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Research Article

AN IMPROVED MICROPROPAGATION PROTOCOL FOR GYMNEMA SYLVESTRE GROWN IN VIETNAM

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A B S T R A C T

Gymnema sylvestre is one of the important medicinal plants used for antiperiodic, diuretic and stomachic treatments. In this study, plant multiplication *in vitro* along with genetic stability analysis of regenerates was achieved using nodal explants. Multiple young shoots were induced from axillary meristem cultured on MS medium containing various concentrations of BAP and NAA. The highest number of shoots (8.62), with average length 0.93 cm, was initially achieved with MS medium containing 2.0 mg/l BAP and 0.5 mg/L NAA. Repeated subculture of newly formed nodal parts after each harvest up to ten passages. Rooting of shoots occurred on 1/2 MS medium supplemented with 1.0 mg/l IAA. The clonal fidelity among the micropropagated plantlets after up to 10 sub-cultures was assessed by RAPD molecular markers. All generated bands from 10 primers were monomorphic among the micropropagated plants compared to mother plant in ten regenerants analyzed. This protocol can be used for propagation of cultivated *G. sylvestre*.

INTRODUCTION

Currently the consumption of many medical species rise rapidly. According to World Health Organization (WHO), 80% population in developing countries uses traditional medicine or medical plants for healthcare. In Vietnam, the market of pharmaceutical materials and pharmaceutical products is very large. There are 63 public hospitals and 7,000 private care centers where patients are treated with traditional medicine using about 300 different medications with average of 3,000 tons a year. *Gymnema sylvestre* belonging to the family Asclepiadaceae is a slow growing, perennial woody climber of tropical and subtropical regions. It is distributed throughout the world including China, Thailand and Vietnam and is one of the precious medicinal plants. The whole plant used as astringent, thermogenic, anti-inflammatory, anodyne, digestive, liver tonic, diuretic, stomachic, stimulant, anthelmintic, laxative, cardiotonic, dyspepsia, jaundice, diabetes, constipation, helminthiasis, cardiopathy, cough, asthma, bronchitis, and conjunctivitis (Ganesan and Xu, 2017).

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In Vietnam, *G sylvestre* is cultivated widely by seeds. However, such production is hampered due to its poor seed viability and low rate of germination (Gupta and Solanki, 2015). Alternative micropropagation methods would be beneficial in accelerating large scale multiplication and production improvement. There are several reports on establishment of multiple shoots from node explants of *G. sylvestre* in MS medium supplemented with various combinations of growth regulators i.e. cytokinin such as benzylaminopurine (BAP) and kinetin (kin) alone or in combination with auxin such as naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA). *In vitro* multiplication of *Gymnema* was first attempted by Reddy *et al*., (1998). Maximum number of shoots was achieved on MS medium containing BAP (5 mg/L) and NAA (0.2 mg/L). Solanki and Gupta (2013) and Shrivastava and Singh (2011) used only BAP 5mg/L and 1.5mg/L, respectively and obtained up to 7 shoots/explant. The combination of NAA and BAP (Amarasingh *et al*, 2011; Reddy *et al*, 1998) or BAP, Kinetin and IAA/IBA (Kumar *et al*, 2015; Narayanaswamy and Rao, 2000; Subathra Devi and Mohana Srinivasan, 2008) also found improvement for certain cultivars.

All microproagated plants were expected to have genetic materials identical to the parent plant. In spite of this, genetic

variation could be observed after long time subculture due to growth regulators, variability of cultivar and/or the age of cultivars in culture. This problem may associate with modification in quality and quantity of medical valuable chemicals of *G. sylvestre*. To govern genetic fidelity, various techniques such as isozymes, cytological, and molecular markers can be done. PCR based molecular markers such as RAPD have been wildly used successfully to determine anomalies and confirm genetic homogeneity among micropropagated medical plants such as *Celastrus paniculatus* (Senapati *et al*., 2013), *Gloriosa superba* (Yadav *et al*., 2013) and *Pittosporum eriocarpum* (Thakur *et al*., 2016).

With this background, the objective of this work was to develop an efficient micropropagation protocol of *G. sylvestre* using nodal explants for large-scale production of plants. An attempt was also made to validate the genetic stability of the regenerants using molecular approaches.

MATERIAL AND METHODS

Plant material initial

Green healthy branches of one-year-old *Gymnema sylvestre* plants, which are free from diseases were collected from the field. The explants (axillary buds) were washed thoroughly under running tap water and then treated with 5% (v/v) commercial dish wash for 15 min, followed by rinsing five times in sterile distilled water. The explants were then surface disinfested with 70% alcohol for 1 min followed by immersion in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 min and finally rinsed 4–5 times with sterilized distilled water in laminar flow chamber.

