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Callus Free *In Vitro* Clonal Propagation Of Giant Milk Weed - *Calotropis Gigantea* (L.) R.Br.

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Abstract

Calotropis gigantea is considered to be a very important species due to the presence of long chain hydrocarbons in its latex, which is chemically equivalent to crude oil, and its adaptability to cultivation in dry and semi-arid regions. It has clusters of waxy flowers that are either white or lavender in colour. A procedure is outlined for the *in vitro* propagation for shoot multiplication and subsequent plant regeneration of the latex-producing plant, *Calotropis gigantea*. Shoot multiplication was obtained by culturing inter nodal and axillary bud explants on Murashige and Skoog medium with different concentration of plant growth regulators (BAP 5 µM, Kin 10 µM, 5 µM, BAP + IAA : 5 µM + 2 µM, Kin + IBA :3 µM + 2 µM, IBA 1 µM, Vermiculite + IBA 1 µM). Large numbers of shoots were produced in different concentration on Kin, and BAP at Kin 10µm and BAP 5µm. BAP 5µm and Kin10 µM hormone concentration were found to be suitable for rapid shoot growth and multiplication. Healthy and sturdy shoots formed were transferred to MS basal, BAP 5 µm with IBA 2µm and Kin 5 µm with NAA 2 µm. The MS Basal, IBA 2µm and NAA 2µm initiated in number of roots. IBA hormone medium is suitable for root initiation in *C. gigantea*. Rhizogenesis- the formation of roots is a crucial step in micropropagation for the formation of complete plantlets. The highest rooting frequency (80%) was observed on MS medium supplemented with IBA (1.47 µM).

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Subsequently, the moisture was reduced by the removal of polythene bags to harden the plants and out of which about 56% survived.

Keywords: Axillary Buds, BAP-6 Benzyl amino Purine, IBA-indole-3-butyric acid, Internodal explants, Kin- Kinetin, Rhizogenesis , Shoot multiplication.

Introduction

The Apocyanaceae (formerly Asclepiadaceae) with 180 genera and 2500-3000 species is distributed mainly in the tropical and subtropical regions of the world (Yamashiro *et al*, 2008; Mahmood *et al*, 2011). 'Asclepios' the Greek god of healing, is the source of the word Asclepiadaceae, the Latin name for the milkweed family. The Asclepiadaceae comprises of plants showing complex floral structures, which are dedicated to the service of highly specialized pollination biology. *Calotropis gigantea* (Asclepiadaceae) is one of the most abundantly available plants in the semi arid and arid conditions of India. *Calotropis gigantea* flowers are showed in two different colors. One is *Calotropis gigantea* [purple] & another one is *Calotropis gigantea* [white]. The plant grows very well in a variety of soils and different environmental conditions. It thrives on poor soils particularly where overgrazing has removed competition from native grasses. It is drought tolerant and the pioneer vegetation in desert soil (Smith, 2002). Sometimes this plant is the only survivor in some areas, where nothing else grows. Presence of latex, extensively branched root system and thick leaves with waxy coverage and pubescence are the xerophytic adaptations (Sharma& Tripathi, 2009). *Calotropis gigantea* (White madar) acts as a strong purgative. It cleans the unwanted waste contained in the stomach. The whole plant is used for skin diseases, boils and sores and as a tonic and purgative in small doses, and as an emetic in larger doses. Propagation through tissue culture provides solution for mass propagation of plants in general and threatened plants in particular. There is a need to conserve plants with medicinal values. In this technique, newly formed shoots or shoot bases serve as explants for repeated proliferation of plants. Micropropagation plays a distinctive role in the conservation of species, particularly hose having pharmacologic value (Afolayan & Adebola, 2004). It offers cost-effective protocols for large scale propagation of medicinal plants collected from the wild without affecting the survival of the species in their natural of the species in their natural habitats (Balaji *et al*, 1988). Aim of this study was to establish a method for shoot multiplication *Calotropis gigantea* through direct organogenesis and seedling organs from axillary buds and intermodal segments. Rooted plants were acclimated in green house conditions and made ready for field transfer.

Materials and Methods

Explants

Calotropis gigantea explants (Axillary buds) and seeds were collected from waste lands in and around Tirunelveli district, Tamil Nadu. The mature and dehusked seeds were assessed by simple germination experiment. Mature seeds of *C. gigantea* white forms were collected and allowed to germinate in pre sterilized petriplates. The seeds were washed under running tap water for 30 min and with 5% (v/v) Teepol for 5 min. After rinsing with sterile DDW, they were surface sterilized in 0.1% $HgCl_2$ for 10 minutes and again rinsed thoroughly with sterile DDW.

