IN VITRO MASS PROPAGATION OF *GYNURA PROCUMBENS* (LOUR.) MERR. - AN IMPORTANT MEDICINAL PLANT

Sweety Majumder, Animesh Biswas, Md. Mahbubur Rahman

Department of Botany, University of Chittagong, Chittagong 4331, BANGLADESH.

sweetymajumder13@gmail.com

ABSTRACT

An improved protocol was developed for direct micropropagation of Gynura procumbens (Lour.) Merr-an important medicinal plant, from different explants (shoot tips and nodal segments) in Murashige and shoog (MS) basal medium supplemented with different growth regulators like, Cytokinins and Auxins either alone or in combinations. Highest rate (100%) of shoot induction was observed on MS medium supplemented with 1.5 mg/l 6-benzylaminopurine (BAP) and 1.0 mg/l α-naphtalene acetic Acid (NAA) from nodal explants. The highest elongation of shoot buds (8.36 cm) was obtained on MS basal medium fortified with 1.0mg/l 6-benzylaminopurine (BAP) and 0.25 mg/l α-naphtalene acetic acid (NAA). The maximum number of roots (6.91±0.50) and root length (7.60cm) was observed in half strength MS medium supplemented with 0.5 mg/l indole-3-butyric acid(IBA).The rooted plantlets were acclimatized and transferred to greenhouse with 97% success. This in vitro propagation protocol should be useful for conservation as well as mass propagation of this medicinal plant.

Keywords: *Gynura procumbens,* Micropropagation and Nodal explants.

INTRODUCTION

Gynura procumbens (Lour.) Merr. is an important medicinal plant, belongs to the family Asteraceae. The plant is indigenous to Malaysia, Indonesia and Thailand. In Malaysia this species has its distribution limited to the western part of peninsular Malaysia. This is not a native plant of Bangladesh. It is commonly known as '*Sambung nyawa'* by the Malays and '*Bai bing ca'* by the Chinese in Malaysia. Its habit is a scrambling or weakly climbing herb with stem up10 to 25 cm tall. This tropical herbaceous medicinal plant, is highly branched with hairy green leaves that are alternately arranged on hairy light purple stem. It produces purple, tubular and bisexual flowers (Wiart, 2002).

This plant is traditionally used for the treatment of eruptive fever, rash, kidney disease, constipation, hypertension, diabetes mellitus, migraines, urinary tract infection, rheumatism, viral diseases of skin (Afandi et al., 2014). The recent pharmacological studies of *G. procumbens* discovered the anti-Herpesvirus activity (Nawawi et al., 1999), anti-hyperglycemic (Hassan et al. 2010), anti-inflammatory (Iskander et al., 2002), antiulcer (Mahmood et al., 2010), anti-hyperlipidemic (Zang & Tan, 2000), anti oxidative (Puangpronpitag et al., 2010) and blood pressure reduction capabilities (Kim et al., 2006). The leaves of *G. procumbens* do not have any toxic effects (Rosidah et al., 2009). Akowuah et al., (2001) discovered that the extract of *G. procumbens* can reduce the blood sugar level of type 2 diabetic rats.

Due to medicinal values, there is a great potential to develop various products from this species. In order to maintain a sustainable raw material supply in manufacturing of *G. procumbens* products, propagation of this plant on a large scale will be a key step. The *in*

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vitro culture techniques can be used as the alternative for the superior planting material and continuous provision of plantlet stocks for large scale field cultivation.

This plant is conventionally propagated by cuttings. Vegetative propagation is slow and further hampered by specific habitat requirements and by poor performance of propagules. This conventional method cannot meet the increasing demand of this plant used as the raw material for the preparation of pharmaceutical, dermaceutical and aromatherapeu products. The *in vitro* micropropagation technique can produce a large scale of raw materials for field cultivation and fulfill the demand. Many important medicinal plant species are now propagated by *in vitro* culture techniques, such as *Cordeauxia edulis* (Yohannes & Firew, 2014), *Caralluma diffusa* (Karthik et al., 2013), *Centella asiatica* (Chandra et al., 2014), *Aloe vera* (Mehta, 2013) *and Vitex negundo* (Usha et al., 2007). This paper describe an efficient protocol for rapid clonal multiplication of this pharmaceutically important plant species through direct organogenesis using nodal explants and shoot tips followed by successful outdoor establishment of regenerated plants.

