Research Paper

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In vitro Propagation of Gymnemasylvestre R. Br. (Retz.) – An Important Antidiabetic Medicinal Plant

Botany

KEYWORDS : Gymnemasylvestre, in-vitro, micropropagation, medicinal plant, MS, B5, WH, NA-, CW- coconut water, DW, PGR, MSM,MSR.

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ABSTRACT

This research work has designed with an objective to develop a cost effective mass multiplication methodin vitro propagation by shoot induction in Gymnemasylvestre R.Br. a valuable medicinal plant. Apical budmeristems were used as explants. Shoots proliferation were achieved on three different medium (MS, White and B5) containing various concentration of BAP. Best shoots initiation (86+0.23%) were achieved on MS withBAP (2mg/l)fromapical bud meristems. The maximum percentages of explants were forming shoots on MS medium.Shoot multiplication and shoots growth were experimented on MS medium containing various cytokinin (BAP and KI) alone and combination with auxins (IAA & NAA) and coconut water (10-50%). A maximum number of shoots (22+ 0.08) and length (8.4+ 0.27) were found on MS with 5mgL-1 BAP + 0.5mgL-1 NAA + 0.2 mgL-1 KI and the other hand high frequency of rooting was observed in explants derived (88+0.23%), maximum number of roots (12±0.26) and root length (10± 0.30 cm) of the plants were noted in the MS medium containing 1.0 mg/l of IBA + 3g/l of charcoal respectively. The plantlets, thus developed were hardened and successfully established in red soil with various plant growth promoting substances like organic compounds. The maximum shoot length (11.48 ± 0.36 cm) and root length (8.50 ± 0.14 cm) were observed with plant grown in red soil + humus rich soil (3:1). The shoot induction protocol developed in this study helps in mass propagation andgermplasm conservation and also for further investigation of medicinally active constituents of this elite medicinal plant.

Introduction-Madhunashini (***Gymnemasylvestre R.Br.***) is a magical antidiabetic plant belongs to the family Asclepiadaceae has a deep rooted in Ayurvedic medicine for over 2000 years. It is anunbranched woody climber reaching approximately 600 m. in dry forest; it is distributedin Asia, Tropical Africa, Malaysia and Srilanka (**Keshavamurthy, 1990**).It is one of the rare species among the medicinal plants (**Flora of Bhopal, 1976**) commonly known as Gurmar in Hindi, Meshashiringi and Madhunashini in Sanskrit, Marathi - dashing, Tamil -adigam, Telugupodapatri, Kannada-Sannagerasehambu (**The Wealth of India, 1956**). It is adapted to different soil texture and climate variation. It grows successfully in sandy loam or light red soil with good organic matter and drainage. It will grow in temperature from 25 ^{0}c-40 ^{0}c. It is planted during the late raining season and prefers dry weather for its successful growth. A large more or less pubescent, woody climber found in the Deccan Peninsula, extending to parts of northern and western India; it is occasionally cultivated as a medicinal plant. Leaves opposite usually elliptic or ovate (1.25-2.0 in.× 0.5-1.25 in) flowers small, lanceolate, up to 3 inches in length (**Asha V.J. et.al., 2010**). ***Gymnemasylvestre***, a plant used in the Ayurvedic medicine of India for the treatment of diabetes mellitus has been known from antiquity also to have an antisaccharin taste effect. The active principles are glycosides (several Gymnemic acids) which shows selective anaesthetic effect (**Warren, 1969**). It is interesting to note that Gymnemaextact given to healthy volunteers does not produce any blood sugar lowering or hypoglycemic effects (**Baskaran K. et.al.1990)**.It is also used in the treatment of asthma, eye complaints, inflammations, family planning and snakebite. In addition, it possesses antimicrobial, antihypercholesterolemic, hepatoprotective and sweet suppressing activities (**Parijat K.** ***et.al.*****, 2007**). It is also act as feeding deterrents to caterpillar, *Prodeniaeridania*:prevesvent dental caries caused by Streptococcus mutans and in skin cosmetics (**Komalavalli N., *et.al.* 2000**). The antidiabetic array of molecules has been identified as a group of vlosely related gymnemic acids after it was successfully isolated and purified from the leaves of ***Gymnemasylvestre*** (**Liu H.M. *et.al.* 1992; Sinsheimer J.E., *et.al.*1965**). Vegetative propagation is only the propagation method, but it is very slow growing process in different climate conditions. In nature less seed germination with poor viability is responsible for its diminishing population size. The poor propagation coupled with over exploitation from natural resources for its diverse medicinal applications ***Gymnemasylvestre*** has been fast disappearing and threatened; therefore, there is urgent need to conserve the plant by biotechnological approach like tissue culture (***Choudhury, 1988)***.The use of *invitro*techniques in germplasm conservation is increasing and has been successfully applied to the conservation of several rare & endangered species, both for propagation and for long-term storage (**Chandra *et.al.*,2006*)***. Little tissue culture work has been reported on ***Gymnemasylvestre*** by **N.Komalavalli and Rao,1997; Reddy *et.al.*,1998; N. Komalavalli& M.V. Rao in 2000; C.Subhathra Devi and V. MohanaSrinivasan in 2008; A.V.Jaybhaya and S.S. Deokule in 2010.** In the present investigation, we report the successful *in vitro* shoot initiation, multiplication and rooting of ***Gymnemasylvestre*** by means of apical shoot bud & meristems.

