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EFFICIENT CALLUS INDUCTION AND INDIRECT PLANT LET PROLIFERATION FROM COTYLEDONARY EXPLANTS OF *GYMNEMA SYLVESTRE* (RETZ) R. BR. EX ROEMER & SCHULTES. AN IMPORTANT MEDICINAL PLANT

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ABSTRACT

Gymnema sylvestre Retz. R.Br. Ex Roemer & Schultes, commonly called as Gudmar" (gud -jaggery, mar-kills) a medicinally important branched woody climber, distributed throughout India in dry forest up to 600 m. belongs to the Asclepiadaceae family commenced 100 years ago, has been employed to control diabetes by traditional medicinal practitioners of India for several centuries. Nowadays focus is being placed on *Gymnema sylvestre* callus induction and plantlet proliferation. In this study the Cotyledonary explants of two weeks old seedlings of *Gymnema sylvestre* were induced for callus induction on MS media containing different combinations and concentrations of growth regulators. Different callusing media containing varying levels of 2.4-D/IAA/NAA (1.0-5.0mg/L) were tested for callus induction response. Maximum (90%) callusing response was obtained from Cotyledonary explants on MS medium containing (3.0mg/L) NAA. After 8 weeks of induction, the calluses were transferred on different regenerated media containing varying level of BAP/Kn/TDZ (1.0-5.0mg/L). Maximum shoot bud differentiation from callus culture was achieved on MS Medium fortified with TDZ (3.0mg/L). Elongation and further development of shoot buds into shoots were achieved on MS medium supplemented with TDZ (3.0mg/L).

This is the first report of *in vitro* callus induction and plantlet regeneration in *Gymnema sylvestre*. The regenerated elongated shoots were transferred to Indole Butyric Acid (IBA) (1.0mg/L–5.0mg/L) for root induction. Rooting was observed within two weeks of culture. Rooted plantlets were successfully hardened under culture conditions and subsequently established in the field conditions. The recorded survival rate of the plants was 86%. Plants looked healthy with no visually detectable phenotypic variations

KEYWORDS: Callus Induction, Plant let Proliferation Indirect Regeneration, Cotyledonary Explants and *Gymnema Sylvestre.*

INTRODUCTION

Natural products remain a prolific source of discovery of new drugs due to their chemical diversity and ability to act on various biological targets (Bhutani and Gohil, 2010; Manonmani and Francisca, 2012). Therefore, search for natural products to cure diseases is becoming an area of great interest, in which plants have been the most important source. The value of the medicinal plants lies in some chemical substances that produce a definite physiological action on the human body (Edeoga *et. al.,* 2005). In fact, herbs have traditionally been considered to be non-toxic, thus have been used for treating various diseases and health related problems (Oduola *et. al.,* 2007). Herbal formulation, a rapidly growing industry (Calapai and Caputi, 2007), controls 30% of the global drug market and draws almost 1 billion dollars in profit every year (Ritchie, 2007: Tsuji and Tsutani, 2008). Herbal medicines are the undeniable root of modern pharmacology also (Oricha, 2009) as it has been estimated that around 75% of all orthodox medicines are of herbal origin (Lam, 2007; Luzhetskyy *et. al.,* 2007).

During the last decade, the changes in life style and junk food habits have resulted in obesity and diabetes in large area of population. For the treatment of such diseases market is flooded with many synthetic antidiabetic medicines but the long term use of these drugs has resulted in many side effects. So that natural plant based products are gaining importance. *Gymnema sylvestre* R. Br. is one of the important medicinal plants of India widely used in the treatment of *diabetes mellitus. Gymnema sylvestre* R.Br. is an imperative remedial woody climber belonging to family Asclepiadaceae- 'The Milk Weed Family'. One special name of this plant species is 'Miracle fruit'. The name '*Gymnema'* probably derives from the Latin word meaning 'naked'

and *sylvestre* means 'from the forest'. It is native to central and western India and can be also found in tropical Africa and in Australia. The leaves are opposite, usually elliptic or ovate. Flowers are small, yellow, in auxiliary and lateral umbel in cymes. The leaves of *Gymnema* are reported to be bitter, astringent and acrid. They temporally paralyze the sensory perception of sweet and for this amazing property it is known as "GUDMAR". It is also known as 'Sugar Destroyer'. *Gymnema* leaves have the mixture of bioactive constituent's tri-terpines and saponins viz. Gymnemic acids, Gymnemagenin and Gurmarin due to them this plant represents antidiabetic property.

