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Callus Induction and *In vitro* Plantlet Regeneration of *Gymnema sylvestre* R. Br. (Retz.) and the Phytochemical Screening of Natural Plants and Callus Cultures

Kaushalya NAN and Senarath WTPSK

Department of Botany, University of Sri Jayewardenepura, Nugegoda, Sri Lanka

Key words: Plantlet regeneration, GC-MS analysis, *Gymnema sylvestre*, Gymnemic acid, Plantlet regeneration, Type II diabetes

Abstract

Gymnema sylvestre is a slow growing perennial medicinal woody climber. It belongs to the family Asclepiadaceae. Gymnemic acid, the major bioactive component of this plant species is used as a remedy for type II diabetes. Propagation of this plant is often difficult and expensive. In the present study, *in vitro* protocols were developed in order to induce callus and regenerate plantlets from different explants of *G. sylverstre*. As secondary metabolites are important in medicinal plants, studies were carried out to screen phytochemicals present in natural plants and callus. The best medium for callus induction from leaf discs was MS supplemented with 5.0 mg/l 2,4-D. Although, nodal segment grown in MS supplemented with 1.0 mg/l BA gave the highest shoot elongation (14.8 ± 0.20), growth regulator free MS also showed a high elongation of shoots (14.2 ± 0.37) and the difference between those two were non-significant. MS supplemented with 3.0 mg/l IBA was best for root induction. Highest survival percentage (62.5) was observed when plantlets were acclimated in a substrate containing a mixture of soil and sand in the proportion of 1 : 2. In the present study, phytochemicals present in callus and the leaves of the naturally grown plants were compared using Gas Chromatography- Mass Spectrophotometer. A total of nine compounds was identified from the leaves of naturally grown plants and nine compounds were identified from the callus. Out of all identified phytochemicals, a total of six compounds were present in both leaves and callus samples suggesting that in addition the plant material, callus may also be used as a supplement raw material to obtain secondary metabolites for the pharmaceutical industry.

*Author for correspondence: <wtpsk2011@yahoo.com>.

Introduction

Gymnema sylvestre R.Br. (Retz.) (Asclepiadaceae) is found in the tropical regions of Africa, Australia and South East Asia. It is occasionally cultivated due to high demand in indigenous medicine. It is rather common in the lowland of Sri Lanka, especially in the dry and intermediate zones (Jayaweera and Senaratne 2006). This is one of the most common plants used for the treatment and prevention of type II diabetes using herbal or indigenous medicine. It increases the insulin producing β-cells of the pancreas and significantly reduces the metabolic effects (Krishna et al. 2012). It also has somatic, diuretic and cough suppressant ability. The plant has been reported to possess antimicrobial, antieruodonic and antiviral effects (Madhurima et al. 2009). *G. sylvestre* leaves contain triterpene saponins characteristic of oleanane and dammarene classes. Oleanane saponins are gymnemic acids and gymnemasapones, while dammarene saponins are gymnesides (Krishna et al. 2012).

Increasing awareness of the side effects of Western drugs have made general public turn towards the herbal medicine, thus the demands for medicinal plants have drastically increased. Due to over exploitation, this plant species has become threatened and is listed in IUCN red data book (Shailasree et al. 2012).

G. sylvestre is a slow growing, perennial woody climber (Shrivastava and Singh 2011). Seeds lose viability in a short period of storage (Reddy et al. l998). Conventional propagation methods are hampered due to its poor seed viability, low rate of germination and poor rooting ability of vegetative cuttings (Komavali and Rao 2000). Therefore, the propagation of this plant species by alternative methods is in demand. In the present study, *in vitro* propagation of *G. sylvestre* through direct organogenesis using nodal segments as explants was investigated as the main objective of the present investigation.

The screening of phytochemicals present in *in vitro* produced callus and leaves from naturally grown plants of *G. sylvestre* has been carried out by extracting the phytochemicals in different solvent systems. Using Gas Chromatography - Mass Spectrophotometer (GC-MS) chemicals present in the leaves of the natural plant population and callus have been identified and compared.