Culture condition and Shoot proliferation

Seedling organs (hypocotyls and epicotyl) and Axillary buds were cultured in sterile culture bottle containing 15 ml of Murashige and Skoog (1962) basal medium with 3% Sucrose, 0.8% Agar and different concentration of hormones for shoot multiplication. Cultures were maintained at 25 ± 1°C, under 16 hrs. light illumination. The effect on shoot multiplication was experimented with different concentrations of auxins (IAA, IBA) and cytokinins (BAP, Kinetin). All the cultures were transferred to fresh medium after 2-3 weeks of inoculation. The frequency and number of shoots formed were evaluated after 6 weeks of inoculation.

Root formation

Individual Microshoots (3-4cm) were excised and transferred to rooting medium comprising of MS basal and half strength MS without any growth regulator or with auxins – IAA/IBA (1µM) for rooting.

Hardening and Acclimation

Well-rooted plants with 5-7 leaves were carefully removed from culture vessels, washed under sterile distilled water to remove the remnants of agar and transferred to pots containing sterile vermiculite. For 2 weeks, the potted plantlets were kept under transparent polythene membrane to ensure high humidity, and then they were kept in open in diffuse light for hardening. After one month, the surviving plants were transferred to pots containing garden soil and maintained in green house for acclimatization.

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Results and discussion

Seedling Organ Culture of *Calotropis gigantea* - White Form

Eight- day- old seedling organs were used for culturing on MS basal medium with different concentration of plant growth regulators such as BAP 10µm, IAA 3µm, NAA 3µm, IBA 1µm, Kin 10µm, Kin 5µm, Kin 2µm. Explants including epicotyls, hypocotyls, axillary buds, stems, leaves were inoculated and incubated. Hypocotyl explants responded quickly by producing direct shoot from the proximal region within 10 days of culture initiation (Figs. c,d-).

Mass propagation

Axillary bud explants were cultured on MS medium fortified with different concentrations of Kinetin - 10 µM, 5 µM separately and it resulted in the development of multiple shoots. Axillary bud explants cultured on medium with cytokinins showed gradual swelling after a week. Shoot regenerated directly without the intervention of callus growth (Fig.-e). When Kinetin was used alone it enhanced shoot multiplications only at certain optimum concentrations (10 µM). Plant regeneration via direct shoot organogenesis has been achieved by culturing axillary bud explants of *Caralluma diffusa* (Karthik Prabu *et al*, 2013).

Table 1 Plant growth regulators used for *Calotropis gigantea* - white form

S. No	MS medium and PGR Combinations	Types of Explants used	Observation
1.	Basal medium	7 day old seedling organs	Slow growth of Shoot buds even after 7 days
2.	BAP (5 µM)	Epicotyls and Axillary buds (Mother plants)	Multiple shoots formed (3 to 5) in 15 days.
3.	Kin (10 µM, 5 µM)	Epicotyls, hypocotyls, axillary buds	More number of multiple shoots formed in 10 µM than 5 µM. from Axillary buds 3 shoots formed after 15 days.
4.	BAP + IAA (5 µM + 2 µM)	Inter nodal segments	Shoots and roots are formed in 10 days.
5.	Kin + IBA (3 µM + 2 µM)	Inter nodal segments	Multiple shoots and roots are produced in 14 days.
6.	IBA (1 µM)	Micro shoots	Many sturdy roots are produced within 7 days.
7.	Vermiculite + IBA (1 µM)	Micro shoots	Slow growth in laboratory conditions.

Shoot Regeneration from Epicotyl and Hypocotyl Segments

The shoots initiated from epicotyl and hypocotyl segments in MS medium containing various concentrations of cytokinin. The shoots emerged from epicotyl and hypocotyls part of explants in the form of large leaf structures and subsequently multiplied in large numbers. All concentrations of cytokinins used facilitated response from axillary bud induction. With respect to number of shoots obtained per shoots, the results were similar