Its leaves are also used in food additives against obesity. *G. sylvestre* also has Stomachic, diuretic and cough suppressant properties. The root of this plant is an antidote for snake bite used by tribals. It was traditionally used in Ayurveda. Due to these unique qualities the plant has been overexploited and the species became endangered.

G. sylvestre produces flowers once a year. Under Sri Lankan conditions, flowering starts early November and lasts for two months. Pods take 2-3 months to mature. A feather like structure attached to the tiny seed takes seeds away from mother plants by means of wind. However, as this structure is easily detachable, widespread distribution is hard to seen. The weight of the seeds varies from 5115-5750 mg per 1000 seeds. At the time of detaching from the mother plant, seeds contain little moisture (4-9%), which in fact hampers the germination. Therefore, number of emerging seedlings is limited in natural habitats, despite the fact that thousands of seeds fall under mother plants. No alternative mode of propagation is naturally found, thus seed propagation is recognized as the sole mechanism of natural regeneration (Arunakumara *et. al.,* 2004).

According to them, a maximum number of roots could be obtained on MS medium supplemented with NAA at 1.0 mg/L. Karthic and Seshadri (2009) used young stem cuttings with an actively growing side branch as explants in their hydroponic experiment conducted with plastic tubes. MS medium containing 1/10 strength of MS salts supplemented with IBA at different concentrations (0.5, 1.0, 2.5 mg/L) and IBA (0.5 mg/L) resulted in the highest rooting (66%) and survival (96%). Based on the findings, they claimed that their protocol can serve as an alternative to the existing *in vitro* and clonal multiplication protocols for *G. sylvestre*. Subathra Devi and Srinivasan (2008) further stressed that *in vitro* propagation of *G. sylvestre* is not very different from that of *G. elegans* and may be applicable for other economically important woody Climbers as well.

The present study described the efficient callus induction and indirect plantlet proliferation from cotyledonary explants of *G. sylvestre* a medicinally important Plants.

METHODOLOGY

Plant material

Seeds of *G. sylvestre* were collected from one single plant grown in the Forest range Officer Waddepally Hanamkonda identified by the taxonomist from Kakatiya University Warangal Telangana State India. The seeds were washed in running tap water for 5 min, treated with 2-3 drops of Tween 40 for 10 min and finally the seeds were washed thrice with sterile distilled water. Seeds were subsequently surface sterilized with 0.1% $HgCl_2$ for 5 min and washed thrice with sterile distilled water. The surface sterilized seeds were cultured on Murashige and Skoog (1962) medium supplemented with 2% sucrose and solidified with 0.8% agar (Himedia) (Ashok *et.al.*, 2002) pH of the medium was maintained at 5.8 samples were grown at a photoperiod of photo period under white fluorescent light of 40-60 mol m-2 s-1 intensity.

For callus induction the explants viz, Cotyledon (0.8–1.0 cm^2) explants from 8-week old seedlings were excised, these explants were inoculated to MS medium supplemented with various concentrations of (1.0- 5.0 mg/L) of auxins such as 2,4–Dichlorophenoxy acetic acid (2,4-D), Indole 3-acetic acid (IAA), Naphthalene acetic acid (NAA) (Table-1) all the explant growth regulators were used as auxine alone in culture media. All media were adjusted to pH 5.8 before addition of 0.8% agar agar and autoclaved at 121^0C and 103 K pa for 20 minutes cultures in 25 x 150 mm cultures tubes. For present investigation, the calli obtained on MS medium supplemented with (3.0 mg/L) NAA for Cotyledonary explants were used after 6 weeks of culture. This callus was regularly sub cultured for three passages on them same fresh medium. The callus induced after 4th passages were used for the present investigations.