Materials and Methods

MS was used in all experiments. Young *G. sylvestre* nodal segments (1.0 cm long) were surface sterilized by washing them first in a mixture of Teepol 2.0% and five drops of Tween 20 in sterile distilled water for 1 hr and then in 10% Clorox for 5 min and finally in 70% ethanol for 1 min. followed by 2 sec dip in a

fungicide solution. Each step was followed by three successive washings in sterile distilled water. Explants were placed vertically on the full and half strength MS supplemented with different concentrations of BA (0.5 - 2.5 mg/l). Growth regulator free MS was used as the control. Cultures were incubated at 25 ± 1ºC in 16 hrs photoperiod. There were 10 replicates in each treatment. Shoot induction was observed at 7 days' interval over a period of 8 weeks. The length of each shoot was measured after 8 weeks of incubation.

Elongated *in vitro* propagated shoots were separated carefully from the culture. Then they were transferred into the MS supplemented with different concentrations of IBA (1.5 - 3.5 mg/l) for root induction. The growth regulator free MS was used as the control. Activated charcoal (1.0 g/l) was added to the medium before adjusting pH to 5.8. There were 10 replicates per treatment. Root induction was observed at 7 days' intervals over a period of 8 weeks. The number of roots per shoot was counted.

When root system was well developed, plantlets were transferred into different potting mixtures (Table 1). Plantlets were removed from the medium without damaging the roots after carefully removing all traces of agar. Plantlets were dipped in a fungicide solution (Thiram® 2.0 g/l) before being planted in sterile potting mixtures. Plantlets were kept in a propagator over a period of 3 weeks and then gradually exposed to the natural environment. There were 10 replicates per treatment. The percentage of survival was assessed after 8 weeks.

Table 1. Different types of potting mixture used in acclimation.

Treatment code	Soil mixture	Ratio
T1	Soil : sand : compost	1 : 1 : 1
T2	Soil : sand	1 : 1
T3	Soil : sand	1 : 2

Young *G. sylvestre* leaves were surface sterilized and placed on the MS supplemented with different concentrations of 2,4-D (4.0 - 5.5 mg/l). Growth regulator free MS was used as the control. Each treatment consisted of 10 replicates and cultures were incubated at 25 ± 1ºC in the dark. The diameter of the callus was measured every week over a period of 10 weeks.

The leaves were collected from the six-months-old plants. Fresh leaves and callus from ten-week-old cultures were air dried first and then in the oven till they attained constant weight. Then the dried samples were powdered and 1.0 g of powdered sample was weighed separately. The phytochemicals in each sample were extracted using the following method. To powdered samples, 95% methanol (50.0 ml) was added, the mixture was refluxed in a water bath for 30 min and then filtered through Whatman® No. 1 filter paper and the process was

repeated. The methanolic extracts obtained by repeated extractions were combined and evaporated. To the resulting semi solid, 5.0 ml of 50% (v/v) methanol and 1.0 ml of potassium hydroxide solution (containing KOH 11.0 g/100.0 ml water) were added and the mixture was refluxed in a 50°C water bath for 1 hr. Then the mixture was allowed to cool and refluxed for another one hour after adding 0.9 ml concentrated hydrochloric acid. The resulting solution was again allowed to cool, the pH was adjusted to 7.83 with potassium hydroxide solution and made up to 50.0 ml with 50 % (v/v) methanol and filtered through Whatman No. 1 filter paper (Pandey and Yadav 2010). The resulting solid was subjected to trituration and this procedure was carried out separately for leaf and callus samples. Finally both samples were filtered through Whatman No. 1 filter paper again and then the filtrates were subjected to GC-MS analysis.

Conditions of the GC - MS experiment are given below:

Column agilent 1909IS- 433 (30 m × 250 μm, thickness 0.25 μm); Amount injection - 2 μl; Injector temperature - 280ºC; Maximum temperature - 325ºC; Column flow - 1.5 ml/min; Hold up time - 1.0947 min; Column pressure - 16.191 psi; Column thickness - 0.25μm; Average velocity - 45.675 cm/s; Detector - Mass Spectrometry; GC - MS conditions used in the analysis are tabulated in Table 2.