Meterial& methods –

Collection of plant material-Healthy, abundant growing and an elite vine (2-3 years old)plants were collected from Kasturi Herbal farm Misrod, Bhopal. Plant Identified by LaghuVanupa jPrasannskarn&Anusandhan Kendra BarkhedaPathani, Bhopal MP.

Preparation and sterilization of Explants- The young actively growing plants of ***Gymnemasylvestre*** were wash thoroughly in running tap water for 15 min to remove all the dust particles adhering to them. Apical shoot bud sections were used as explants for this experiment. Explants were cut and reduced to length of 2cm, retaining the apical dome (1cm).Explants were immersed in water with a little amount of fungicide-Bavistin for 1 to 3 hours and then rinse with tap water five to six time. Then explants weredipped in distilled water with few drops of wetting agent, labolene for ten minutes. It was immediately rinsesin distilled water five time rinses in distilled water to remove traces of labolene. Further surface sterilization treatment was conducted in a laminar air flow chamber. The explants were sterilized with 70 % Ethyl alcohol for 30 sec and dipped into 0.1% (w/v) fresh prepared mercuric chloride solution for 1-3min, and then washed with double DW two times. There after explantswere carefully transferred to sterile blotting paper placed over sterile Petri plate to remove excess water & then inoculated vertically into the culture establishment medium (MS, B5 and WH)using sterile forceps under aseptic conditions. The new shoots induced from the *in vitro* cultures were used as explants for further experiment.

Bud break –Apical bud explants were cultured on B5 (**Gamborg*et.al.*, 1968**),**whites medium (1963**)and MS (**Murashige&Skoog, 1962**) full and half basal andsupplementedwith various concentrations of BAP (mg L^{-1} w/v) (table-1), optimize salt and hormones required for shoot sprouting. Therefore, surface sterilized explants were transferred aseptically to sterile glass plate. Then undesirable and dead portions of basal portion of the explants were removed. The nodal ex-

plants were placed in an erect position in the culture bottle containing medium with the help of sterile forceps. Then lid was closed carefully and sealed with Klin film. The same procedure was used for all the explants. The culture bottles were kept in the growth room at 25±2°C, with a photoperiod of 16 h daylight and 8 h night breaks under the cool white fluorescent light. The explants with bud proliferation cultures were transferred to culture tubes containing fresh media after 15 days.

Multiplication- Theinitiated stem explants were inoculated onMS medium with various combinations ofcytokinins (BAP & KI), alone and in combinations with different concentration ofauxins(NAA & IAA) andCW (coconut water) (table-2).Shoots were carried out byrepeated sub-culturing on MSmedium. Multiple shoots andcluster were transferred from theculture bottle to a sterile glassplate. These culture bottles were incubated at25±2°C for 16-8h of day and night under the low temperature with white fluorescent light. These steps were repeated at every 25-30 daysintervals and shoot multiplication rate and length was observed.