Culture Media and Culture Conditions

The calli pieces approximately 0.5-10 cm from Cotyledonary explants derived fresh friable callus cultures were transferred on to regeneration medium containing MS basal salts supplemented with different concentrations of BAP/Kn/TDZ. The pH of the media was adjusted to 5.8 either with 0.1 N HCL or 0.1N Na OH, solidified with 0.8% Difco-bacto agar and autoclaved at 121^0C under 15 psi for 15-20 minutes. All the cultures were incubated at 25^0C with 16h photo period under white fluorescent light of 40-60 mol m-2 s-1 intensity.

The shoots proliferated from cotyledonary explants; they were excised and cultured on a rooting medium consisting of MS medium supplemented with different (0.5-2.0 mg/L) IAA or IBA. The rooted plantlets were gently removed from the flasks and the roots were washed in tap water to remove traces of agar.

Acclimatization

Plants with roots were transferred during two weeks, after washing of the agar with distilled water and to pots with a mixture of soilrite (1:1). Potted plantlets were

covered with transparent polythene membrane to ensure high humidity and watered every three days with half strength MS salts solution for two weeks in order to acclimatize plants to field conditions. After two weeks the acclimatized plants were transferred to pots containing normal garden soil and maintained in greenhouse under natural day length conditions.

RESULTS

Callus induction ability of cotyledonary explants was investigated by using varying concentrations of different auxins individually. Callus proliferation was initiated at the cut surfaces of the explants studied and later it covered the entire surface For callus induction the explant viz. Cotyledon (0.6-0.8cm^2) from 6 weeks old axenic seedling were excised and inoculated to MS medium supplemented with various concentrations of 2,4-D, IAA and NAA (1.0-5.0 mg/L). Callus proliferation was initiated at the cut surface of the cotyledon explants .The results are presented in (Table-1).

Table. 1: Morphogenetic response of Cotyledonary explants of *Gymnema sylvestre* on MS medium with different concentrations of 2, 4-D, IAA and NAA.

Hormone cone (mg/L)	% of cultures responding	Morphology	Callusing response
2.4 – D			
1.0	53	White compact	++
2.0	60	White compact	+++
3.0	80	White compact	+++
4.0	70	Creamy compact	++
5.0	58	Creamy compact	+
IAA			
1.0	58	White compact	+
2.0	60	White friable	++
3.0	68	White friable	++
4.0	50	Green nodular	+
5.0	45	Green nodular	+
NAA			
1.0	80	White friable	++
2.0	82	White friable	+++
3.0	86	White friable	+++
4.0	78	Creamy friable	+++
5.0	70	Creamy friable	++

Relative amount of Callus formation: - - = No, + = low, ++ = moderate, +++ = high

Table. 2: Effect of BAP, Kn and TDZ on induction of shoots proliferation from Cotyledon derived callus cultures of *Gymnema sylvestre*.

Hormone concentration (mg/L)	% of cultures responding	Average No. of shoots / Explants ± (SE)*	Average length of shoots (cms) ± (SE)*
BAP			
1.0	50	8.6 ± 0.32	1.0 ± 0.32
2.0	53	12.3 ± 0.07	2.4 ± 0.23
3.0	60	14.5 ± 0.32	2.4 ± 0.12
4.0	55	12.3 ± 0.43	1.6 ± 0.32
5.0	48	06.0 ± 0.35	1.4 ± 0.25
Kn			
1.0	56	9.2 ± 0.35	1.2 ± 0.35
2.0	60	10.8 ± 0.35	2.0 ± 0.24
3.0	64	13.0 ± 0.35	2.3 ± 0.13
4.0	58	12.8 ± 0.22	1.2 ± 0.32
5.0	50	8.2 ± 0.34	1.0 ± 0.24
TDZ			
1.0	55	7.2 ± 0.25	1.3 ± 0.42
2.0	63	9.8 ± 0.55	2.0 ± 0.53
3.0	65	10.0 ± 0.45	2.6 ± 0.12
4.0	62	11.8 ± 0.72	1.2 ± 0.52
5.0	57	7.5 ± 0.64	1.0 ± 0.35

*** Mean ± Standard Error**

Table. 3: Rooting ability of regenerated shoots from cotyledonary explants culture of *Gymnema sylvestre* cultured on MS medium supplemented with IAA and IBA.