MATERIALS AND METHODS

Collection of plant materials and surface sterilization

The vigorous apical shoot buds or young shoot cutting of *Gynura procumbens* were collected from a mature plant growing in the Medicinal Plants Garden of the Bangladesh Forest Research Institute at Chittagong. After removing the leaves, the shoot tips and nodal segments with dormant axillary buds, were washed thoroughly under running tap water for 30 minutes, followed by treated with liquid detergent (Tween 20) for 10 minutes and dipping in 5% (v/v) savlon solution for 10 minutes. The materials were then washed 6-7 times with distilled water. After rinsing with 70% ethanol for less than 60 seconds, they were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 minutes and washed with sterile double distilled water 4-5 times after each surface disinfection treatment under aseptic conditions. The surface sterilized explants were cut into small pieces (0.5-1.0cm) with a sterilized surgical blade and then inoculated onto the culture media.

Culture medium and conditions

The Culture medium used for the present work was full strength MS basal medium (Murashige and Skoog) with 3% (w/v) sucrose and 0.8% (w/v) agar. The medium was supplemented with various concentrations of cytokinins such as, BAP and Kn (6-furfurylamine) either alone or each in combination with auxins , like, IAA (Indole-3-acetic acid) and NAA. The pH of medium was adjusted to 5.8 before autoclaving at $121^{o}C$ for 30 minutes under $1.1kg/cm^2$ pressure. All the cultures were kept in culture chamber at $25\pm2^{o}C$ under 14/10 hours (light/dark) photoperiod with a light intensity of 2000-3000 lux supplied by cool white fluorescent tubes and with 60-65% relative humidity.

Multiplication of shoot buds

Sterilized nodal explants and shoot tips were cultured on MS basal medium containing 3% sucrose (w/v), 0.8 % agar (w/v) and various concentrations of BAP and Kn (1.0-3.0 mg/l) either individually or in combinations with IAA (0.5-1.0 mg/l) or NAA(0.5-1.0mg/l) were used for shoot proliferation. After two weeks the clumps of shoots were sub cultured on MS medium containing 3 % (w/v) sucrose, 0.8 % (w/v) agar with suitable growth regulators for multiplication and maturation of the shoots.

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Shoot Elongation

Proliferated multiple shoot buds were divided into small clusters of one to two shoots. They were sub cultured on shoot elongation medium containing various concentrations and combinations of cytocynins (BAP and Kn) and auxins (IAA and NAA).The cultures were incubated at 25 ± 2°C under 14/10hour light/dark photoperiod.

Rooting of microshoots

For *in vitro* rooting, the elongated shoots of 6-8 cm length were excised from each culture passage and transferred to half strength MS fortified with different concentrations and combinations of IAA, IBA (Indole-3-butyric acid) and NAA. Data were recorded on percentage of rooting, mean number and length of roots after 4 weeks of inoculation**.**

Hardening and Acclimatization

The regenerated plantlets with well developed roots were removed from culture tubes, washed under running tap water to remove agar and transferred to sterilized soil and compost mixture(1:1) in small plastic pots and maintained under 30±2 °C and relative humidity 80-85%. After 3-4 weeks the plantlets were subsequently transferred to larger pots & gradually acclimatized to outdoor condition. The percentage of survival was recorded one month after transfer

Data analysis

All the experiments were conducted with minimum of 15 replicates per treatment. 10- 15 explants were used per treatment in each replication and each experiment was repeated thrice. Data are the Mean ± SE for the indicated number of separate experiments. Observations were recorded on the frequency and the number of shoots, shoot length, number of roots and root length respectively. Data were recorded after 25-30days of interval.

RESULTS AND DISCUSSION

Different concentrations of cytokinins like, BAP (1.0-3.0 mg/l) or Kn (1.0-3.0 mg/l) either alone or in combinations with auxins such as, IAA (0.5-1.0 mg/l) or NAA (0.5-1.0 mg/l) were used in MS basal medium and observed on morphogenetic responses of both explants (Shoot tips and nodal explants) of *G. procumbens* (Table-1).When both explants were cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators, a differential response with regard to shoot induction was observed (Figure1-A&B). Of the two explants such as, shoot tips and nodal explants, nodal segments were found to be the best sources of multiple shoot buds induction. A number of author also suggested the same type of explants for propagation of other important medicinal plants such as, *Pterocarpus santalinus* (Warakagoda & Subasinghe, 2013), *Plumbago zeylanica* (Ceasar et al., 2013) and *Boerhaavia repens (*Rownaq & Hossain, 2010). The highest proportion (100%) of multiple shoot buds induction was observed from nodal explants on MS basal medium supplemented with 1.5 mg/l BAP+1.0 mg/l NAA. The minimum number (1.05±0.16) of shoots was obtained from shoot tips on MS fortified with 3.0 mg/l Kn+1.0 mg/l 1AA. In terms of elongation, the multiple shoot buds that produced directly from the different types of explants underwent elongation when cultured on MS with various concentration and combination of cytokinins and auxins (Table-2). Data on elongation of shoot buds were recorded after 4 weeks of inoculation. Maximum elongation (8.36cm) of shoot duds took place in media supplemented with 1.0 mg/l BAP + 0.25 mg/l NAA after four weeks of culture (Table 2) (Figure-1 C,D & E). On the contrary, lowest elongation (1.73cm) was recorded in MS medium that contained 3.0 mg/l Kn + 0.5 mg/l NAA. Such direct organogenesis were