Rooting - The sterilized explants were cut into smallPieces (4-5cm). For root induction, MS basal medium was supplemented with IBA andCharcoalthe initial pH of the medium was adjusted to 5.8 with various combinations (table- 3). Theexplants were implanted aseptically on the culture media. After 45 days in rooting medium, the rooted micro shoots were removing from the culture medium and harden in soil and organic mixture (table-4).

Culture condition and statistical analysis-The explants were inoculatedon different types of media (MS, Whites and B5) with or without any growth hormone. The Basal medium was amended with 3 % (w/v) sucrose and 0.8 % agar, adjust between 5.4-5.8 pH (1N HCl or 1N NaOH). The medium was autoclaved at a temperature of 121 ºC and a pressure of 15 psi for 15-20 minutes, and poured into culture bottles or test tube (6o mm in diameter and 18mm in height). All cultures were incubated in culture room at 27± 2 ^{0}C cool white fluorescent light of 3,500 Lxu, at plant level provided with 60-65 % relative humidity. After 10- 15 days apical bud meristems were initiated. Explants were sub cultured after 15-15 days onMS medium with different growth hormons (cytokinins and auxins) and coconut water. After 45- 60 days shoots were multiply and rooting represent. The effects of different treatments quantified and Data Series of experiments were conducted and analyze statistically. Data were showed some prosperous effect, included in the table and repeated in mean±SE of 10 explants per treatment and repeated three times Mean values were not significantly different more than one.

Results and Discussion- In order to establish an efficient in vitro micro propagation system for ***Gymnemasylvestre*** from apical bud explants were observed on different medium and various concentrations of PGR.

Influence of medium and shoot initiation -Apical bud explants incubated on three different basal medium and with different concentration of BAP for shoots proliferation, but MS full basal was the best with BAP(2 mgL^{-1}) obtained maximum number of shoots sprouting (86±0.23%), but little number of shoots and short shoot length were observed in 10±0.30 days. The minimum number of shoots buds which sprouted on whites medium with BAP (2mgL^{-1}) (56 ± 0.21%) and B5 medium(2mg/l) (45±0.30%) (table-1). The shoots bud sprouting on MS, White and B5 medium, no significant difference similar response was also observed by Komalavalli and Rao (2000), C. Subathra Devi et.al. (2008). Previous workcarried out by Bharti Sharma &Y.K. Bansal studies at in vitro conditions using apical buds (2010). MS, Whites and B5 mediumwith BAP is notsufficient for maximum length and multiple shoots of ***Gymnemasylvestre.***

Effect of Plant growth regulators on shoots multiplications- MS medium were supplemented different concentration of cytokinin (BAP and KI) alone and combination with auxins (IAA & NAA) and CW (10-50%) obtained multiple shoots. Namely the highest numbers of multiple shoots (22± 0.08) and length (8.4± 0.27) were found on MS with 5mgL^{-1} BAP + 0.5mgL^{-1} NAA + 0.2 mgL^{-1} KI. MS with 20% coconut water +BAP-2mgL^{-1} + 0.5mgL^{-1} NAA were also observed maximum no. of shoots (19±0.30) and shoot length (6.0±0.21) with 80% shoot proliferation. The combinations and their concentrations were mentioned in Table 2. *In vitro* propagation of plants belonging to Asclepidiaceae has also been shown to have optimum overall growth on MS medium (**Chi Won and John, 1985; Patnaik and Debata, 1996; Komalavalli and Rao, 1997**). According to this study high salt requirement needed for shoots sprouting and proliferation of ***Gymnemasylvestre*** and cytokinin with auxin is best combination for multiplication and shoots length. Thus the degree of growth and differentiation varied considerably with the medium constitution **(Shekhawat*et.al.* 1993; Das *et.al.,* 1996**). **Whereas Reddy *et.al.* (1998)** reported that Kinetin at high concentration, reduced in suppression of shoot sprouting and domination of callus growth.