Growth Hormones (mg/L)		Percentage of response	Average no of roots (S.E)*
IAA	IBA		
00	00	23	1.0 ± 0.12
0.5	-	60	2.3 ± 0.37
1.0	-	70	3.2 ± 0.38
2.0	-	73	5.6 ± 0.38
-	0.5	54	4.3 ± 0.36
-	1.0	73	8.3 ± 0.87
-	2.0	70	6.3 ± 0.36

***Mean ± Standard Error**

Effect of 2, 4-D/IAA/NAA

On 2,4-D supplemented medium early induction was observed in all concentration of 2,4-D. High amount of callus was induced at (2.0 and 3.0 mg/L) 2,4-D. Different callusing response was recorded in cotyledon explants at all the concentrations of 2,4-D. Morphology of callus was found to be varied at different levels of 2,4-D. White compact callus was found at all concentration of 2,4-D. Low amount of callus was induced at high concentrations of 2,4-D. Highest percentage (80%) of response was observed at 3.0mg/L 2,4-D.

On IAA supplemented medium moderate amount of callus was observed at (2.0 and 3.0 mg/L IAA.) Low amount of callus was observed at (1.0, 4.0 and 5.0 mg/L) IAA. Morphology of callus was also found to be varied at different levels of IAA (Table-1). White compact callus was induced at (1.0 white friable callus obtained at 2.0 and 3.0 mg/L IAA.) Green nodular callus was observed at (4.0 and 5.0mg/L IAA.) 68% callusing response was recorded in cotyledon explants at (3.0 mg/L) concentration of IAA.

Effect of NAA on callusing ability of cotyledon explants is shown in (Table-1). High percentage (86%) of response was observed at (3.0 mg/L) NAA. Responding callus was also varied at different levels of NAA. Whereas moderate amount of callus was observed at (1.0 and 2.0mg/L) NAA. High amount of callus was induced at (3.0 and 4.0mg/L) NAA. A white friable type of callus was induced at (1.0, 2.0 and 3.0 mg/L) NAA. At high concentration (4.0 and 5.0 mg/L) NAA induced Creamy friable callus with (78% and 70%) were cultures responded.

Data on multiple shoot induction from cotyledonary callus explants cultured on MS medium fortified with different concentrations of BAP/Kn/TDZ alone is presented in (Table -2) for root induction from regenerated cotyledonary explants cultured on MS medium supplemented with different concentrations of IAA and IBA is presented in (Table-3) (Fig a. b & c)

Effect of BAP/Kn/TDZ

Callus-mediated shoot regeneration is an indirect method for plant regeneration. To probe into the formation of adventitious shoot from callus, 35-day-old calli were transferred to plant regeneration medium. At 45 days of plant regeneration, the number of adventitious shoots per callus and the rate of adventitious shoot induction were counted as shown in (Table-1).

The media containing BAP/Kn/TDZ could induce the formation of adventitious shoots the rate of adventitious shoot induction showed that a trend declined with the increased.