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also reported in other important medicinal plant species such as *Santalum album* (Janarthanam & Sumathi, 2011), *Plumbago zeylanica* (Mahendra et al., 2014), *Eclipta alba* (Sharma et al., 2013), *Tribulus terrestris* (Raghu et al., 2010), *Curculigo orchioides* (Francis et al., 2007) and *Cicer arietinum* (Ugandhar et al., 2012).

Table 1. Effect of different concentrations and combinations of plant growth regulators in MS basal medium on shoot proliferation from shoot tips and nodal segments of *Gynura procumbens*

PGRs (mg/l)				*Shoot tips*			*Nodal segments*		
BAP	*Kn*	*IAA*	*NAA*	*Time (Days) required for shoot initiation*	*% of explants forming shoots*	*Average* No. of shoots per culture (mean±SE)*	*Time(Days) required for shoot initiation*	*% of explants forming shoots*	*Average* No. of shoots per culture (mean±SE)*
1.0	-	-	-	10-25	40.00	2.00±0.10	10-25	55.20	2.74±0.11
1.5	-	-	-	10-25	61.25	2.13±0.22	10-25	68.10	2.87±0.09
2.0	-	-	-	10-25	57.00	2.37±0.06	10-25	75.00	4.51±0.13
3.0	-	-	-	10-25	25.00	1.79±0.33	10-25	60.21	1.90±0.12
1.0	-	0.5	-	10-20	55.00	3.05±0.31	10-20	69.00	3.67±0.15
1.5	-	0.5	-	10-20	62.00	4.15±0.04	10-15	73.00	4.85±0.10
2.0	-	1.0	-	10-25	50.10	3.57±0.16	10-25	65.00	3.79±0.31
3.0	-	1.0	-	10-25	30.00	2.13±0.25	10-25	45.21	2.50±0.20
1.0	-	-	0.5	10-20	75.20	4.00±0.05	10-20	80.00	6.50±0.01
1.5	**-**	**-**	**1.0**	10-15	83.10	5.41±0.32	**10-15**	**100**	**9.10±0.16**
2.0	-	-	1.0	10-15	80.00	5.01±0.10	10-15	90.00	7.31±0.15
3.0	-	-	1.0	10-15	50.10	4.57±0.25	10-15	60.00	5.61±0.01
-	1.0	0.5	-	10-20	55.00	3.17±0.16	10-15	68.00	3.51±0.16
-	1.5	0.5	-	10-20	60.00	4.00±0.05	10-20	75.10	4.68± 0.33
-	2.0	1.0	-	10-20	40.15	2.35±0.28	10-20	50.00	3.00±0.05
-	**3.0**	**1.0**	-	**10-20**	**25.00**	1.05±0.16	10-25	30.10	1.60± 0.23
-	1.0	-	0.5	10-25	40.00	2.51±0.33	10-20	50.00	2.80±0.25
-	1.5	-	0.5	10-25	55.00	2.30±0.11	10-20	66.10	2.66±0.15
-	2.0	-	1.0	10-25	35.00	2.15±0.02	10-25	45.00	2.30±0.08
-	3.0	-	1.0	10-25	31.00	2.10±0.34	10-20	40.00	2.50±0.09

* Values are the mean of three replicates each with 15 explants

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Table 2. Effect of different concentrations and combinations of cytokinins and auxins on elongation of multiple shoot buds *of G. procumbens*