Effect of growth hormones on Rooting-The plant growth regulators not only control the shoot

Bud formation but also influence the root. In accordance with this, the effect of IBA and Charcoal on root induction was carried out. Among them, root proliferation (88±0.23%), maximum number of roots (12±0.26) and root length (10± 0.30 cm) of the plants were noted on the MS medium containing 1.0 mg/l of IBA + 3g/l of charcoal respectively. when the medium supplemented with IBA 2.0 mgL^{-1} and 2 g/l charcoal on MS medium also the maximum number roots (9.25±0.20), root length(7.56±0.82cm) and root percentage (82 ±0.32) were produced. similarly maximum root length (7.12±0.09cm), roots no.(10 ± 0.23) observed on MS + 0.5mg/l IBA + 2.5 g/l charcoal. According to this study IBA + Charcoal is best combination for rooting. The otherconcentrations of IBA and Charcoal could be responsemoderate to minimum level (Table-3). The micro shoots within 40 days were not observedRooting. However a single root emerged after 60 days in the presence of IBA that continued its linear growth with further production of branches when isolated micro shoots were maintained on media containing IBA + charcoal. **Komalavalli and Rao (2000)** reported that IBA was most effective for root induction and survival in the field with minimal callus formation. IBA improved the overall growth of roots and reduced the time duration of root induction with charcoal. There are about 90% shoots could be induced to root on MS with high concentration of IBA within 60 days with more length and number of roots per shoots. **R. Karthic and S. Seshadri (2009)**also reported that MS medium containing IBA (0.5, 1.0, 2.5 mgL^{-1}) the best for root proliferation.

Hardening of plantlets-The rooted plantlets were kept under mist chamber for 10-15 d. Then, it was transferred to different sterile soils Mixtures with various ratios such as humus rich soil, red soilin sterile polythene bags. The maximum shoot length (11.48 ± 0.36 cm) and root length (8.50 ± 0.14 cm) were observed with plant grown in red soil + humus rich soil (3:1) respectively.The Minimum shoot length (4.0±0.09 cm) and root length (3.25±0.12 cm) were recorded with plant grown in alone red soil. The survival was recorded as 75-85% in the transplantation. The other soil mixtures induced moderate to minimum shoot and root growth of plants (Table-4). Thus, it has been reported that the growth of plants in the soils was controlled by various plant growth promoting substances like organic compounds. Previous workers carried out similar transplantation studies at in vitro conditions using various plants such as ***TerminaliaarjunaRoxb. (*Thomas *et al.,* 2003*); WithaniasomniferaDunal.(*Sivanesan&Murugesan, 2005*); Morusindica L. (*Kavyashree*et al*., 2005);*Hyptissuaveolens*Poit. (*Jain &Chaturvedi, 2005*); Musa acuminata-Colla (*Anilkumar&Sajeevan, 2005*) and Gymnemasylvestre R.Br.,(*S.Padmapriya, and K.Kumanan*, et al.,* 2010*)***. After 10-15 days Plantlets growing proper they were transferred in to field.

Conclusion- In the present study, a fruitful protocol was set up for***Gymnemasylvestre*** through shoot induction and successful

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multiple shoot. The results of this study shown that tissue culture techniques can play an important role in clonal propagation of elite genotypes of ***Gymnemasylvestre***which has diverse medicinal applications and eventually due to over exploitation this plant is facing local extinction. This protocol can be exploited for commercial propagation and conservation of potential rare medicinal plant resources.

Table 1.Effects of various media supplemented with various concentration of BAP on in vitro shoots bud initiation from meristems of Gymnemasylvestre R.Br. after six weeks of culture.