Table 2. GC-MS system conditions

	Rate (C/min)	Value (ºC)	Hold time (min)	Run time (min)
Initial	0.0	100	5.0	5.0
Ramp	5.0	275	10.0	50.0

Results and Discussion

The results of the experiment are given in Table 3. For nodal segments of *G. sylvestre*, the best growth regulator concentration for direct shoot induction in MS was observed in the presence of 1.0 mg/l BA which gave the highest shoot elongation (14.8 ± 0.20). The presence of 0.1 mg/l NAA and 1.0 mg/l BAP was best medium for shoot induction from nodal explants (Amarasinghe et al. 2011) which conform the results of the present study. The presence of higher concentrations of BAP (1.5 mg/l) in the medium suppresses the shoot development (Komalavalli and Rao 2000) confirming the results of the present study, where the mean shoot length was drastically reduced in the presence of ≥ 1.5 mg/l BA. However, the growth regulator free MS (control) also showed high shoot elongation (14.2 ± 0.37) and the difference between those two treatments is not significant. When half strength of MS was used as the basal medium, it was observed that the best growth regulator concentration for direct shoot induction

from nodal segments was 1.5 mg/l BA (10.4 ± 0.81) and it is significantly lower than MS.

When the shoot elongation in MS and half strength of MS was compared, it was observed that lowering the basal salt concentrations significantly lowered the ability of shoot elongation. Full strength MS was responding better than half strength MS. Although pair-wise comparison of the data indicated that, MS basal medium supplemented with 1.0 mg/l BA is the most suitable medium, the results suggested that the growth regulator free MS medium is the best for shoot induction and elongation from nodal segments of *G. sylvestre* (Fig. 1a).

Table 3. Influence of various concentrations of BA on direct shoot induction from nodal segments of *G. sylvestre* after 8 weeks of incubation. There were 10 replicates.

Basal	Mean shoot length (mm) ± SE						5%
medium	0.0 (Control)	0.5	1.0	1.5	2.0	2.5	LSD
MS	14.2 ± 0.37	14.4 ± 0.51	14.8 ± 0.20	13.6 ± 0.25	12.4 ± 0.40	10.6 ± 0.51	0.01
½MS	7.4 ± 0.25	8.4 ± 0.51	9.2 ± 0.37	10.4 ± 0.81	9.0 ± 0.32	8.2 ± 0.37	0.01
5% LSD	0.01	0.02	0.01	0.02	0.01	0.02	-

Table 4. Influence of various concentrations of IBA on rooting of *in vitro* shoots of *G. sylvestre* after 8 weeks of incubation. There were 10 replicates in each treatment.

IBA concentration (mg/l)	Percentage of root forming plantlets	Mean root length (mm) ± SE
0.0 (control)	0	0.0
1.5	0	0.0
2.0	0	0.0
2.5	60	3.5 ± 1.1
3.0	100	6.2 ± 0.9
3.5	80	6.0 ± 1.0[a]
5% LSD	-	1.2

[a] = Callus at the base of the shoot.

The results are summarized in Table 4. Regenerated shoots were rooted on MS medium without supplementing any growth regulator (Reddy et al. 1998). However, in contrast present study indicated that the growth regulator free MS (Control) did not induce roots *in vitro.* ANOVA using Kruskal-Wallis Test indicated that all the treatments were significantly different from each other.

High frequency of rooting (50%) was obtained in axillary node explants derived shoots on half strength MS supplemented with IBA (3.0 mg/l (Komalavalli and Rao 2000). In the present study the best growth regulator concentration for *in vitro* root induction was observed in MS supplemented with 3.0 mg/l IBA giving 100% success in root induction (Plate 1b). This indicates that the decreased levels of basal salt concentrations may have affected the

percentage rooting (Komalavalli and Rao 2000). Presence of lower levels of IBA (≤ 2.0 mg/l) did not induce roots in *in vitro* propagated shoots. Increased concentrations of higher than 3.0 mg/l IBA not only retarded the root induction but also induced callus at the base of the shoot. Thus, it could be suggested that MS supplemented with 3.0 mg/l IBA in MS would be the best medium for rooting of *in vitro* shoots of *G. sylvestre.*

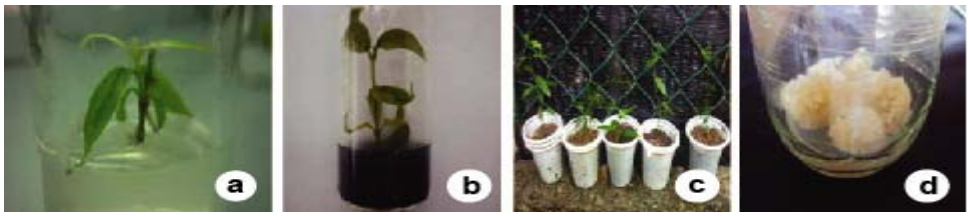

Fig. 1 a. Shoot induction in growth regulator free MS, b. Rooting of elongated shoots in MS + 3.0 mg/l IBA, c. Acclimated plantlets in (1 : 2) sand : soil mixture, d. Callus induction in MS + 5.0 mg/l 2,4-D.