PGRs (mg/l)				*Average*initial length(cm) of individual shoot(±SE)*	*Average*length(cm) of shoots after 30days of culture(±SE)*
BAP	*Kn*	*IAA*	*NAA*		
0.5	-	0.25	-	2.50±0.15	4.33±0.12
1.0	-	0.25	-	3.15±0.17	6.10±0.22
2.0	-	0.50	-	3.37±0.10	4.45±0.15
3.0	-	0.50	-	2.08±0.21	3.41±0.05
0.5	-	-	0.25	3.11±0.40	6.10±0.28
1.0	-	-	**0.25**	**3.88±0.20**	**8.36±0.10**
1.5	-	-	0.50	3.40±0.15	7.11± 0.05
2.0	-	-	0.50	3.01±0.22	5.21±0.45
3.0	-	-	0.50	2.36±0.12	3.21±0.14
-	0.5	0.25	-	2.25±0.31	4.22±0.28
-	1.0	0.25	-	2.85±0.11	4.10±0.35
-	2.0	0.50	-	2.51 ± 0.15	3.84 ±0.33
-	3.0	0.50	-	2.00± 0.06	3.23± 0.27
-	1.0	-	0.25	2.11±0.44	3.05±0.20
-	2.0	-	0.50	1.90±0.35	2.62± 0.31
-	**3.0**	-	**0.50**	**1.25±0.01**	**1.73±0.11**

* Values are the mean of three replicates each with 15 explants

Well elongated shoots were excised and transferred to the root induction medium. Full and half strength MS medium fortified with different concentrations of auxins (IAA, IBA and NAA) were used for root induction. Rooting was observed from the cut ends of shoots within 3 - 4 weeks in most media tested. Among the three auxins tested, the number and length of roots varied in both medium (Table-3). Half strength MS medium fortified with different concentrations of auxins shows better root formation when compared to full strength MS medium with various concentrations of auxins. The best response was observed with 1/2 MS + 0.5 mg/l IBA (Figure1-F&G). The highest number (6.91±0.50) and length of root (7.60cm) was obtained on half strength MS medium supplemented with 0.5 mg/l IBA. Among the three auxins, IBA was found to be comparatively more effective than IAA and NAA. The influence of auxins for induction and proliferation of root has been reported in many medicinal plants *viz. Mentha pulegium* (Khadija & Noureddine, 2012), *Salvia guaranitica* (Echeverrigaray et al., 2010), *Boerhaavia diffusa* (Wesely et al., 2010), *Turnera ulmifolia* (Kalimuthu et al., 2014) and *Cucumis Anguria* (Jeyakumar & Kamaraj, 2015).

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Table 3. Data on the development of roots in elongated multiple shoot buds of *Gynura procumbens* when grown on 0.8% agar solidified rooting media

PGRs	*mg/l*	*Full strength MS media*		
		% of Rooting	*Number* of roots per shoot*	*Average *Length of roots(cm)(mean±SE)*
	0.25	35	2.34±0.15	3.10±0.15
IAA	0.50	47	3.40±0.20	4.20±0.11
	1.0	51	3. 51±0.14	4.00±0.07
	0.25	40	3.16±0.33	4.15±0.52
IBA	0.50	60	4.50±0.11	5.07±0.20
	1.0	56	4.07±0.30	4.11±0.15
	0.25	30	2.16±0.30	3.05±0.18
NAA	0.50	40	2.89± 0.07	3.40±0.51
	1.00	**25**	**2.00±0.05**	**2.45±0.10**
		Half strength MS media		
	0.25	60	4.75±0.32	5.64±0.41
IAA	0.50	75	5.13±0.16	6.05±0.30
	1.00	80	5.80±0.10	6.41±0.04
	0.25	90	6.28±0.11	7.10±0.25
IBA	**0.50**	**100**	**6.91±0.50**	**7.60±0.22**
	1.00	75	5.84±0.17	6.75±0.01
	0.25	50	4.33±0.12	5.32±0.30
NAA	0.50	70	4.77±0.19	5.61±0.15
	1.00	65	3.60±0.07	4.55±0.20

* Values are the mean of three replicates each with 15 explants.

For acclimatization, well rooted plantlets were gently removed from the test tubes and thoroughly washed with running tap water to remove traces of medium and transferred to plastic pots having soil and compost (1:1) (Figure1-H). The pots were kept under semi controlled temperature 30±2°C and light (3000 lux) in a growth chamber with 80-85% humidity. Then the plants were transferred to earthen pots filled with soil containing organic manure for further growth. Through this process of acclimatization, regenerated plantlets were established under *ex vitro* conditions. About 97% of plantlets survival under *ex vitro* environment was observed after hardening of *G. procumbens*.