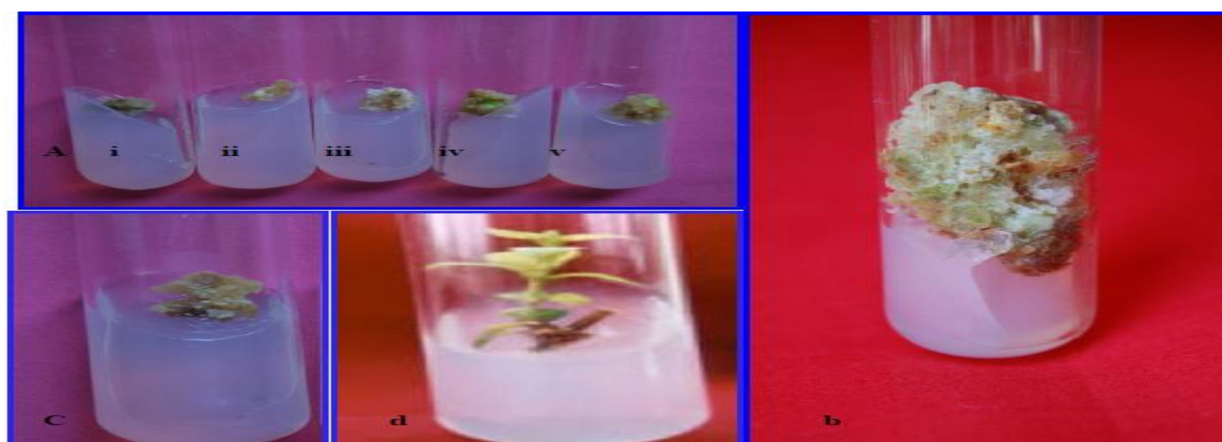

Fig-I Indirect Plantlet proliferation from Cotyledonary of *Gymnema sylvestre* a), Maturation of callus on MS+ (1.0 to 5.0 mg/L) i.ii.iii.iv and v NAA (b) callus induction on MS + 4.0 mg/L IAA after six weeks (c) Callus induction on MS+ 4.0mg/L 2.4-D after three weeks (d) Formation of shoots on MS+NAA (4.0mg/L) + TDZ (4.0) mg/L.

Concentration of (1.0-5.0 mg/L) BAP/Kn/TDZ. During the present investigation, variation combinations of (1.0-5.0 mg/L) were tried (Table-2). The number of shoot buds included increased when callus was sub cultured on MS- medium containing BAP/Kn/TDZ. The entire calluses became compact and later shoot buds differentiated within 4-6 weeks. Maximum number of shoot buds proliferated (14.5+ 0.32) at (3.0mg/L) BAP. At the concentration of (3.0mg/L) Kn and TDZ induced (13.0 + 0.35) and (10.0+ 0.45) of shoot buds were obtained. Lower concentrations (1.0-2.0mg.L) and higher concentrations (4.0-5.0mg.L) of BAP/Kn/TDZ decreased the number of shoot buds. The different concentrations of BAP (1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) were added to the MS medium. Lower level of BAP (5.0mg/L) induced less number of shoots at (3.0 mg/L) BAP 60% cultures responded and maximum number of shoots/explant (14.5 + 0.32) were recorded. At the concentration of BAP was increased to (4.0 and 5.0 mg/L) the percentage of responding was decreased (Table-2) Fig-1-d).

DISCUSSION

Interaction of auxins and cytokinins plays vital role in cell division, growth, development, differentiation and the formation of plant organs (Shrivastava and Banerjee, 2008; Purkayastha *et. al.,* 2010; Jha *et. al.,* 2007). The present results showed that different combination of plant hormone could induce the formation of callus in Cotyledon explants of *G. sylvestre.*

The regeneration capacity through indirect shoot organogenesis from Cotyledon, derived callus was tested either. Shoots number per explant was influenced strongly by culture medium and application of growth regulators. In our protocol NAA induced organogenic callus from Cotyledonary explants. The hormonal supplement was selected because it was optimum for callus formation among many tested combination. The Cotyledonary explants showed 86% callus formation after 6 weeks of culture when placed in media supplemented with auxins NAA (3.0mg/L) singly. However, the response of the axillary buds decreased (70%) with increase in concentration of NAA in the medium. Dode *et. al.,* (2003) had also successfully reported micropropagation of *O. basilicum* using *in vitro* geminated plants. Sub culturing of callus on to fresh medium containing the same concentrations of growth regulators resulted in the emergence of shoot buds. Multiple shoot buds were initiated on the callus cultured on MS medium supplemented with cytokinins (BAP/KnTDZ).

