IN VITRO PLANT MULTIPLICATION OF *GYNURA PROCUMBENS* AND CHEMICAL ANALYSIS OF REGENERANTS

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Yuwalak Phaidee

IN VITRO PLANT MULTIPLICATION OF _GYNURA PROCUMBENS_ AND CHEMICAL ANALYSIS OF REGENERANTS

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ABSTRACT

Gynura procumbens Merr. is widely used for the treatment of inflammation skin rash, rheumatism, and viral diseases of the skin in traditional medicine. There for, to increase the supply, *in vitro* direct and indirect shoot multiplication techniques were developed in this research. The effects of plant growth regulators (TDZ, BA, NAA, and 2,4-D), duration of culture, and explant types were investigated for multiple shoot induction *via* direct and indirect shoot organogenesis. Multiple shoots were directly induced from node explants cultured on MS with 9.08 μM TDZ for 6 weeks prior to transfer to culture on MS medium without plant growth regulator for 8 weeks. The maximum shoot regeneration rate was 202.50±20.75 shoots/responding explant with a response rate of 100%. An efficient protocol for indirect shoot induction of *G. procumbens* was culturing node explants on MS medium with 8.87 μM BA and 0.45 μM 2, 4-D for 8 weeks prior to transfer to culture on MS medium with 4.44 μM BA for 4 weeks and further culture on MS medium without plant growth regulator for 4 weeks. After the total of 16 weeks of culturing, maximum shoot induction rates of 71.28±18.11 shoots/responding explant with a responding rate of 100% were achieved. Indirect shoot organogenesis of leaf and internode explants was also obtained, however, with lower induction and response rates than those of node explants. Rooting was spontaneously revealed on MS medium without PGR. Rooted plantlets were successfully transferred to a mixture of sand and rice shell ash with 95% survival. The regenerants grew as normal under field conditions. The amounts of stigmasteryl and sitosteryl-3-*O*-β-D-glucopyranosides from 2-5 months-old plant materials were between 0.42 to 0.74 mg/g dried weight (calculation based on stigmasteryl-3-*O*-β-D-glucopyranoside). The established protocols can be used as a guideline for the production of high quality plant material of *Gynura procumbens* for the herbal industry.

KEY WORDS: _GYNURA PROCUMBENS_ / HPLC ANALYSIS / MEDICINAL PLANT / MICROPROPAGATION / ORGANOGENESIS / TISSUE CULTURE

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การขยายพันธุ์ แป๊ะตำปึงโดยวิธีในหลอดทดลอง และการวิเคราะห์ทางเคมีของกล้าเพาะเลี้ยง
(*IN VITRO* PLANT MULTIPLICATION OF *GYNURA PROCUMBENS* AND CHEMICAL ANALYSIS OF REGENERANTS)

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บทคัดย่อ

การพัฒนาวิธีการขยายพันธุ์โดยวิธีในหลอดทดลองสำหรับแป๊ะตำปึง (*Gynura procumbens*) ซึ่งเป็นสมุนไพรที่ใช้ในการรักษาอาการอักเสบ โรคไขข้อ ปวดกล้ามเนื้อ และติดเชื้อไวรัสผิวหนัง โดยวิธีกระตุ้นการสร้างยอดโดยตรงและกระตุ้นการสร้างยอดผ่านแคลลัส ทำการศึกษาผลของสารควบคุมการเจริญเติบโต (TDZ, BA, NAA, และ 2,4-D), ระยะเวลาการเพาะเลี้ยง และชนิดชิ้นส่วนพืช ต่อการกระตุ้นการสร้างยอดใหม่ ในการกระตุ้นการสร้างยอดโดยตรงพบว่าชิ้นส่วนข้อของแป๊ะตำปึงที่วางเลี้ยงในอาหารสูตร Murashige and Skoog (MS) ที่เติม 9.08 µM TDZ นาน 6 สัปดาห์ แล้วย้ายเลี้ยงต่อในอาหารสูตร MS ที่ไม่มีสารควบคุมการเจริญเติบโต นาน 8 สัปดาห์ สร้างยอดเฉลี่ยมากที่สุด 202.50±20.75 ยอดต่อชิ้นส่วน และมีอัตราการตอบสนองต่อการสร้างยอด 100% สำหรับการกระตุ้นการสร้างยอดโดยผ่านแคลลัสจากชิ้นส่วนข้อของแป๊ะตำปึงในอาหาร สูตร MS ที่เติม 8.87 µM BA ร่วมกับ 0.45 µM 2,4-D นาน 8 สัปดาห์ แล้วย้ายเลี้ยงต่อในอาหารสูตร MS ที่เติม 4.44 µM BA นาน 4 สัปดาห์ และอาหารสูตร MS ที่ไม่มีสารควบคุมการเจริญเติบโต อีก 4 สัปดาห์ จำนวนยอดเฉลี่ยสูงสุดคือ 71.28±18.11 ยอดต่อชิ้นส่วน และมีอัตราการตอบสนองการสร้างยอด 100% ชิ้นส่วนใบและปล้องสามารถกระตุ้นให้สร้างยอดใหม่ได้ด้วยวิธีเดียวกันนี้ แต่มีอัตราการสร้างยอดน้อยกว่าชิ้นส่วนข้อ ยอดใหม่ที่ได้สามารถสร้างรากได้เองเมื่อย้ายลงในอาหารสูตร MS ที่ไม่มีสารควบคุมการเจริญเติบโต เมื่อย้ายกล้าเพาะเลี้ยงลงในวัสดุเพาะชำทรายและขี้เถ้าแกลบ ต้นกล้าเพาะเลี้ยงมีอัตรารอดสูงถึง 95% และเจริญเติบโตได้ตามปกติในสภาพแปลงปลูก เมื่อวิเคราะห์ปริมาณของ stigmasteryl-3-*O*-β-D-glucopyranoside และ sitosteryl-3-*O*-β-D-glucopyranoside (Ga) ในกล้าเพาะเลี้ยงอายุ 2-5 เดือน โดยวิธี HPLC พบว่า ในผงแห้งของส่วนใบและลำต้น 1 กรัม มีปริมาณ Ga อยู่ระหว่าง 0.42-0.74 มิลลิกรัม (คำนวณจาก stigmasteryl-3-*O*-β-D-glucopyranoside) จากการวิจัยนี้สามารถกำหนดวิธีการขยายพันธุ์แป๊ะตำปึงโดยวิธีในหลอดทดลองสำหรับผลิตต้นกล้าจำนวนมากและสามารถนำไปใช้เพื่อการผลิตวัตถุดิบในอุตสาหกรรมยาสมุนไพรต่อไป

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ABBREVIATIONS

BA	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
DW	Dried weight
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
KIN	Kinetin
MS	Murashige and Skoog (1962) medium
NAA	α-naphthalene acetic acid
PGR	Plant growth regulator
S.E.	Standard error of mean
S.D.	Standard deviation
TDZ	Thidiazuron
cm	centimeter (s)
m	meter (s)
mg	milligram (s)
ml	milliliter (s)
µl	microliter (s)
µM	micromolar

CHAPTER I
INTRODUCTION

Gynura procumbens Merr. (Asteraceae), a decumbent perennial herb, is widely used as a traditional medicine in Thailand and Southeast Asia. It has been used for treating inflammation, rheumatism, and viral diseases of the skin (1). Stigmasteryl and sitosteryl 3-*O*-β-D-glucopyranosides are active compounds discovered in *G. procumbens* with anti-viral activity against herpes simplex type 1 and 2 (2). Furthermore, the plant demonstrated anti-inflammatory (3), anti-diabetic (4), anti-hyperlipidaemic (4), anti-hypertensive activities (5). Proliferative effect of human lymphocyte *in vitro* (6), and anti tongue-carcinogenic effect (7). Due to antiherpes simplex activity, the herbal industry has been interested in this plant as antiviral herbal medicine. Therefore, the research on production and quality of plant material is warrant.