For natural growth plants, seeds were harvested, pretreated with 96% ethyl alcohol to improve their wettability and then were submerged in 0.4% solution of fungicide "Ditan M" for 30 min to minimize the fungal infection. Seeds were surface sterilized in 15% NaClO solution (commercial bleach) for 15 min. Finally the seeds were rinsed twice with sterile distilled water and placed on MS media without growth regulators at 16-hour photoperiod. Single node seedlings formed after two months.

Node culture

The surface sterilized explants or geminated seedling were aseptically trimmed into 1–1.2 cm prior fragments containing an axillary bud and placed on MS media supplemented with various combinations of plant growth regulators (PGRs), 30g/L sucrose, 8g/L agar and pH=5.7. A series experiments was conducted to compare the plant regeneration capacity of *G. sylvestre* under the present of variable concentrations of 6-benzylaminopurine (BAP; 0.5 – 2.5 mg/L) or kinetin (0.5 to 2.0 mg/L) alone and in combination with constant concentrations of BAP (1.5 mg/L) and naphthaleneacetic acid (NAA; 0.5 to 2.0 mg/L) or indole-3-acetic acid (IAA; 0.5 to 2.0 g/L). After four weeks of incubation observations were recorded for average number of shoots per explant; average shoot length (cm); and number of full leaves per shoot. Developed shoots (2-3 cm) were rooted in ½ MS and 1.0 mg/L IAA.

DNA Extraction and genetic stability analysis

DNA was extracted from fresh leaves of micropropagated and field grown plants by the CTAB method (Doyle and Doyle, 1987). The crude DNA was purified with RNase A followed by washing thrice with phenol/chloroform/isoamyl alcohol (25: 24: 1 v/v/v) and subsequently with chloroform/isoamyl alcohol (24:1 v/v). DNA samples were quantified using a NanoDrop 1000 instrument (Thermo Fisher Scientific, USA) and diluted to 25 ng/µl for RAPD-PCR analysis.

Twenty arbitrary 10-base primers (Operon Technologies Inc., Alameda, USA) from B series were used for polymerase chain reaction (PCR). Amplification reactions were performed with total volume of 25 µl containing 15 ng of the primer, 10× assay buffer, 2.0 µl of 1.25 mM each of dNTPs, 0.5 units of Dream*Taq* DNA polymerase (Thermo Fisher Scientific, USA), 2.5 mM $MgCl_2$, and 25 ng of genomic DNA. DNA amplification was performed in a thermal cycler (Gene Amplification PCR System 9700, Applied Biosystems, CA, USA). The first step consisted of holding the samples at 94^0C for 5 min for complete denaturation of the template DNA following by 35 cycles having three ranges of temperature (92^0C for 1 min for denaturation of template DNA, 49^0C for 1 min for primer annealing and 72^0C for 2 min for primer extension). Then, PCR reaction was completed at 72^oC for 7 min. The PCR products were electrophorezed in 1.5% agarose gel in TAE buffer for 2.0 h at 80 V. The size of the amplicons was determined using size standards 1kb DNA Ladder (Thermo Fisher Scientific, USA). DNA analysis was visualized under UV light and photographed using a gel documenting system (Bio-Rad, CA, USA). RAPD analysis using each primer was repeated twice.

Data Analysis

The experiments were repeated 3 times with 30 samples each. Monitoring parameters were observed and monitored after 4 weeks. The experimental data was processed using IRRISTAT 4.0 and Excel.

RESULTS

Regeneration from node explants

Shoot regeneration of *G. sylvestre* was induced from node explants on media containing BAP, kin or the combination with NAA or IAA. The shoots initiation from the axillary buds was observed after one or two weeks of cultivation. Media containing 0.5 –2.5 mg/L BAP as the sole PGR was found to induce shoot formation at all concentrations (Table 1).

Table 1 Effect of BAP on shoot multiplication of Gymnema sylvestre from node explants

BAP mg/L	Shoot/explant	Shoot length (cm)	No of Leaf /shoot
0	1	2.15	3.22
0.5	2.51	2.17	3.3
1.0	2.89	2.24	3.23
1.5	3.58	2.12	3.25
2.0	5.01	1.95	3.29
2.5	4.97	1.99	3.25
CV%	0.4	0.80	0.80
LSD (5%)	0.24	0.15	0.48

Shoot number increased with increasing BAP concentration and highest at 2.0 mg/l and 2.5 mg/l. The optimum level of BAP was 2.0 mg/l which yielded an average of 5.1 regenerated plants per explant. The shoot height as well as number of leaves/shoots seem unaffected by BAP concentration in culture medium.

