glutamine (100 mg/l) in various combinations. However, the callus proliferated 2-3 times and appeared nodular in at BAP and L-glutamine containing medium, but the quantity of callus declined with increase at BAP levels. The nodular structures of the light green friable callus on further subculture on to MS medium lacking growth regulators individually differentiated into shoot buds within 12 to 15 days (Fig. 3). These buds grew into long green healthy shoots after another three weeks. When the shoots were removed and further subcultured in growth regulator free MS medium, root formation was noticed at the base of the differentiated shoots after 7 days of subculture (Fig.4).

Four weeks old plantlets with well developed root system were removed from the culture medium, hardened and acclimatized. The *in vitro* raised shoots grew vigorously and developed without any visible deformities. Almost 90% survival of the transplanted plantlets of *Wedelia chinensis* was observed in soil (Fig 5). Averages of 40 good sized plants were produced per explant within a period of three months.

Peroxidase

Only one band was observed in the upper (+ve) region of the gel with Rf value 0.34. No band was seen in the lower region (-ve). There was no difference in the intensity of bands between donor plants and regenerated plants of *Wedelia chinensis* (Fig 6).

For cytological study

Both the donor plants and regenerated plants had 2n=50 chromosomes. Difference in chromosome morphology between donor plants and tissue culture raised plants of *Wedelia chinensis* were not detected.

Discussion

Callus induction was obtained in 90% of the explants cultured in MS medium supplemented with 0.5-2.5 mg/l BAP and 0.5-1 mg/l NAA. MS medium fortified with NAA (0.5 mg/l) and BAP (1.0 mg/l) is the best medium for the callus initiation and further growth. The combined activity of BAP and NAA is likely to be responsible for accelerating the rate of cell division in this taxon. BAP alone was not responsible for callus induction from the explant or shoot regeneration from the callus. When the callus were subcultured on MS medium containing BAP and NAA was found to inhibit shoot regeneration. Although for several members of the Asteraceae, kinetin was found to be the most effective hormone in shoot regeneration media (Flick *et al*. 1983). BAP which has been used as a source of cytokinin in the present study has also

been used successfully to induce somatic embryogenesis in several plants (Chand & Singh, 2001;. Anand *et al.* 2001; Prakash *et al.* 2001).

The use of L-glutamine in the embryo proliferation medium was very critical and effective at a concentration of 100 mg/l. Increase in concentration (more than 100 mg/l) in the medium restricted embryo proliferation. The nitrogen content of the culture medium has been reported to influence the morphogenic effects of growth substances. L-glutamine that has been reported critical for embryogenesis among the amino acids used as a source of nitrogen (Thorpe, 1994; McKersie & Brown, 1996; Shetty & McKersie, 1993; Sinha *et al*. 2000) was also found to be effective nitrogen containing molecules for the development of shoot buds in this species.

However, the leaf derived primary callus did not show any morphogenic response when they were repeatedly subcultured on the same medium. Similar effects were reported by Hutchinson *et al.* (1994) in connection with callus induction and plant regeneration from mature zygotic embryos of tetraploid *Alstroemeria*. But when the calli was transferred to growth regulator free MS medium, they showed shoot bud initiation followed by rooting but underlying cause is not very clear. However, it may be mentioned that the method followed is suitable for rapid proliferation and multiplication.

Isozyme profile showed no variation between donor and *in vitro* raised plants through callus culture. Although, isozymes have been used extensively for genomic modification and for identification of varieties and cultivars (Livneh & Vardi, 1998).

REFERENCES

Ajithkumar D. and Seeni S. (1998) Rapid clonal multiplication through *in vitro* axillary shoot proliferation of *Aegle marmelos* (L) Corr., A Medicinal Tree. *Plant Cell Rep.* **17:** 422-426.

Anand RP, Ganapathi A, Vengadesan G, Selvaraj N, Anabazhagan VR and Kulothungan S (2001). Plant regeneration from immature cotyledon-derived callus of *Vigna unguiculata* (L.) Walp (cowpea). *Curr. Sci.,* vol. **80(5):** 671-674.

Azad MAK, Yokota S, Ohkubo T, Andoh Y, Yahara S and Yoshizawa N (2005) *In vitro* regeneration of the callus of *Hybanthus enneaspermus* L. Muell., a rare ethnobotanical herb. *Plant Cell Rep.* **18:** 873-878.