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to that reported in other Asclepiadaceae members as such as *Ceropegia juncea* (Nikam & Savant, 2009) the rare *Hoya wightii* spp. *palniensis* (Lakshmi *et al*, 2010) *Gymnema sylvestre* (Komalavalli & Rao, 2000) and in the Plantaginaceae *Isoplexis canariensis* (Arrebola *et al*, 1997). The number of shoots produced per explants varied with the type of explants and different combinations of auxins and cytokinins. Number of shoots was influenced by explants used in the experiment. Large numbers of shoots were produced in different concentration on Kin, and BAP at Kin 10µm and BAP 5µm (Fig.-f-i). A maximum 4 or 8 shoots multiplied in after 40 days of subculture and in one cycle of growth conditions. The explants (epicotyls, hypocotyls, stem, and leaf) were exposed to individual auxin and cytokinin (BAP, IAA, IBA, and KIN) at different concentration. BAP 5µm and Kin10 µm hormone concentration were found to be suitable for rapid shoot growth and multiplication. It has been reported earlier that the cytokinin/auxin ratio is critical rather than their independent presence for the induction of callus or shoot differentiation responses (Tripathi *et al*, 2013) Hypocotyl explants were better than epicotyls for shoot multiplication. When epicotyl explants were used, less number of shoots multiplied in all concentration of plant growth regulators. Multiple shoots were formed in epicotyls and hypocotyls at cut ends. Hypocotyl segments quickly responded and produced large number of shoots. (Pradhan *et al*, 1998) also archived a high rate (76%) of callusing when exposing cut ends of hypocotyl explants of *Dalbergia latifolia*. In a study using a number of latex-producing plants, (Tideman & Hawker, 1982) demonstrated callus initiation.

4. *In vitro* root Induction

Healthy and sturdy shoots formed were transferred to MS basal, BAP 5 µm with IBA 2 µm and Kin 5 µm with NAA 2 µm. The MS Basal, IBA 2µm and NAA 2µm initiated in number of roots (Figs.- j,k). Roots initiated on MS basal medium were frivolous and easily breakable than those formed in IBA hormone medium. IBA hormone medium is suitable for root initiation in *C. gigantea*. Rhizogenesis- the formation of roots is a crucial step in micropropagation for the formation of complete plantlets. Auxin induce root differentiation and their role in root development is well documented (Scott, 1972). In the present experiment, regenerated shoots, transferred to MS medium fortified with IBA and NAA (above 0.24 and 0.27 µM) showed well developed roots when compared to IAA (above 0.28 µM). The highest rooting frequency (80%) was observed on MS medium supplemented with IBA (1.47 µM). Intermittent callus formation was noticed on MS medium supplemented with NAA (1.62 µM). This may be due to the residual cytokinin in shoots (Nemeth, 1979). Adventitious roots were induced only by NAA at 0.27 µM on MS medium. The roots formed at the higher concentrations of NAA on MS medium were thick, stumpy and green in colour. These roots easily detach from the micro shoots during transplantation and therefore were not suitable for complete plantlet formation. The rooted shoots were transferred to paper cups containing vermiculite (Figs.- l-n). Initially each pot was covered with a polythene bag to maintain high moisture. Subsequently, the moisture was reduced by the removal of polythene bags to harden the plants and out of which about 56% survived. These plants were successfully planted in the green house till maturity indicating the potential use of this system similar to that of *H. wightii* ssp. *palniensis in vitro* regeneration reported earlier by (Lakshmi *et al*, 2010).

5. Acclimatization

Plantlets were transferred to full or half strength MS medium for acclimatization. Half strength MS medium was finer to full strength MS medium. Root growth was successfully achieved on plantlets cultured on half strength MS medium with 1µM IBA. Reduction of MS salt solution to ½ strength enhances root formation in shoot lets. The favorable effects of low concentration of macro and micro nutrients on rooting are probably due to the decreased requirements of nitrogen for rhizogenesis (Scott, 1972). Half strength MS medium fortified with auxin at lower concentrations facilitated better rooting. Similarly, IBA has been used successfully to obtain maximum rooting frequency of *Decalepis hamiltonii* (Obul Reddy, 2001). Plantlets with a proper root system were ready for field transfer. They were transferred to plastic pots containing sterile garden soil and vermiculite mixture under diffuse light (16.8 h photoperiod) conditions. Potted plantlets were covered with a transparent polythene membrane to ensure light humidity and watered every alternative day with half strength MS salt solution for two weeks. Polythene membranes were removed after two weeks in order to acclimatize plants to field conditions.

6. Conclusions

Using the above mentioned medium composition a rapid regeneration protocol for large scale propagation of *Calotropis* could be developed and multiplied. The technology would be useful for propagation, extraction of secondary metabolites and for conservation of genetically rich populations of either taxonomically distinct form of *Calotropis*. Thus the findings of the present study were tested repeatedly to ensure cent percent reproducibility so that the protocols would be of immense use for needy plant breeding organizations, biotechnologist, commercial and industrial programs.