Gopi and Vatsala (2006) initiated the callus cultures from nodal segments and leaf explants of *G. sylvestre* on MS medium containing basic salts and (30.0g/L) sucrose supplemented with different concentrations (0.10, 0.25, 0.5, 1.0, 2.0 and 5.0 mg/L) of 2, 4- D, NAA, IAA, IBA, Kn and BA, and found that main components of the active principles namely Gymnemic acids and Gymnemagenin were present in sufficiently large amounts in the cultured undifferentiated cells. Kanetkar *et. al.,* (2006) also reported that *in vitro* callus with the active compounds, Gymnemic acid and Gymnemagenin presenting in sufficiently large amount in the cultured undifferentiated cells. According to them, the production of Gymnemic acid is significantly higher in callus treated with 2,4-D and KN. Furthermore, the blue light increases Gymnemic acid accumulation up to 4.4-fold as compared with fluorescent light treatment. Aneesa *et. al.,* (2010) estimated the Gymnemic acid content in callus cultures as (2.52 mg/g) DW, while Kumar *et. al.,* (2010) estimated as 5.09% w/w. Elicitation in *Gymnema* callus culture (static culture) has been investigated by Bakrudeen *et. al.,* (2009), under abiotic stress conditions (light & salts) and reported that the maximum gymnemic acid content (53.94 mg/g DW) could be obtained after 45 days of culture under blue light exposure.

According to Reddy *et. al.,* (1998), MS medium supplemented with 6-Benzyladenine (BA) at 5.0 mg/L and 1-napthalene acetic acid (NAA) at 0.2 mg L-1 could induce 7 shoots per explant. They further reported that the best root induction resulted in half strength MS medium without growth regulators. In order to find out the best sterilization procedure, different treatments using $HgCl_2$ and NaOCI or with both were employed with two types of explants (leaf and stem) (Amarasinghe *et. al.,* 2011). The highest survival (70%) of each explant type was recorded with 0.5% $HgCl_2$ for l0 minutes exposure time. They have tested the different levels of Benzylaminopurine (BAP) and Kinetin (KA) along with NAA (0.1 mg/L) on shoot proliferation, and the best results were achieved with BAP at 1.0 mg/L.

Komalavalli and Rao (2000) also observed the best shoot proliferation on MS medium containing similar concentrations of BAP, KA, and NAA. However, they have additionally added Malt extract (100 mg/L), and Citric acid (100 mg/L) into the medium. According to them, a maximum number of shoots (7) could be induced from 30-day old seedling axillary node explants. Moreover, they observed high frequency of rooting (50%) on half strength MS medium supplemented with Indole butyric acid (IBA) at 3 mg/L. These plantlets were hardened and successfully established in natural soil, thus based on their findings, they claimed that use of axillary nodes for micropropagation is beneficial than other explant types. Though Subathra Devi and Srinivasan (2008) also reported that the MS medium supplemented with IBA (3 mg/L) is the best medium for root induction, their best shoot proliferation was recorded on MS medium containing BA (1 mg/L), IAA (0.5 mg/L), Riboflavin (100 mg/L), and Citric acid (100 mg/L). Sharma and Bansal (2010) used apical bud as the explant to achieve successful micropropagation of the species. They obtained multiple shoots on MS medium containing BA and KA, and when sub-cultured on half strength MS medium with IAA, a high number of shoots were rooted. Furthermore, 85% of the rooted shoots were found to be survived in the field. Stem and nodal

segments as well as basal, middle and terminal cuttings have been successfully employed with MS medium supplemented with different concentrations of various growth regulators (Tafokou, 2010).

A procedure of *G. sylvestre* regeneration via indirect organogenesis and a successful adaptation of plants to greenhouse conditions were developed. NAA being more effective for callus induction than IAA. Plant regeneration via indirect shoot organogenesis was achieved from the cotyledonary –derived callus on MS medium supplemented with (3.0 mg/L) BAP. High rate of multiplication (60%), maximum shoot bud proliferation (12.0 shoots per explants) observed on M.S. medium supplemented with (3.0 mg/L) BAP. The optimal medium for rooting was MS containing (1.0 mg/L) IBA. The plants were successfully *in vivo* acclimatized. This *in vitro* regeneration system can be used for effective screening and propagation of elite clones of *G. sylvestre*.

CONCLUSIONS

Ideal medium for callus establishment through Cotyledonary explants was MS-medium supplemented with NAA (3.0 mg/L) callus culture was achieved. Maximum shoot bud differentiation from callus culture was achieved on MS-medium supplemented with BAP/Kn/TDZ (3.0 mg/l). Elongation and further development of shoot buds into shoots were achieved on MS-medium fortified with BAP/Kn/TDZ (3.0 mg/L). Therefore, this medium was designated as "Shoot bud differentiation and elongation medium".

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