Micropropagation is an alternative method of vegetative propagation, which is well suited for the multiplication of elite clones. It is accomplished by several means, i.e., direct multiplication of shoots from different explants such as shoot tips or axillary buds or indirect formation of adventitious shoots or somatic embryos *via* callus induction (8). These tissue culture techniques provided a large numbers of uniform regenerated plants which can be used for medicinal plant production. The development of micropropagation in Asteraceous medicinal plants has been reported using direct and indirect shoot regeneration. These include *Artemisia judaica* (9), *Carthamus tinctorius* (10), *Chamomilla recutita* (11), *Cichorium intybus* (12), *Echinacea pallida* (13), *Echinacea purpurea* (14), *Pentanema incicum* (15), *Petasites hybridus* (16), *Santolina canescens* (17), *Saussurea involucrata* (18), *Saussurea obvallata* (19), *Spilanthes acmella* (20), and *Vernonia cineria* (21). Thus micropropagation of *G. procumbens* by using direct and indirect shoot regeneration is well suited for multiplication of elite clones and plant raw material production.

In this thesis, effective protocols for *in vitro* mass propagation of *G. procumbens* using direct and indirect shoot organogenesis were established. The effects of different concentrations of plant growth regulators (auxins and cytokinins), and explant types (node, internode, and leaf) were evaluated. Contents of a mixture of stigmasteryl and sitosteryl-3-*O*-β-D-glucopyranosides (Ga) in the regenerants at different growth stages under field conditions were analysed using HPLC and the calculation based on stigmasteryl-3-*O*-β-D-glucopyranoside.

CHAPTER II
OBJECTIVES

1. To study factors affecting shoot multiplication of *G. procumbens.*
2. To establish protocols for shoot multiplication of *G. procumbens via* direct and indirect shoot regeneration.
3. To analyse the active marker compound content in field-grown micropropagated plants.

CHAPTER III
LITERATURE REVIEWS

1. *Gynura procumbens* Merr.

1.1 Botanical characteristics

Gynura, a small genus of Asteraceae, consists of about 50 species of annual and perennial herbs and subshrubs. The plants are widely distributed in the warm region of Asia, Africa, East Indies, and Australia. *Gynura procumbens* Merr is found in various parts of Asia and widely used in Thailand and Southeast Asia as a traditional medicine (2).

Gynura procumbens (Figure 1), is a decumbent perennial herb; stem angular striate. Leaves spirally arranged; obovate, obovate-oblong or spathulate, 5-8 cm long, 2.5-3.5 cm wide, apex acute or obtuse, base attenuate, margin laxly, irregularly crenate or serrate, venation inconspicuous, pubescent on both surfaces; petiole about 1 cm long, pubescent. Inflorescences in heads, 2-7 in loose terminal corymbs; head consists of more than 60 flowers, 1-1.5 cm long, 7-10 mm wide; peduncle short hairy unequal long; involucral bracts arranged in 2 series, linear, about 8 mm long, 1 mm wide, with translucent margins, during anthesis cohering into a cylindrical tube outer ones 3-5 mm long, linear, free; corolla tubular, orange-yellow, about 8 mm long; stamens 5, style divided into 2 long arms, short hairy; achenes linear, 3-4 mm long, with numerous white pappus-hairs (22).

Figure 1 ***Gynura procumbens*** **Merr.**

A: Whole plant

B: Inflorescence

1.2 Ethnomedical uses

G. procumbens was traditionally used as an anti-inflammatory remedy for skin rash and inching (23, 24). In Thailand, the aerial part is used as a topical therapy for the treatment of inflammation, rheumatism, and viral diseases of the skin (1). In China, the dried leaves were rubbed with oil and mashed as a salve for rash (1) In Indonesia, the aerial and some other parts were used to treat fevers, skin rashes and as a remedy for ringworm infection (3). In some part of Southeast Asia, the leaves of *G. procumbens* have been used as a remedy for kidney diseases, eruptive fevers, rash, hypertension, diabetes mellitus, and hyperlipidemia (1).

1.3 Chemical studies

Compounds which have been isolated and identified from *G. procumbens* include caffeoylquinic acids, flavonoids, phytosterols, phytosterol glucosides, glycoglycerolipids, and ceramindes are shown in table 1 (2).

Table 1 Substances isolated from *G. procumbens* (2).

Group	**Name**
Caffeoylquinic acids	3,5-di-*O*-caffeoylquinic acid 4,5-di-*O*-caffeoylquinic acid 5-*O*-caffeoylquinic acid (chlorogenic acid)
Flavonoids	Kaemferol-3-*O*-alpha-L- rhamnosyl (1→6) β-D- glucopyranoside Kaemferol-3-*O*-alpha-L-rhamnosyl(1→6) β-D-galactopyranoside 3-*O*-β-D-glucopyranosyl-4′,5,7-trihydroxyflovone 3-*O*-β-D-glucopyranosyl-5,7-3′,4′-tetrahydroxyflovone 3,5,7,4′-tetrahydroxyflovone (kaempferol)
Phytosterols	(22E,24S)-24α-ethlcholest-5,22-dien-3β-ol (Stigmasterol) (24S)-24α-ethylcholest-5-ene-3β-ol (Dihydrostigmasterol)
Phytosterol glucosides	3-*O*-β-D-glucopyranosyl-22E,(24S)-24α-ethlycholest-5,22-diene (stigmasteryl glucoside) 3-*O*-β-D-glucopyranosyl (24S)-24α-ethylcholesta-5-ene (sitosteryl glucoside)
Glycoglycerolipids	1,2-bis-dodecanoyl-3-α-D- glucopyranosyl-*sn*-glycerol
Ceramides	1-(1′,5′dihydroxy-docosanyl)-2′′-(2-hydroxy-tetracosanamide)-*sn*- glycerol

A mixture of 3-*O*-β-D-glucopyranosyl-22E, (24S)-24α-ethlcholest-5,22-diene and 3-*O*-β-D-glucopyranosyl (24S)-24α-ethlcholesta-5-ene possessed strong antiviral activity (2). The two compounds differed only in the side chain by one double bond (2). Structure of stigmasterol-3-*O*-β-D-glucopyranoside and sitosteryl -3-*O*-β-D-glucopyranoside are shown in figure 2.