Reference

Yamashiro T, Yamshiro A, Yokoyama J, Maki M, Morphological Aspects and Phylogenetic Analyses of Pollination Systems in the *Tylophora-Vincetoxicum* Complex (Apocynaceae-Asclepiadaceae) in Japan. Biological Journal of The Linnean Society 2008) 93: 325 - 341.

Mahmood T, Tariq A, Nazar N, Abbasi BH, Saqlan Naqvi SM, Comparative Assessment of Genetic Variability in *Cryptolepis buchananii*, *Tylophora hirsuta* and *Wattakaka volubilis*. Pak. J. Bot 2011; 43 (5): 2295-2300.

Smith NM. Weeds of the Wet/Dry Tropics of Australia- a Field Guide. Environment Centre NT 2002: p 112.

Sharma AP, Tripathi BD. Assessment of Atmospheric PAHs Profile Through *Calotropis gigantea* R. Br. Leaves in the Vicinity of an Indian Coal-Fired Power Plant Environ Monit Assess 2009;149:477 - 482.

Afolayan AJ, Adebola PO. "In Vitro Propagation: a Biotechnological Tool Capable of Solving the Problem of Medicinal Plants Decimation in South Africa". Afr J Biotechnol 2004; 3: 683 - 687.

Bajaj YPS, Furmanowa M, Olszowska O. "Biotechnology of micropropagation of medicinal and aromatic plants". In: Biotechnology in Agriculture and Forestry, 4, Medicinal and Aromatic Plants I. Bajaj. Y.P.S., [Ed.]. Springer, Berlin, Heidelberg, New York. 1988: pp 60 - 103.

Karthik Prabu M, Samydurai P, Subbaiyan B, Thangapandian V, Binu T. *In vitro* propagation of a rare succulent medicinal plant *Caralluma diffusa* (Wight) N.E.Br. Research in Plant Biology 2013; 3(1): 08-17.

Nikam TD, Savant RS. Multiple shoot regeneration and alkaloid cerpegin accumulation in callus culture of *Ceropegia juncea* Roxb. Physiol Mol Biol Plants 2009; 15(1):71-77.

Lakshmi RS, Franklin Benjamin, JH, Senthil kumar T, Murthy, GVS, Rao MV. *In vitro* propagation of *Hoya wightii* ssp. *palniensis* K.T. Mathew, a highly vulnerable and endemic species of Western Ghats of Tamil Nadu, India. African Journal of Biotechnology 2010; 9 (5): 620-627.

Komalavalli N, Rao MV. *In vitro* Micropropagation of *Gymnema sylvestre*- a Medicinal Plant. Plant cell tissue organ culture 2000; 61: 97-105.

Arrebola ML, Socorro O, Verpoorte R. Micropropagation of *Isoplexis canariensis* (L.) G. Don. Plant Cell Tiss Organ Cult 1997;49:117–119.

Tripathi PK, Awathi S, Kanojiya S, Tripathi V, Mishra DK. Callus Culture and *In Vitro* Biosynthesis of Cardiac Glycosides from *Calotropis gigantea* (L.) Ait. *In Vitro* Cellular and Developmental Biology – Plant 2013; 49 (4):455-460.

Pradhan SP, Conrad JR, Paterek JR, Srivastava VJ. Potential of Phytoremediation for Treatment of PAHs in Soil at MGP Sites. Soil Sediment Contam 1998; 7(4): 467-480.

Tideman J. Hawker JS. In vitro propagation of latex producing plants. Ann Bot 1982; 49:273-279.

Scott TK. Auxins and Roots. Ann Res Plant Physiol 1972; 23: 235-258.

Nemeth G. Benzyladenine Stimulated Rooting in Fruit Tree Root Stocks Cultured *in vitro*. Z. Pflanzenphysiol. 1979; 95:389-396.

Obul Reddy B, Giridhar P, Ravishankar GA. *In vitro* Rooting of *Decalepis hamiltonii* Wightii & Arn., an Endangered Shrub, by Auxins and Root-Promoting Agents. Curr Sci 2001; 81(11): 1479-1482.