Medium used PGR's in mgl^{-1}			Medium	% of shoots initiated±SE	Mean no. of shoots / explants±SE	Mean shoots length (cm) ±SE	Days required for shoots induction ±SE
Medium type	Medium Strength	$BAPmgL^{-1}$					
WH	½	0.0	WH1	0±0	0± 0	0 ±0	0± 0
	1	0.0	WH2	06±0.12	0 ±0	0 ±0	28.4± 0.31
	1	0.2	WH3	13± 0.28	0± 0	0± 0	18.1 ±0.27
	1	0.5	WH4	25 ±0.06	1.0 ±0.08	0.4± 0.13	25.6 ±0.09
	1	1.0	WH5	42 ±0.23	1.5± 0.23	0.5± 0.21	30.0 ±0.23
	1	2.0	WH6	56 ±0.21	1.3 ±0.01	0.8 ±0.15	15.7 ±0.16
MS	½	0.0	MS1	0± 0	0 ±0	0 ±0	0 ±0
	1	0.0	MS2	15± 0.31	0 ±0	0 ±0	36.2± 0.29
	1	0.2	MS3	70± 0.21	1.0 ±0.16	0.5± 0.11	20.1 ±0.19
	1	0.5	MS4	59± 0.33	1.4 ±0.21	1.0 ±0.29	16.5 ±0.31
	1	1.0	MS5	65± 0.19	1.8± 0.31	1.5±0.20	24.9 ±0.26
	1	2.0	MS6	86± 0.23	2.1± 0.11	2.5 ±0.27	10.0 ±0.30
B5	½	0.0	B5-1	0 ±0	0 ±0	0 ±0	0 ±0
	1	0.0	B5-2	0 ±0	0 ±0	0 ±0	0 ±0
	1	0.2	B5-3	14± 0.28	0 ±0	0 ±0	40.0± 0.08
	1	0.5	B5-4	34 ±0.31	1.0 ±0.20	0.2± 0.04	29.3 ±0.27
	1	1.0	B5-5	22± 0.10	0 ±0	0 ±0	20.5 ±0.34
	1	2.0	B5-6	45± 0.30	1.2± 0.03	0.5± 0.16	17.9 ±0.06

Table 2.Effect of Different type and concentration of Plant growth regulators with MS medium on in vitro shoots multiples of Gymnemasylvestre after 30 days.

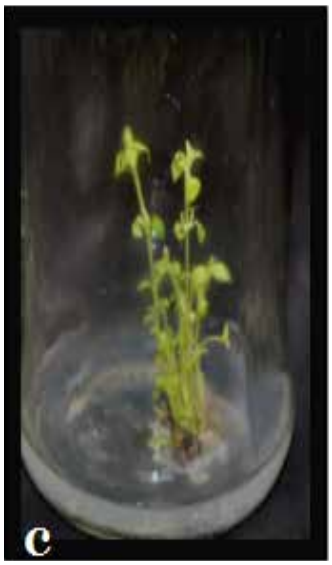
Plant Growth Regulators composition (mg/l)				Coconut water	Medium	Frequency of shoots regeneration % ±SE	Mean no. of shoots/ explants ±SE	Mean shoots length (cm) ±SE	Basal Callus
BAP	NAA	KI	IAA						
0.0	0.0	0.0	0.5	10%	MSM_1	0 ±0	0±0	0.±0	-
0.2	0.0	0.1	0.1	20%	MSM_2	18 ±0.19	1.0± 0.06	2.7± 0.27	+
0.2	0.1	0.2	0.0	30%	MSM_3	48 ±0.27	2.3 ±0.21	3.1 ±0.31	-
0.5	0.1	0.0	0.0	40%	MSM_4	26 ±0.13	1.7 ±0.30	2.5± 0.08	+
0.5	0.0	0.2	0.1	50%	MSM_5	54 ±0.29	4.6 ±0.19	3.0 ±0.13	++
1.0	0.0	0.0	0.2	20%	MSM_6	38 ±0.16	7.8 ±0.33	4.7 ±0.18	-
1.0	0.0	0.0	0.5	10%	MSM_7	60 ±0.31	16 ±0.21	5.4 ±0.16	-
2.0	0.5	0.1	0.0	20%	MSM_8	80 ±0.27	19±0.30	6.9±0.21	+
2.0	0.1	0.0	0.0	30%	MSM_9	51 ±0.07	10 ±0.21	3.7 ±0.28	-
2.0	1.0	0.5	0.0	10%	MSM_{10}	63 ±0.18	12.5 ±0.09	5.7 ±0.20	++
3.0	0.0	0.5	0.5	10%	MSM_{11}	58 ±0.21	14 ±0.12	6.2 0.19	+
3.0	0.2	0.0	0.0	20%	MSM_{12}	35 ±0.13	9.5 ±0.32	4.5 ±0.32	-
3.0	0.5	0.1	0.0	30%	MSM_{13}	57 ±0.32	15.0 ±0.22	7.0± 0.06	+++
4.0	0.1	0.5	0.0	0%	MSM_{14}	70 ±0.14	16.7 ±0.31	5.0 ±0.17	-
4.0	0.2	0.0	0.0	10%	MSM_{15}	42 ±0.09	11.8 ±0.12	4.4 ±0.09	+
4.0	0.0	0.2	0.5	20%	MSM_{16}	68 ±0.15	8.6 ±0.30	3.5 ±0.29	+
5.0	0.5	0.2	0.0	0%	MSM_{17}	70 ±0.30	22±0.08	8.4±0.27	++++
5.0	0.2	1.0	0.0	10%	MSM_{18}	40 ±0.16	05±0.12	3.2±0.31	