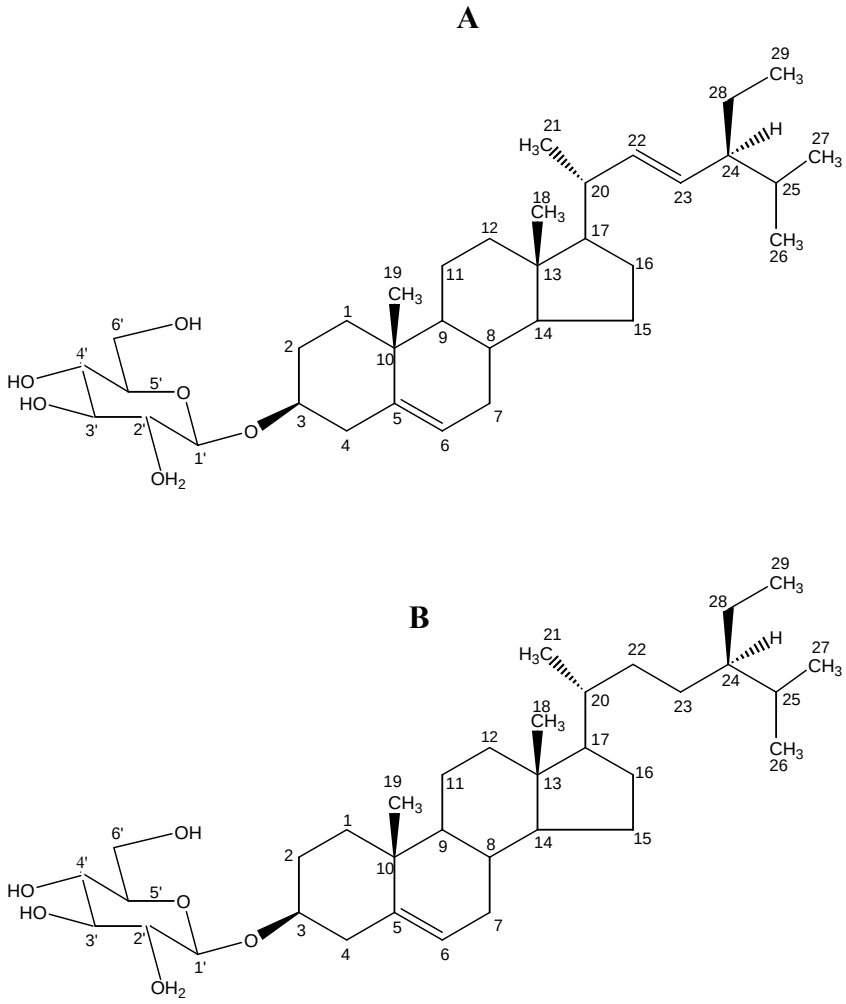

Figure 2 Structure of stigmasterol-3-*O*-β-D-glucopyranoside and sitosteryl –3-*O*-β-D-glucopyranoside (2).

A: 3-*O*-β-D-glucopyranosyl-22E, (24S)-24α-ethlcholest-5, 22-diene ($C_{35}H_{58}O_6$)

B: 3-*O*-β-D-glucopyranosyl-(24S)-24α-ethlcholesta-5-ene ($C_{35}H_{60}O_6$)

1.4 Biological Studies

1.4.1 Anti-inflammatory activity

An ethanolic extract of *G. procumbens* has antiinflammatory action and to relate the activity to particular fractions using a croton oil-induced mouse ear inflammation model. After the extract was partitioned between water and ethyl acetate, and fractioned with a series of solvents, the hexane and toluene fractions showed significant inhibitions of 44.6% and 34.8%, respectively. These two fractions had similar activities to 4 mg/ear of hydrocortisone (inhibition 35.0%) (3).

1.4.2 Antiviral activity

The ethanolic extract of the aerial plant powder of *G. procumbens* showed the antiviral activity against herpes simplex virus type 1 and herpes simplex virus type 2. A mixture of 3,5- and 4,5-di-*O*-caffeoylquinic acid from water-methanol (1:1) fraction showed the virucidal activity against HSV-2 with IC_{50} of 96.0 μg/ml and antireplicative activity against HSV-2 with IC_{50} of 61.0 μg/ml, respectively. Methanol fraction showed antiviral activities in the virucidal and post-treatment test. The isolated compounds include a mixture of β-sitosterol and stigmasterol showed the virucidal activity against HSV-2 with IC_{50} of 250 μg/ml. The compound mixture of β-sitosterol 3-*O*-β-D-glucopyranosyl and stigmasterol 3-*O*-β-D-glucopyranosyl showed the virucidal activity against HSV-2 with IC_{50} of 50 μg/ml and also exhibited pre-treatment anti-HSV-1 and HSV-2 (2).

Table 2 Substances isolated from *G. procumbens* and their antiviral activities (2).

Group	Name	Antiviral activity (IC_{50} µg/ml)
Caffeoylquinic acids	3,5-di-*O*-caffeoylquinic acid	Active Post-treatment (61.0) Virucidal (96.0)
	4,5-di-*O*-caffeoylquinic acid	
	5-*O*-caffeoylquinic acid (chlorogenic acid)	ND
Flavonoids	Kaemferol-3-*O*-alpha-L- rhamnosyl (1→6) β-D- glucopyranoside	Inactive
	Kaemferol-3-*O*-alpha-L-rhamnosyl (1→6) β-D- galactopyranoside	
	3-*O*-β-D-glucopyranosyl-4′,5,7-trihydroxyflovone	Inactive
	3-*O*-β-D-glucopyranosyl-5,7-3′,4′-tetrahydroxyflovone	ND
	3,5,7,4′-tetrahydroxyflovone (kaempferol)	Inactive
Phytosterols	(22E,24S)-24α-ethlcholest-5,22-dien-3β-ol (Stigmasterol)	Active Virucidal (250.0)
	(24S)-24α-ethylcholest-5-ene-3β-ol (Dihydrostigmasterol)	
Phytosterol glucosides	3-*O*-β-D-glucopyranosyl-22E,(24S)-24α-ethlcholest-5,22-diene	Active Pre-treatment (ND) Virucidal (50.0)
	3-*O*-β-D-glucopyranosyl (24S)-24α-ethlcholesta-5-ene	
Glycoglycerolipids	1,2-bis-dodecanoyl-3-α-D- glucopyranosyl-*sn*-glycerol	Active Virucidal (40.0)
Ceramides	1-(1′,5′dihydroxy-docosanyl)-2′′-(2-hydroxy-tetracosanamide)-*sn*- glycerol	Inactive