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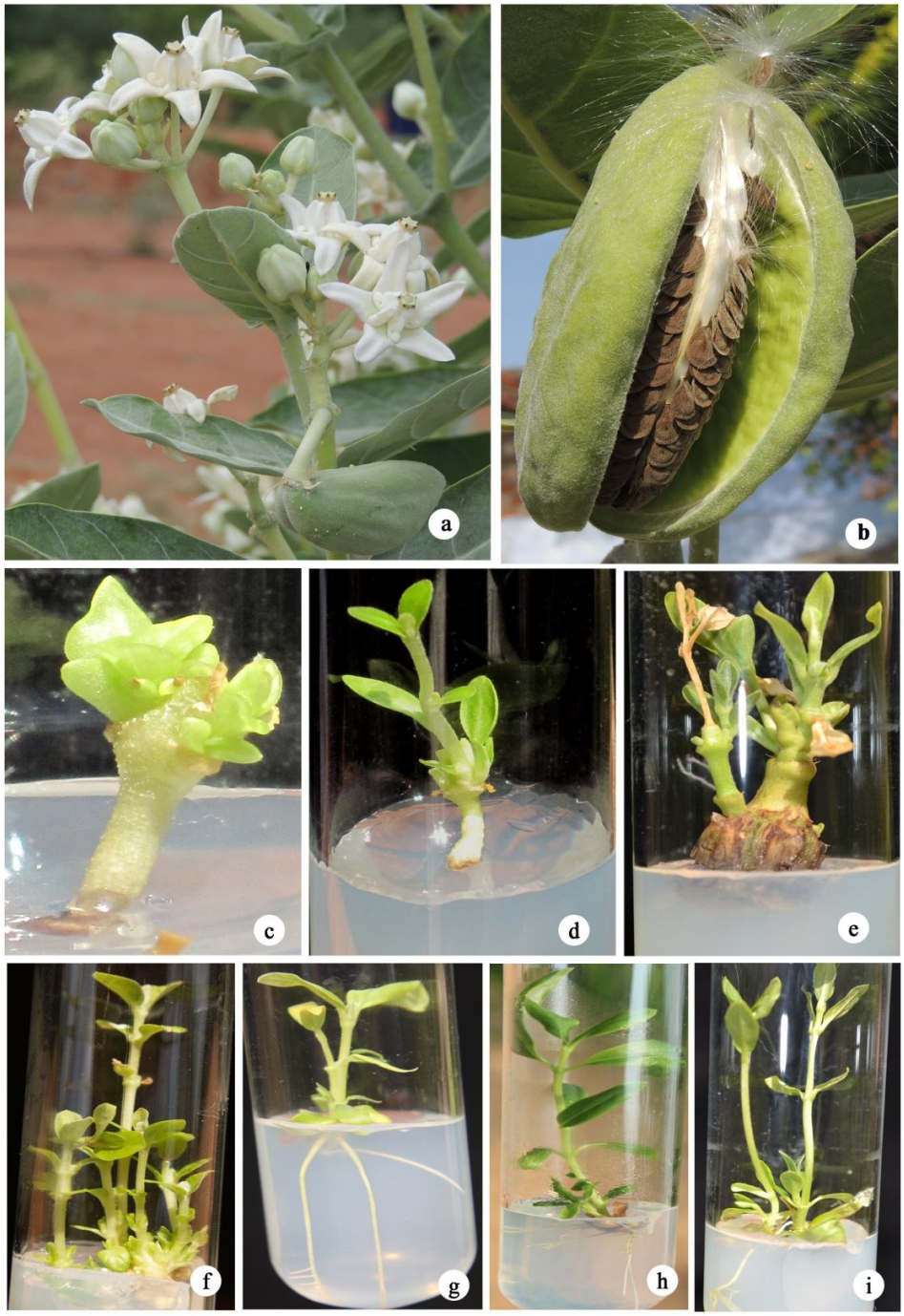

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Fig.a. Flowering and fruiting Twigs of *Calotropis gigantea* White form

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Fig.b. Mature fruits and seeds of *Calotropis gigantea*

Fig.c. Starting stages of hypocotyls segments on MS basal medium

Fig.d. Response of hypocotyls segments on MS medium +BAP, IAA. Shoot initiation and multiplication of shoots.

Fig.e. Mother explant response in initiation and shoot multiplication BAP 5µM.

Fig.f.- Hypocotyl segments on MS medium +BAP IAA. Shoot bud shoot multiplication and elongation after 20 days of culture.

Figure h-i- MS medium combined hormone response (BAP+IAA) Shoot multiplication and root formation.

Figure j-k- Well developed Micro shoots and roots MS Medium +Kinetin, IBA. Ready for hardening.

Figure l-n. Rooted plantlets transferred to polycups for acclimatization.