Bose TK and Chowdhury B (1991). Tropical Garden plants in Colour, Vol. 1 (Horticulture and Allied Publishers, Calcutta), 147.

Carman J (1990). Embryogenic cells in plant tissue cultures: occurrence and behaviour. *In Vitro Dev. Biol.,* **26:** 746-753.

Chadha, Y.R. (1976) The wealth of India, Raw materials (eds) CSIR, New Delhi, X, 568.

Chand S and Singh AK (2001). Direct Somatic embryogenesis from zygotic embryos of a timber yielding leguminous tree, *Hardwickia binata* Roxb. *Curr. Sci.,* Vol. **80(7):** 882-887.

Craig W, Wiegand A, O'Neill CM, Mathias RJ, Power JB and Davey MR (1997). Somatic embryogenesis and plant regeneration from stem explants of *Moricandia arvensis. Plant Cell Reports,* **17:** 27-31.

Cramer S and Bridgen P (1997). Somatic embryogenesis and shoot proliferation of *Mussaenda* cultivars, *Plant Cell Tissue and Organ Culture,* **50(2):** 135-138.

Eapen S and George L (1992). Somatic embryogenesis and plant regeneration in inbred and hybrid lines of *Pennisetum americanum* (L) K. Schum. Indian J. Exp. Biol., Vol.- 30, 792-795.

Faisal M, Ahmad N and Anis M (2003) Shoot multiplication in *Rauvolfia tetraphylla* L. using thidiazuron. *Plant Cell Tiss. Organ. Cult.* **80:** 187-190.

Flick CE, Evans DA and Sharp WR (1983) (Yamada Y (eds) Handbook of plant cell-culture). Macmillan, New York, 1, 13.

Hassan AKMS and Roy SK (2005) Micropropagation of Gloriosa superba L. through high frequency shoot proliferation. *Plant Tissue Cult. and Biotech.* **15 (1):** 67-74.

Hislop EC and Stahmann MP (1971). Peroxidase and ethylene production by barley leaves infected with *Erysiphe graminis* f.sp. *hardei. Physiol. Plant Pathology,* 1: 297.

Hutchinson HJ, Tsujita JM and Saxena PK (1994). Callus induction and plant regeneration from mature zygotic embryos of a tetraploid *Alstroemeria (A. pelegrina X A. psittacina). Plant Cell Rep*., **14:** 184-187.

Jasrai YT, Goswami MT, Deshpande SR, Misra A and Parthapasenan G (1999). Micropropagation of an ornamental shrub-*Mussaenda luteola* Linn. Plant Tissue Culture and Biotechnology: Emerging Trends, Universities press (India), 106-111.

Livneh O and Vardi E (1998). Molecular Genetic Markers. In: Hybrid cultivar development (S.S. Banga and S.K. Banga -eds.). Narosa publishing house, New Delhi, India, Pp-201.

Martin K. P. (2003). Plant regeneration through somatic embryogenesis on *Holostema ada-kodien* Schult. a rare medicinal plant. *Plant Cell Tiss. Orga. Cult.* **72:** 79-82.

McKersie BD and Brown DCW (1996). Somatic embryogenesis and artificial seeds in forage legumes. *Seed Sci Res.*, **6:** 109-126.

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

Prakash E, Khan SV, Meru E and Rao KR (2001). Somatic embryogenesis in *Pimpinella tirupatiensis* Bal. and Subr., an endangered medicinal plant of Tirumala hills. *Curr. Sci*., vol **81(9):** 1239-1242.

Prakash E, Sha PS, Sairam P and Rao KR (1999). Regeneration of plants from seed-derived medicinal woody plant *Phellodendron amurense* Rupr. Through excised leaves. *Plant Cell Tiss. Orga. Cult.* **80:** 43-50.

Shetty K and McKersie BD (1993). Proline, thioproline and Potassium mediated stimulation of Somatic embryogenesis in alfalfa (*Medicago sativa* L.) *Plant Sci.,* **88:** 185-193.

Sinha RK, Majumdar K and Sinha S (2000). Somatic embryogenesis and plantlet regeneration from leaf explants of *Sapindus mukorossi* Gaertn: A soapnut tree, *Curr. Sci.,* **78(5):** 620-623.

Thorpe TA (1994) (eds vasil IK and Thorpe TA) Kluwer Academic Publishers. The Netherlands, 17.

Zimmerman JL (1993). Somatic embryogenesis: a model for early development in higher plants. *Plant Cell*, **5:** 1411-1423.