- Positive control: Aciclovir 50 µg/ml
- \- = inactive at subtoxic concentration (MNYD/2)
  (inhibition of plaque forming < 50%)

- \+ = active but IC_{50} not to be determined
- MNTD = maximum non toxic dose
- NP = not perfomed

Inactivation = Plant extract was incubated with the virus 1 h before the infection of monolayer cell line (test for virucidal action)

Pre-treatment = Plant extract was added to the monolayer cell line 24 h before virus infection (test for prevention of viral adsorption and /or replication)

Post-treatment = Plant extract was added to the monolayer cell line 1 h after virus infection (test for prevention of viral replication)

1.4.3 Antihyperglycaemic and anti-hyperlipidaemic activities

An ethanolic leaf extract, at single doses of 50, 150 and 300 mg/kg orally, significantly suppressed the elevated serum glucose levels in diabetic rats; 150 mg/kg was found to be the optimum hypoglycaemic dose. The extract however did not significantly suppress the elevated serum glucose levels in normal rats, unlike glibenclamide. When the optimum dose was given to diabetic rats for 7 days, the extract significantly reduced serum cholesterol and triglyceride levels in these rats (4).

1.4.4 Antihypertensive activity

An aqueous extract of *G. procumbens* was tested to spontaneously hypertensive rats for 4 weeks. Oral administration of 500 mg/kg of *G. procumbens* extract resulted in significantly lower blood pressure in the hypertensive rats compared to a control group ($P<0.05$). Furthermore, the rats received the extract had significantly lower serum lactate dehydrogenase, creatine phosphate kinase, and increased nitric oxide, a known vasodilator, compared with the control group ($P<0.05$). The results suggest that oral administration of the aqueous etract of *G. procumbens* may be useful for prevention and treatment of hypertension through increasing NO production in blood vessels (5).

1.4.5 Human lymphocyte activity

Sriwanthana et al. reported that an aqueous extract from leaves of *G. procumbens* at the concentrations of 1-100 μg/ml increased human lymphocyte proliferation *in vitro* (6).

1.4.6 Anticarcinogenic activity

An ethanolic extract of *G. procumbens* leaves could inhibit the progression of 4 nitroquinoline 1-oxide (4 NQO) induced rat tongue carcinogenesis in the initiation phase (7).

1.4.7 Anti-proliferative on human mesangial cell

An aqueous extract of *G. procumbens* inhibits mesangial cell proliferation and the inhibition may be mediated by the suspression of platelet-derived growth factor (PDGF-BB) and transforming growth factor (TGF-β1) expression and the modulation of cyclin-dependent kinase1 and cyclin-dependent kinase 2 expression (25).

2. *In vitro* propagation of medicinal plant

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells. Tissue culture is alternatively called cell, tissue and organ culture through *in vitro* condition. It can be employed for large-scale propagation of disease-free clones and gene pool conservation (8). *In vitro* propagation offer many advantages over the conventional propagation methods. It comprises two main types:

1. Direct morphogenesis which produced the regenerated plants from tissue or organs (explants) removed from the mother plants. It provides uniform plants with genetic identity (26).

2. Indirect morphogenesis which produced the regenerated plants from unorganized cell (suspension) or callus established on the explants. It may result in somaclonal variation (26).

The amounts and kinds of plant growth regulators which are included in the culture medium largely determine the success of the micropropagation. Root and shoot initiation, and the process of differentiation from unorganised callus tissue, are closely regulated by the relative concentrations of auxins and cytokinins in the medium. An auxin is required by most plant cells for the division and root initiation. At high concentrations, auxin can suppress morphogenesis. Cytokinins promote cell

division, shoot proliferation, and shoot morphogenesis. Moreover, an explant can develop a callus as a wound response that consists of unorganized, dividing cells (27).

There are a number of researches on *in vitro* propagation of Asteraceous medicinal plants. Different types and concentration of auxins and cytokinins and various explants types were summarized in tables 3 and 4.

Table 3 Direct shoot regeneration of Asteraceous medicinal plants.

Plant	Explant source	Media	Response	Ref.
Cichorium intybus	Le	MS+ 2.32μM KIN+ 1.71μM IAA MS+ 0.046μM TDZ+ 5.71μM IAA MS+ 2.85μM IAA	Shoots formation Roots formation	(12)
Eclipta alba	No	MS+ 4.44μM BA MS+ 2.22μM BA+ 1.44μM GA MS+ 4.9μM IBA	Shoots formation Roots formation	(28)
Petasites hybridus	Le, Pe, Ib	MS+ 17.6μM BA+ 0.54μM NAA MS+ 8.8μM KIN+ 0.54μM NAA	Shoots formation Shoots formation Roots formation	(16)
Santolina canescens	Sh	MS+ 1.33μM BA+ 0.32μM NAA MS+ 2.68μM NAA	Shoots formation Roots formation	(17)
Spilanthes acmella	Ab	MS+ 8.87μM BA MS+ 2.22μM BA	Shoots formation Shoots formation Roots formation	(29)
Spilanthes acmella	No	MS+ 3.33μM BA MS+ 4.90μM IBA	Shoots formation Roots formation	(20)
Stevia rebaudiana	Sh, No, Le	MS+ 8.87μM BA+ 5.7μM IAA 1/2MS+ 4.90μM IBA	Shoots formaton Roots formation	(30)
Typhonium flagelliforme	Bd	MS+ 1.33μM BA + 2.46μM IBA MS+ 0.54-5.37μM NAA	Shoots formation Roots formation	(31)
Vernonia cinerea	No	MS+ 20.9μM BA MS (PGR-free)	Shoots formation Roots formation	(21)

Ab: Axillary bud, Bd: Bud, Ib: infloresence bud, Le: leaf, No: Node, Pe: Petiole, Sh: Shoot tip
BA: 6-benzylaminopurine, GA:Giberellic acid, KIN: Kinetin, NAA: α-naphthalene acetic acid, IAA: Indoleacetic acid, IBA: Indole-3-butyric acid, TDZ: Thidiazuron, MS: Murashige and Skoog (1962) media

Table 4 Indirect shoot regeneration of Asteraceous medicinal plants.