The highest percentage of survival (62.5) was observed in the substrate with a high proportion of sand (soil : sand :: 1 : 2) (Table 5). Reddy et al. (1998) reported that for acclimation, plantlets were removed from the rooting medium 8 weeks after root initiation, and transferred to fresh tubes containing autoclaved tap water. After 8 - 10 days, plantlets were subsequently transferred to plastic pots (9 × 9 cm) containing autoclaved soilrite covered with perforated polythene bags to maintain humidity; and were kept under culture room conditions for about 7 days. After three weeks, polythene bags were removed and pots were transferred to the garden, and placed under the shade till the new leaves appeared. Then they were planted under normal garden conditions. The transplantation success was about 75%. In the present study, substrate mixture with soil : sand (1 : 3) enhanced the surviving ability (Plate 1c).

Table 5. Percentage of survival in different potting mixtures used in acclimation after 8 weeks. There were 10 replicates in each treatment.

Treatment code	Percentage of survival
T1	0
T2	0
T3	62.5 %

An increasing portion of sand in the mixture enhances the surviving ability as smaller amount of water was retained in the substrate (Senarath *et al.* 2007). Plantlets are unable to compete with soil microbes and to cope with the harsh environmental conditions especially immediately after removal of the plantlets

from *in vitro* conditions. After removal of the plantlets from the propagator, they were exposed to natural environment gradually. Yet, it was observed that especially during the rainy season, the survival ability of the plantlets was poor thus making it essential to keep the plantlets in a propagator for a long period if the rainy season persisted.

In trial experiments lower concentrations of BAP, Kn and also 2,4-D have been used but no callus induction was observed except in the presence of 4.0 mg/l 2,4-D. Thus increased level of 2,4-D has been used in this experiments reported here. Callus induction was observed in all the treatments with over 4.5 mg/l 2,4-D after 3 weeks of incubation. Calli were pale yellow and hard indicating that those were not embryonic calli. The best growth regulator concentration for callus induction from leaf disc explants of *G. sylvestre* was 5.0 mg/l 2, 4-D in MS. This was significantly higher than all other treatments and a significant difference in callus diameter was observed among all treatments (Fig. 1d). Leaf disc explants cultured on growth regulator free MS medium (Control) did not induce any callus. The increased concentrations of 2,4-D over 5.0 mg/l drastically reduced callus induction (Table 6). BAP is more effective in producing callus than Kn (Gopi and Vatsala 2006) but the amount produced was less. In contrast, in the present study it was observed that the presence of 2,4-D accelerates the callus induction making it possible to obtain large quantities of phytochemicals.

Table 6. Influence of various concentrations of 2, 4-D on callus formation from leaf explants of *G. sylvestre* after 10 weeks of incubation. There were 10 replicates in each treatment.

Concentration of 2,4-D (mg/l)	Mean callus diameter (mm) ±SE
0.0 (Control)	0.0 ± 0.0
4.0	11.4 ± 2.6
4.5	18.3 ± 0.3
5.0	21.0 ± 0.4
5.5	18.8 ± 0.3
6.0	17.9 ± 0.3
LSD 5%	0.01

Many secondary metabolites have a complex and unique structure and their production is often enhanced by biotic and abiotic stress conditions. The production of gymnemic acid is significantly higher in callus treated with 2,4-D and Kn (Ahmed *et al.* 2009). Although there are studies based on the phytochemical screening of fresh leaf samples of *G. sylvestre*, there are no comparisons were made between *in vitro* regenerated plants and callus until the

present study was undertaken. In the present study, the phytochemicals present in the callus samples have been compared with those of the fresh leaf.