Similar to BAP, kinetin also influence shoot formation from node, but not for shoot length. The highest shoot number was at 0.5 to 1.5 mg/L (Table 2). This number tends to decrease as kinetin concentration increased to 2.0 mg/l. Although, the shoot length does not affect, the number of leaves per shoot significantly decreased (from 2.25 and 3.22 to 1.23 leaves/shoot) at a concentration of 2.0 mg/l.

Table 2 Effect of kinetin on shoot multiplication of *G. sylvestre* from node explants

Kin mg/L	Shoot/explant	Shoot length (cm)	No. of leaves/shoot
0	1	2.15	3.22
0.5	2.25	2.12	3.21
1	2.43	2.17	2.25
1.5	2.38	2.19	3.2
2	1.23	2.20	1.23
CV%	0.6	0.8	0.3
LSD (5%)	0.23	0.18	0.18

The use of BAP or kinetin obviously accelerates shoot multiple from axillary buds. However, more shoots were observed in the medium supplemented with BAP than kinetin. When application of BAP in combination with NAA the number of shoot regenerated from the single node significant increased, while it seems inhibited by IAA (Table 3). The optimum level of BAP and 0.5 mg/L NAA yielded an average of 8.23 regenerated plants per explant in 4 weeks.

Table 3 The effect of BAP combined with NAA or IAA on shoot multiplication of *G. sylvestre* from node explants

NAA mg/L	Shoot/ explant	shoot length (cm)	Leaf no/shoot	IAA mg/L	Shoot/ explant	shoot length (cm)	No. of leaves/shoot
0.0	5.01	3.95	3.29	0.0	5.01	1.95	3.29
0.5	8.23	3.93	3.24	0.5	5.07	1.93	3.24
1.0	6.48	3.11	3.25	1.0	5.12	1.90	3.22
1.5	3.41	3.34	3.26	1.5	4.87	2.00	3.27
2.0	1.02	3.52	3.22	2.0	4.68	2.11	3.26
CV%	1.40	0.90	0.40	CV%	0.20	1.00	0.50
LSD (5%)	1.50	0.20	0.23	LSD (5%)	0.14	0.18	0.27

About 90% of shoots regenerated in MS medium supplemented with IAA alone at 1.0 mg/l formed an average of 2.2 roots per explant. Therefore a micropropagation protocol was established for *G. sylvestre* using nodes of healthy field plants (Fig 1). The highest number of shoots was produced in MS culture medium added 2mg/L BAP and 0.5 NAA. Almost regenerated shoots form roots in culture medium with present of 1.0 mg/L IAA.

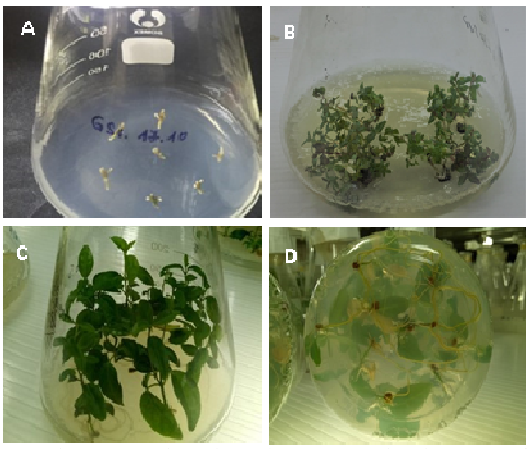

Figure 1 Micropropagation *of G. sylvestre* grown in Vietnam A) shoots initiated from the node; B) Shoot multiplication after 4 weeks in MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/l NAA; C) Developed shoots in ½ MS containing 1.0 mg/L IAA after 3-4 weeks; D) Root formation.

RAPD analysis

In order to confirm the genetic integrity of micropropagated *G. sylvestre* maintained in culture over a period of 12 months, RAPD analysis was carried out from one-month-old plantlets. A total of 150 plantlets over a period of 12 months were analyzed taking a minimum of 15 plants randomly at each culture period. Ten selected RAPD primers utilized in this study gave rise to a total of 49 scorable bands, ranging from 1250 bp to 2500 bp in size (Table 4). The number of bands for each primer varied from 3 to 6, with an average of 5 bands per RAPD primer.

Table 4 RAPD banding pattern of both micropropagated and field grown mother plant

Primer	Sequence	Total band	Band ranges (bp)
OPB-03	CATCCCCCTG	5	300 - 2000
OPB-04	GGACTGGAGT	6	200 - 1200
OPB-05	TGCGCCCTTC	6	300 - 750
OPB-06	TGCTCTGCCC	3	350 - 700
OPB-08	GTCCACACGG	4	500 - 2000
OPB-10	CTGCTGGGAC	4	200 - 1500
OPB-12	CCTTGACGCA	4	500 - 1500
OPB-13	TTCCCCCGCT	5	150 - 2000
OPB-15	GGAGGGTGTT	6	450 - 2500
OPB-18	CCACAGCAGT	6	250 - 1000

The RAPD banding pattern showing monomorphic bands obtained among 10 regenerants from a 12-month-old culture is shown in Figure 2 for RAPD markers. No RAPD polymorphism was observed in the micropropagated plants (Fig 2).