Plant	Explant source	Media	Response	Ref.
Artemisia judaica	Hy	MS+ 1.00µM TDZ MS+ 1.00µM IBA	Callus formation Shoots formation Roots formation	(9)
Carthamus tinctorius	Hy, Co, Ro, Le	MS+ IAA + NAA + BA + KIN MS+ 4.43µM BA MS+ 2.8-5.7µM IAA	Callus formation Shoots formation Roots formation	(10)
Chamomilla recutita	Fl (ray and disk)	MS+ 8.87µM BA + 1.07 µM NAA MS+ 26.8µM NAA+ 11.5µM KIN MS+ 8.87µM BA+ 1.07µM NAA	Callus formation Shoots formation	(11)
Cichorium intybus	Le	MS+ 2.0µM IAA + 5.0µM BA+ CH MS+ 2.0µM IBA	Callus formation Shoots formation Roots formation	(12)
Echinacea pallida	Le	MS+ 26.6µM BA + 0.11µM NAA MS (PGR-free) or MS+ 0.49-9.80µM IBA	Callus formation Shoots formation Roots formation	(13)
Echinacea purpurea	Le	MS+ 4.44µM BA+ 0.054µM NAA MS+ 2.46 or 4.90 µM IBA	Callus formation Shoots formation Roots formation	(14)
Pentanema indicum	Le, St	MS+ 4.44 µM BA + 4.90µM IBA MS+ 17.76µM BA + 4.90µM IBA MS+ 9.80µM IBA	Callus formation Shoots formation Roots formation	(15)
Saussurea involucrata	Le	MS+ BA + NAA MS+ 10µM BA+ 2.5µM NAA MS+ 2.5µM IAA	Callus formation Shoots formation Roots formation	(18)
Saussurea obvallata	Le	MS+ 2.5µM BA + 1.0µM NAA MS+ 5.0µM BA + 1.0µM NAA ½MS+ 2.5µM IBA	Callus formation Shoots formation Roots formation	(19)

Table 4 Indirect shoot regeneration of Asteraceous medicinal plants (Continue).

Plant	Explant source	Media	Response	Ref.
Spilanthes acmella	No	MS+ 8.87μM BA + 5.71μM IAA MS+ 2.46μM IBA	Callus formation Shoots formation Roots formation	(20)
Vernonia cinerea	Le	MS+ (0.418-41.8μM) BA + (0.53-13.4μM) NAA or IAA MS+ 20.9μM BA+ 5.3μM NAA MS (PGR-free)	Callus formation Shoots formation Roots formation	(21)

Co: Cotyledon, Fl: Flower, Hy: Hypocotyl, Le: Leaf, No: Node, Ro: Root, St: Stem
BA: 6-benzylaminopurine, CH: casein hydrolysate, IAA: Indoleacetic acid, IBA: Indole-3-butyric acid, KIN: Kinetin, NAA: α-naphthalene acetic acid, TDZ: Thidiazuron, MS: Murashige and Skoog (1962) media

The effect of several plant growth regulators and the different types of explants are responded to callus, shoots, and roots formation. Node, leaf, petiole, shoot tip, and axillary bud were used in direct shoot multiplication. For indirect shoot multiplication was induced via callus on hypocotyl, leaf, cotyledon, root, flower, and node explants.

In direct shoot formation experiments, BA, KIN, TDZ alone or BA in combination with GA, NAA, IAA or IBA were used (12, 16, 17, 20, 21, 28-31). For indirect organogenesis, calli were induced using combinations of auxins (NAA, IAA, and IBA) and cytokinins (BA or KIN) or TDZ alone (9-15, 18-21). Shoots were induced by transferred the calli to media with increased proportion of cytokinins or cytokinin alone (10, 11, 15).

The use of tissue culture technology for the vegetative propagation of plants is the most widely used application of the technology. Micropropagation can be realized by somatic embryogenesis and cultivation of shoot tips or axillary buds and adventitious shoots. Through plant micropropagation, high quality seedlings which are virus-free and disease-resistant, and high yields of medicinal components can be attained. The development and application of micropropagation techniques for medicinal plants are importance for preservation, rapid production and study of valuable herb species. Micropropagation is an alternative method of commercial propagation and is being used widely for the commercial propagation of a large

number of plant species. It is well suited for the multiplication of elite clones. In addition, these techniques are an important role in the production for plant cultivation and quality of medicinal plant raw materials (8, 27, 32).

3. Chemical analysis of plants regenerated from *in vitro* propagation

Chromatography is a method in which the components of mixture are separated on an adsorbent column in a system. The absorbent material, or stationary phase has taken many forms including paper, thin layers of solids attached to glass plates, immobilized liquids, gels, and solid particles packed in columns. The flowing component of the system, or mobile phase, is either a liquid or gas. Concurrent with development of the different absorbent materials has been the development of methods more specific to particular classes of analyses (33).

High-performance liquid chromatography (HPLC) is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped though a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and detector (33).

It is used extensively in the pharmaceutical industry in applications ranging from content uniformity assays to pharmacokinetic studies. The chemical industry relies on HPLC in the quality control of raw materials, intermediates, and finished products. In environmental laboratories, HPLC is used for analysis of pesticides and other contaminants in soil and water. Applications of HPLC in the food industry include the analysis of pesticide residues or the content of nutrients or additives (34).

HPLC is a powerful technique for fingerprinting biologically active extracts and comparisons can be drawn with chromatograms and UV spectra stored in an electronic library. This is currently very important for the quality control of herbal medicines for with appropriate standards in reproducibility of extra quality must be met. It is the method of choice for the pharmaceutical industry because of its excellent separating power, speed and reproducibility (35).

The phytochemical profile of micropropagated plants was similar to that of intact plants of *Catalpa ovata* (36) and *Saussurea involucrata* (18). Moreover, the amounts of active compounds of micropropagated plants were higher than that of intact plants in *Hyacinthus orientalis* (37), *Cichorium intybus* (38), *Scrophularia yoshimurae* (39), and *Solanum nigrum* (40).

CHAPTER IV
MATERIALS AND METHODS

1. Materials

1.1 Plant materials

Gynura procumbens plants were provided by Siriruckhachati medicinal plant garden, Faculty of Pharmacy, Mahidol University. They were grown in pots under greenhouse conditions. The plants were used as mother plants for tissue culture experiment. For a field experiment, *in vitro* regenerated plants were used as plant seedlings.

1.2 Materials used for tissue culture experiment

Chemicals

- 95% Ethanol, commercial grade (Merck)
- Distilled water (BDH)
- Sodium hypochlorite solution (13%) (BDH)
- Sodium hydroxide (J.T. Baker)
- Tween® 80 (Merck)
- Ammonium nitrate (NH_4NO_3) (Merck)
- Potassium nitrate (KNO_3) (Merck)
- Calcium chloride dihydrate ($CaCl_2.2H_2O$) (Merck)
- Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Merck)
- Potassium dihydrogen phosphate (KH_2PO_4) (Merck)
- Manganese (II) sulfate monohydrate ($MnSO_4.\ H_2O$) (Merck)
- Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) (Merck)
- Boric acid (H_3BO_3) (Merck)
- Potassium iodide (KI) (Merck)

- Sodium molybdate dihydrate ($Na_2MoO_4.2\ H_2O$) (Merck)
- Copper (II) sulfate pentahydrate ($CuSO_4.5\ H_2O$) (Merck)
- Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$) (Merck)
- Pyridoxine hydrochloride ($C_8H_{11}NO_3.HCl$) (Sigma)
- Nicotinic acid ($C_6H_5NO_2$) (Sigma)
- Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS.HCl$) (Sigma)
- Glycine ($C_2H_5NO_2$) (Sigma)
- Iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) (Merck)
- Sodium EDTA ($C_{10}H_{14}N_2Na_2O_8.\ 2H_2O$) (Merck)
- Myo-inositol ($C_6H_{12}O_6$) (Sigma)
- Sucrose (Merck)
- Agargel® (Sigma)
- BA (6-benzylaminopurine) (Sigma)
- NAA (α-naphthalene acetic acid) (Sigma)
- TDZ (N-phenyl-N-1,2,3-thiadiazol-5-ylurea (Sigma)
- 2,4-D (2,4-dichlorophenoxyacetic acid) (Sigma)