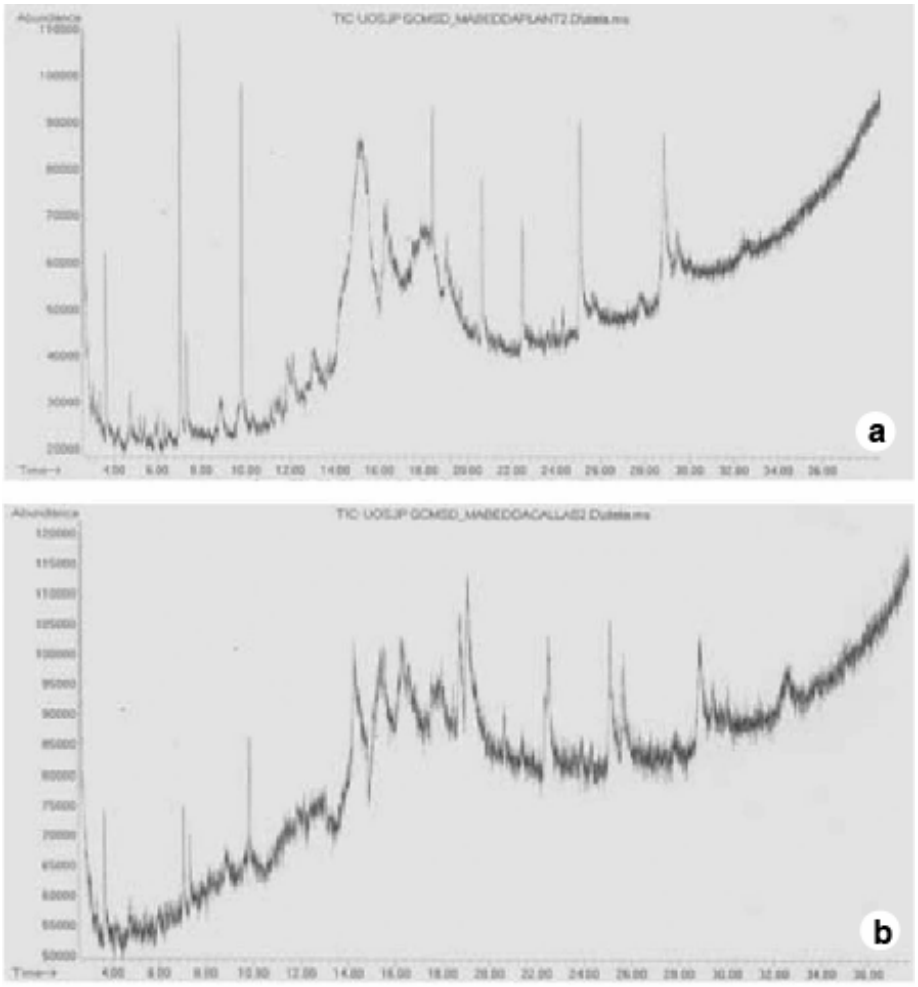

Fig. 2a. GC-MS chromatogram of the leaf sample and (b) callus sample of *G. sylvestre.*

The observation of chromatograms for the callus and the leaves from naturally grown plants are given in Fig. 1. The results of the GC-MS analyses on methanolic extract of the leaves and callus of *G. sylvestre* indicated there are six phytochemicals which were found to be common in both samples (Table 7). In contrast, the isozyme (peroxidase) profile showed no difference between parent and *in vitro* raised plants (Reddy *et al.* 1998).

Table 7. Phytochemicals identified in leaf samples by GC-MS analysis.

Compound	Molecular weight (g/mol)	Fresh leaves similarity (%)	Callus similarity (%)
2-pyrrolidinone	85	80	59
2-Methyl-2,3-dihydrobenzofuran	120	87	72
β - sitosterol	414	-	90
Cyclononasiloxane, octadecamethyl	666	-	83
2-furancarboxaldehyde, 5-hydroxy methyl	126	81	58
2-methoxy-4-vinylphenol	150	95	90
2-propenoic acid,3-(4-hydroxy-phenyl)-methyl ester	178	97	49
n-hexadecanoic acid	256	98	92
7,10,13- hexadecatnenoic, methyl ester	264	76	
Benzeneacetic acid, alpha, 3, 4-tris [(trimethylsilyl)oxy] –	472	-	43
Octasiloxane	578	87	-
Octadecanoic acid	284	38	-

The presence of n-hexadecanoic acid and octadecanoic acid in the leaves of *G. sylvestre* was reported by Parinala and Ramasubramaniaraja (2010) conforming the results of the present study. When compared the leaf and callus samples more than 80% similarity were found in them. The leaves of *G. sylvestre* contain triterpene classes of oleanane saponins (gymnemic acids, gymnemasaponins) and dammarene saponins (gymnemasides). It was difficult to detect those compounds using GC-MS as gymnemic acid is a large molecule and it can be dissociated into a number of small functional groups.

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