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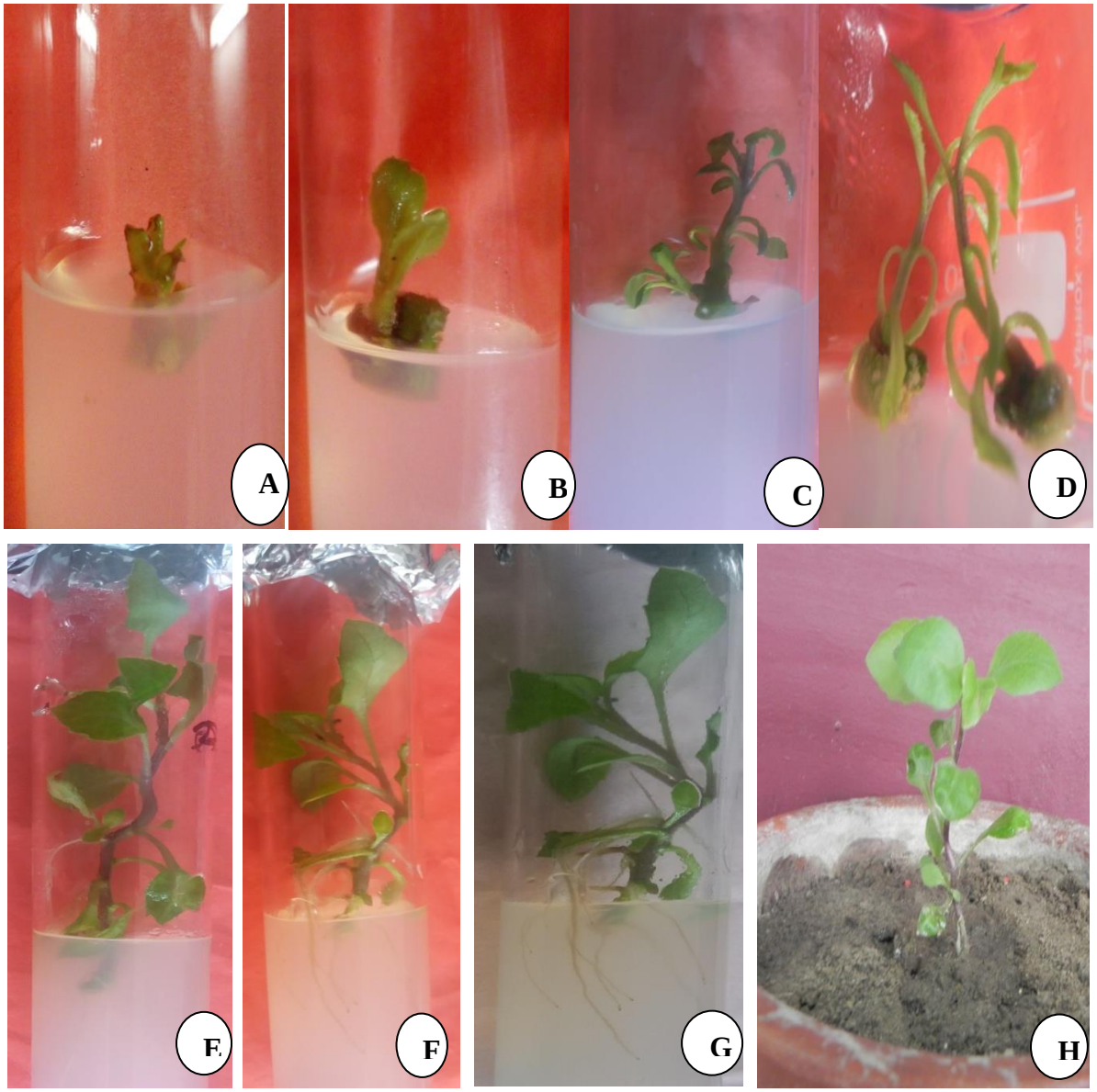

Figure 1. (A-H) Different steps of *in vitro* micropropagation and establishment of *Gynura procumbens* through direct organogenesis: A&B- Multiple shoot buds induction from shoot tips and nodal segments; C,D&E- Elongation of multiple shoot buds ; F&G- Rooting of *in vitro* shoots; H- Hardening and acclimatization of *in vitro* regenerated plants.

CONCLUSION

In conclusion, the present study describes a simple, reproducible and efficient protocol for mass propagation of this important medicinal plant species**.** The protocol can be suitably exploited for mass multiplication on a large scale for commercial production. It would also ensure a continuous supply of plants in limited time and space for this valuable medicinal plant, thereby ruling out the enslavement on natural source to execute the growing demands for the pharmaceutical industry.

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(株) リナアンドルナインターナショナル, 筑西市,日本
ISSN: 2186-8476, ISSN: 2186-8468 Print
www.ajsc. leena-luna.co.jp