Apparatuses

- Balance model AG204 (Mettler toledo®)
- pH meter model MP220 (Mettler toledo®)
- Magnetic stirrer model AGIMATIC-E (Selecta®)
- Autoclave model SS-325 (Tomy®)
- Laminar air flow cabinet model H1 (Clean®)
- Refrigerator (Mitsubishi®)

1.3 Materials used for field cultivation and harvesting

- Name tags
- Measuring tools
- Cultivated trays
- Sand and rice shell ash (1:1)
- Mixed soil

1.4 Materials used for chemical analysis

Chemicals

- A mixture of stigmasteryl 3-*O*- β-D- glucopyranoside and sitosteryl 3-*O*-β-D-glucopyranoside (40:60) (Ga) was provided by Assoc. Prof. Dr. Weena Jiratchariyakul, Department of Pharmacognosy, Faculty of pharmacy, Mahidol University
- Methanol, AR grade (Burdick & Jackson)
- Water, Sterile water for injection

Apparatuses

- Mill Type MM 200 (Retsch®)
- Hot air oven (Memmert®)
- Pipetman (Gilson Medical Electronic, France)
- Milliopore membrane filter (0.45 μm) (Millipore®)
- Ultrosonicate bath Sonorex, Germany)
- High Performance Liquid Chromatography (Shimadzu)
  - Column 4.6 x 250 mm 5 μm HYPERSIL® BDS C18
  - Pump (LC-10AD)
  - System controller (SCL-10A)
  - Detector (SPD-10AV)
  - Auto injector (SIL-10AD)

2. Methods

2.1 *In vitro* propagation of *Gynura procumbens*

2.1.1 Basic media and media preparation

The composition of basic media based on MS medium as shown in table 5. A stock solution of microelements excluding $FeSO_4.7H_2O$ and Na_2EDTA was prepared at 100-fold concentration. Organic components (except myo-insitol) were prepared as 500 or 2000-fold concentrates, and the solutions were stored at 4 ºC.

A double concentrated MS stock solution without iron was prepared by dissolving macronutrients and myo-inositol one by one and adding the stock solutions of microelements and organic compounds. After adjusting volume, the stock solution was divided into portions of 500 or 1,000 ml and stored at -20 ºC.

A solution of Fe-EDTA chelate (x100) was prepared by dissolving $FeSO_4.7H_2O$ (2.78 mg/l) and Na_2EDTA (4.1 mg/l) separately in water. The solutions were heated, mixed together, cooled, volume adjusted, and stored in the dark at 4 ºC.

Plant growth regulators were brought into solution in a few drops in 1N NaOH. The volume was adjusted by water. The stock solutions were kept in a refrigerator (4 ºC) until required but not longer than 1 month.

For experiment, media were prepared by dissolving sucrose (3%) in water, adding the MS stock solution, the Fe-EDTA solution, and aliquots of plant growth regulator stock solution. Agargel® (0.55%) was used as a gelling agent. All media were adjusted pH to 5.8 and dispensed to the bottles before autoclaving.

Table 5 Composition of MS medium

Compounds	mg/l
Macroelements	1,650
Ammonium nitrate (NH_4NO_3)	1,900
Potassium nitrate (KNO_3)	440
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	370
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	170
Potassium dihydrogen phosphate (KH_2PO_4)	
Microelements	
Iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$)	27.8
Sodium EDTA ($C_{10}H_{14}N_2Na_2O_8.\ 2H_2O$)	41.0
Boric acid (H_3BO_3)	6.2
Manganese (II) sulfate monohydrate ($MnSO_4.\ H_2O$)	16.9
Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	8.6
Potassium iodide (KI)	0.83
Sodium molybdate dihydrate ($Na_2MoO_4.2\ H_2O$)	0.25
Copper (II) sulfate pentahydrate ($CuSO_4.5\ H_2O$)	0.025
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	0.025
Organic substances	
Myo-inositol ($C_6H_{12}O_6$)	100
Glycine ($C_2H_5NO_2$)	2.0
Nicotinic acid ($C_6H_5NO_2$)	0.5
Pyridoxine hydrochloride ($C_8H_{11}NO_3.HCl$)	0.5
Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS.HCl$)	0.1

2.1.2 Surface sterilization

G. procumbens shoots of 20-cm length were immersed in 70% ethanol solution for 1 minute and washed through in running tap water for 30 seconds, then sterilized for 20 minutes in 1,000 ml of 2% sodium hypochlorite solution containing 100 µl of Tween® 80. After pouring out the solution, explants were rinsed 3 times with sterile distilled water. The sterile plant material was used as initial explants. One week after inoculation, the microbial contamination was observed (Figure 3).

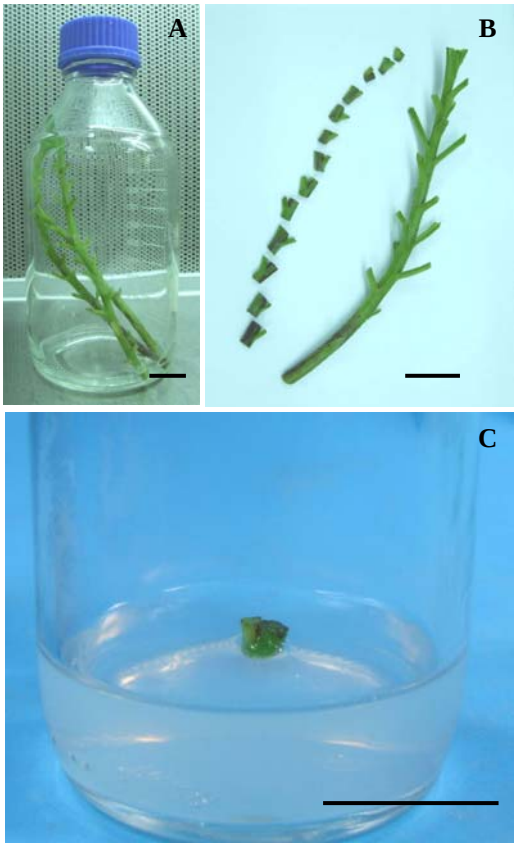

Figure 3 Surface sterilization of *G. procumbens*

(A) Shoots after surface sterilization, (B) Sterilized shoots were excised into a single node explant, (C) A node explant on MS medium without PGR. Scale bars: 2 cm

2.1.3 Direct shoot regeneration experiments

2.1.3.1 Mother plants

In direct shoot regeneration experiments, explants were derived from two types of mother plant, *in vivo* and *in vitro*.