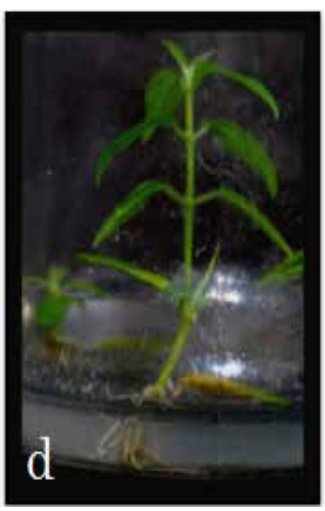
Table 3.Effect of different types and various concentration of growth hormones with MS medium on rooting of Gymnemasylvestre derived in vitro shoots after 60 days, values are mean ±SE of 10 replicates.

IBA mg/l	Charcoal g/l	Medium	Percentage of shoots rooted ±SE	Mean no. of roots / explants ±SE	Mean root length (cm) ±SE
0.0	0.0	MSR_1	0± 0	0 ±0	0 ±0
0.0	1.0	MSR_2	22± 0.31	1.4± 0.10	0.4± 0.22
0.2	1.0	MSR_3	49 ±0.20	3.0 ±0.33	1.6 ±0.08
0.2	0.0	MSR_4	42 ±0.27	4.2 ±0.21	3.4 ±0.21
0.2	2.0	MSR_5	54 ±0.15	5.5 ±0.09	4.1 ±0.27
0.5	0.0-	MSR_6	33 ±0.18	3.7 ±0.16	5.2 ±0.31
0.5	2.5-	MSR_7	76 ±0.21	10.0 ±0.23	7.12 ±0.09
1.0	0.0	MSR_8	50±0.06	4.5 ±0.30	6.0 ±0.23
1.0	3.0-	MSR_9	88 ±0.23	12.0 ±0.26	10.0 ±0.30
1.0	0.0	MSR_{10}	60 ±0.28	5.0 ±0.22	6.5 ±0.07
2.0	0.0	MSR_{11}	47 ±0.13	5.8 ±0.09	6.9 ±0.29
2.0	2.0-	MSR_{12}	71 ±0.24	9.25 ±0.20	7.56± 0.22
2.0	0.0-	MSR_{13}	69 ±0.32	8.0 ±0.29	5.5 ±0.15

Table 4.Growth response of plant at different soil mixtures

S. no.	Soil Mixtures	Conc. %	Shoots length (cm)	Roots length (cm)
1.	Red soil alone	NA	4.0± 0.09	3.25 ±0.12
2.	Red soil +Humus rich soil	3:1	11.48± 0.36	8.50 ±0.14
3.	Humus rich soil	NA	6.5 ±0.31	7.2 ±0.33
4.	Red soil +Humus rich soil	1:1	5.7±0.11	6.0±0.27
5.	Red soil +Humus rich soil	1:2	8.0±0.32	5.0±0.15





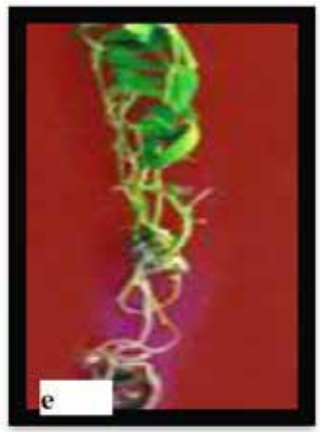


Figure.1. *In vitro***micropropagation of *Gymnemasylvestre* a.** *Invitro* **shoot sprouting b. in vitro initiation of apical bud meristem MS-6 c. in vitro multiplication on MSM-17 medium d. in vitro rooting on MSR-9 medium e. hardinging in soil mixture f. maximum shoot and root length in red soil+ humus rich soil after 30 days.**

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