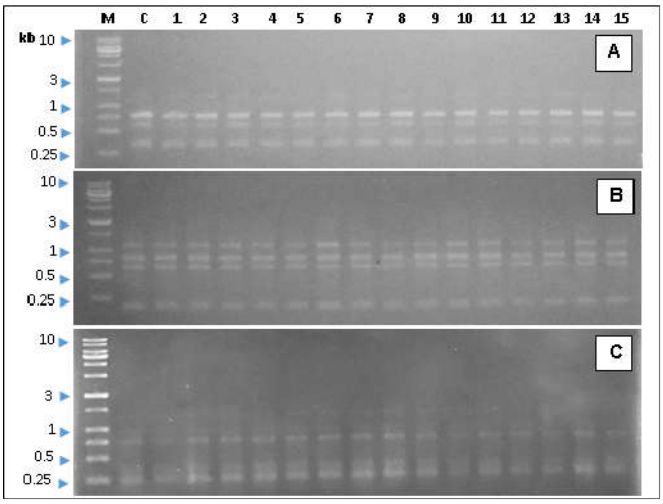

Figure 2 Representative banding pattern in micropropagated plants and field grown mother plant of *G. sylvestre.* Banding pattern with primer OPB06 (A), OPB10 (B) and OPB18 (C) used in RAPD-PCR analysis with the total DNA from mother plant (Lane C) and 15 different micropropagated plants (lane 1-15). Size of DNA bands were compared with a 1 kb DNA ladder (Thermo Fisher Scientific) in lane M.

DISCUSSION

This study was aimed to develop a micropropagation scheme for the production of genetic stability plants for cultivation. Nodes or axillary buds of field plants are most suitable as the initial materials. Despite the micropropagation of *G. sylvestre* can be from germinated seeds (Subathra and Srinivasan, 2008; Komalavalli and Rao, 2000), micropropagation of selected field plants is desirable since micropropagation from germination seeds may face high levels of genetic variability - a limiting factor for its commercial use.

In plant tissue culture, cytokine and auxin/cytokinin ratio can play a critical role to induce the morphogenic response in higher plants (Sakakibara, 2006). In our study, cytokine BAP and kinetin alone can promote multiple shoot formation from axillary buds of *G. sylvestre* regardless their concentrations tested. However, it was noticeable that *in vitro* shoot multiplication response in MS medium with combination of BAP and NAA was comparatively better as compared to BAP alone. The highest number of plant regeneration was produced in 2.0 mg/L BAP and 0.5 mg/L NAA. A lot of previous studies have confirmed the effectiveness auxin and cytokinin to rapid shoot multiplication of variety of plants. In *G. sylvestre*, Reddy *et al* (1998) and Amarasingh *et al* (2011) showed the similar results when using BAP and NAA combination. While Manonmani and Francisca (2012), Shrivastava and Singh (2011) and Syedy and Nama (2018) observed BAP alone was most suitable. Apart from BAP and NAA, Komalavalli and Rao (2000) found the addition effect of kinetin in multiple shoot induction of *G. sylvestre*. Recently, Kumar *et al* (2015) reported BAP and Kinetin in combination with IBA showed better growth rate in terms of number of shoot, length of shoots and number of leaves. The different requirement of plant growth regulators for micropropagation of *G. sylvestre* may be resulted of different genotypes used.

For the first time, the genetic stability of the micropropagated *G. sylvestre* plants was analyzed at DNA level using RAPD markers. This is one of the most important prerequisites in the micropropagation of any plant species. A most common problem encountered with plant micropropagation is the presence of somaclonal variation among subculture cycles from a single mother line. Therefore, it is necessary to examine the improved protocol to confirm true-to-type clonal fidelity. In the present study, 20 RAPD markers were used. Amongst them 10 RAPD markers produced scorable bands which are monomorphic and similar to the mother plant confirming the genetic uniformity within regenerants of at least ten cycles. Genetic integrity by RAPD analysis has been reported earlier in many medicinal plant species such as *Zingiber rubens* (Mohanty *et al*. 2011), *Capparis spinosa* (Carra *et al*, 2012); *Trichosanthes dioica* (Kumar *et al*, 2016).

CONCLUSIONS

To conclude, the present study produces an improved protocol for *in vitro* propagation of *G. sylvestre* using node explants and the culture system would be suitable for commercial applications and overcome problems in conventional methods of propagation.

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