- *In vivo* mother plant

The sterilized shoots of *G. procumbens* were dissected into single-node explants of 1 cm length and used as initial explants for experiment 1.

- *In vitro* mother plant

In vitro shoots of *G. procumbens* obtained from the experiment 1 were dissected into single-node explants of 0.5-1 cm length and used as initial explants in experiments 2-3.

2.1.3.2 Plant growth regulator treatments

- Shoot Induction Media

Experiment 1: The effects of BA (4.44-35.52 μM) alone and in combination with 2.69 μM NAA on shoot regeneration were examined. To determine an optimum duration of PGR incubation, the experiments 1.1 and 1.2 were performed to compare the periods of 4 and 6 weeks in PGR-supplemented MS media.

Experiments 2 and 3: To determine an optimum concentration of plant growth regulators for direct shoot regeneration, various concentrations of BA (4.44-71.04 μM) and TDZ (4.54-72.64 μM) alone and in combination with 2.69 μM NAA were tested.

- Shoot Proliferation Medium

Experiment 1: After 4 or 6 weeks of PGR-treated cultures, regenerated shoots were excised into a single shoot and inoculated on MS medium without plant growth regulators for 6 and 4 weeks in experiments 1.1 and 1.2, respectively.

Experiments 2 and 3: Six weeks after inoculation, clusters of regenerated shoots were transferred on MS medium without PGR for 4 weeks. Then,

the regenerated shoots were separated into single shoots and transferred to culture on MS medium without PGR for further 4 weeks.

2.1.3.3 Replication and observation

A total number of 10-15 replications with one explant were used for each treatment in all experiments. The frequency of explants responding to form shoots, number of regenerated shoots per responding explant, shoot length, rooting, and browning were observed.

The methodical details of experiments 1-3 are shown in table 6

Table 6 Mother plants, explant type, plant growth regulators, and culture duration used in the direct shoot regeneration experiments.

Experiment	Mother plant	Explant type	Media	Plant growth regulator (μM)									Culture duration (weeks)
				I	II	III	IV	V	VI	VII	VIII	IX	
1.1 Effect of BA and NAA	*In vivo*	node	Shoot Induction Media	BA 4.44	BA 8.87	BA 17.76	BA 35.52	BA 4.44 NAA 2.69	BA 8.87 NAA 2.69	BA 17.76 NAA 2.69	BA 35.52 NAA 2.69	PGR-free	4
			Shoot Proliferation Medium	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	6*
1.2 Effect of BA and NAA	*In vivo*	node	Shoot Induction Media	BA 4.44	BA 8.87	BA 17.76	BA 35.52	BA 4.44 NAA 2.69	BA 8.87 NAA 2.69	BA 17.76 NAA 2.69	BA 35.52 NAA 2.69	PGR-free	6
			Shoot Proliferation Medium	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	4*

* The regenerated shoots were separated into a single shoot and transferred to culture on PGR-free MS medium.

Table 6 Mother plants, explant type, plant growth regulators, and culture duration used in the direct shoot regeneration experiments (Continue).

Experiment	Mother plant	Explant type	Media	Plant growth regulator (μM)											Culture duration (weeks)
				I	II	III	IV	V	VI	VII	VIII	IX	X	XI	
2. Effect of BA and NAA	*In vitro*	node	Shoot Induction Media	BA 4.44	BA 8.87	BA 17.76	BA 35.52	BA 71.04	BA 4.44 NAA 2.69	BA 8.87 NAA 2.69	BA 17.76 NAA 2.69	BA 35.52 NAA 2.69	BA 71.04 NAA 2.69	PGR-free	6
			Shoot Proliferation Medium	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	4* +4**
3. Effect of TDZ and NAA	*In vitro*	node	Shoot Induction Media	TDZ 4.54	TDZ 9.06	TDZ 18.16	TDZ 36.32	TDZ 72.64	TDZ 4.54 NAA 2.69	TDZ 9.06 NAA 2.69	TDZ 18.16 NAA 2.69	TDZ 36.32 NAA 2.69	TDZ 72.64 NAA 2.69	PGR-free	6
			Shoot Proliferation Medium	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	4* +4**

* The clusters of regenerated shoots were transferred to PGR-free MS medium.

** The regenerated shoots were separated into a single shoot and transferred to new medium with the same compositions.

2.1.4 Indirect shoot regeneration experiments

2.1.4.1 Mother plants and explant types

In vitro shoots of *G. procumbens* from the experiment 1 were dissected into single node and internode explants of 1 cm length and leaf explants (approximately 0.5 x 0.5 cm in size) were used as explants in experiments 4-6.

The effects of explant types (node, internode and leaf) on callus induction were examined in experiment 4. For experiments 5-6, internode and leaf explants were used as explant for callus induction.

2.1.4.2 Plant growth regulator treatments

- Callus Induction Media

The explants were inoculated on MS medium supplement with different plant growth regulators (2,4-D, BA, TDZ and NAA).

Experiment 4: Concentrations of BA (4.44 and 8.87 μM) and 2,4-D (0.45-9.05 μM) were tested for callus induction.

Experiments 5 and 6: The effects of BA (4.44-71.04 μM) and TDZ (4.54-72.64 μM) alone and in combination with 2.69 μM NAA on callus induction of internode and leaf explants were examined.

- Indirect Shoot Proliferation Medium I and II

To induce shoots, green compact calli were transferred to MS media supplemented with 4.44 μM BA (Indirect Shoot Proliferation Medium I) for 4 weeks.

Then, the regenerated shoots were excised into a single shoot and transferred to MS medium without plant growth regulators (Indirect Shoot Proliferation Medium II) for 4 weeks.

The methodical details of experiments 4-6 are shown in table 7.

2.1.4.3 Replication and observation

In experiments 4-6, a total number of 8-15 replications with 1 explant were used for each treatment. The frequency of explant responding to callus

formation, shoot formation, number of regenerated shoots per explant, shoot length, rooting, and browning were observed.

To evaluate the callus formation, callus intensity was recorded in 4 classes according to the following: 0 = none, 1 = little, 2 = medium, 3 = maximum (Figure 4).

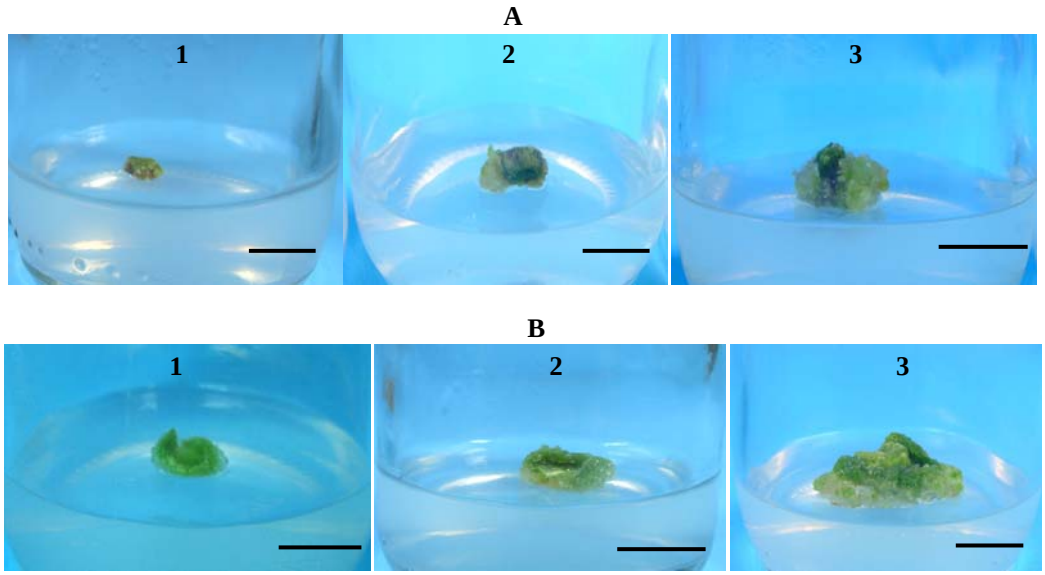

Figure 4 Three classes of callus intensity in internode and leaf explants
(A) Internode explant, (B) Leaf explant, Scale bars = 1 cm

Table 7 Mother plants, explant types, plant growth regulators, and culture duration used in the indirect shoot regeneration experiments.

Experiment	Mother plant	Explant type	Media	Plant growth regulator treatment (μM)									Culture duration (weeks)
				I	II	III	IV	V	VI	VII	VIII	IX	
4. Effect of BA and 2,4-D	*In vitro*	Node, internode, and leaf	Callus induction	BA 4.44 2,4-D 0.45	BA 4.44 2,4-D 2.26	BA 4.44 2,4-D 4.52	BA 4.44 2,4-D 9.05	BA 8.87 2,4-D 0.45	BA 8.87 2,4-D 2.26	BA 8.87 2.4-D 4.52	BA 8.87 2,4-D 9.05	PGR-free	8
			Indirect Shoot Proliferation I	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	4
			Indirect Shoot Proliferation II	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	4

Table 7 Mother plants, explant types, plant growth regulators, and culture duration used in the indirect shoot regeneration experiments (Continue).

Experiment	Mother plant	Explant type	Media	Plant growth regulator treatment (μM)											Culture duration (weeks)
				I	II	III	IV	V	VI	VII	VIII	IX	X	XI	
5. Effect of BA and NAA	*In vitro*	Internode and leaf	Callus induction	BA 4.44	BA 8.87	BA 17.76	BA 35.52	BA 71.04	BA 4.44 NAA 2.69	BA 8.87 NAA 2.69	BA 17.76 NAA 2.69	BA 35.52 NAA 2.69	BA 71.04 NAA 2.69	PGR-free	4
			Indirect Shoot Proliferation I	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	4
			Indirect Shoot Proliferation II	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	4
6. Effect of TDZ and NAA	*In vitro*	Internode and leaf	Callus induction	TDZ 4.54	TDZ 9.06	TDZ 18.16	TDZ 36.32	TDZ 72.64	TDZ 4.54 NAA 2.69	TDZ 9.06 NAA 2.69	TDZ 18.16 NAA 2.69	TDZ 36.32 NAA 2.69	TDZ 72.64 NAA 2.69	PGR-free	4
			Indirect Shoot Proliferation I	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	BA 4.44	4
			Indirect Shoot Proliferation II	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	PGR-free	4

2.1.5 Culture conditions

In all experiments, the cultures were maintained in growth room at 25ºC under 16/8-h (light/dark) photoperiod of 37.5 µE $m^{-2}s^{-1}$.

2.1.6 Acclimatization

The regenerated plants from experiment 2 were placed in temperature room for 3-5 days. Then, they were washed and placed into cultivation trays containing sand and rice shell ash (1:1). The plants were grown in the acclimatized chamber under greenhouse conditions for 6 weeks. These plants were used as seedlings for a field experiment.

2.2 Field experiment for chemical analysis of the regenerants

2.2.1 Field experiment

The regenerants were grown in the plot (3 x 9 m), spaced at 45 x 45 cm at Siriruckhachati medicinal plant garden, Salaya Campus, Mahidol University. The plantlets were transplanted on November, 2006 and grown until April, 2007 for five months. Every month, eight plants were harvested. Observations were recorded on plant height, diameter, leaf size, number of leaves and flowers, fresh weight, and dry weight.

2.2.2 HPLC analysis

Aerial parts (leaves and stems) of plant materials were washed and dried in hot-air oven at 50 ºC for 48 h and ground into powder with a Retsh® mill (Type MM 200). A total of 800 mg of powdered drug was sonicated with 9 ml of methanol for 15 min and the volume adjusted in 10-ml volumetric flask. Each plant extract was filtered through a 0.45-µm milliopore membrane filter. A 20 µl volume of this extract was injected into a reversed-phase HPLC system. All analyses were conducted with a Shimadzu HPLC system consisting of a series LC-10AD pump and a series SPD-10 AV detector. The separation was carried out using a Hypersil® BDS C18 column (4.6 mm x 250 mm) with mobile phase consisting of methanol and water (100:5). UV

detection was at 210 nm. A mixture of stigmasteryl and sitosteryl-3-*O*-β-D-glucopyranoside in the ratio of 40:60 was used as external standard (24).

CHAPTER V

RESULTS

1. *In vitro* propagation of *Gynura procumbens*

1.1 Surface sterilization

The sterilization protocol by treating *G. procumbens* shoots with 2% sodium hypochlorite for 20 minutes was effective. One week after inoculation, the frequencies of bacterial and fungal contaminations of sterilized explants were only 0.74% and 7.41%, respectively.

1.2 Direct shoot regeneration

Two different sources of mother plants, *in vivo* and *in vitro*, were used. *In vivo* mother plants were used in BA and NAA treatments (experiment 1), and *in vitro* mother plants were used in BA, TDZ, and NAA treatments (experiments 2 and 3).

In the experiment 1, *G. procumbens* node explants from *in vivo* mother plant were incubated on MS medium supplemented with BA (4.44-35.52 µM) alone and combination with 2.69 µM NAA for 4 and 6 weeks in experiment 1.1 and 1.2, respectively. Then they were transferred to culture on MS medium without PGR for 6 and 4 weeks in experiment 1.1 and 1.2, respectively.

The experiment 1.1 demonstrated that the frequency of explant responding to shoot formation in all treatments was 100% within the first 4 weeks of culture. Four weeks after culture initiation, the maximum shoot regeneration rate was 12.69±1.83 shoots/response explant with average shoot length of 0.73±0.05 cm obtained when the explants were cultured on MS with 35.52 µM BA. Among the BA and NAA treatments, the maximum shoot regeneration rate was 11.93±0.66 shoots/response explant with an average length of 0.86±0.06 cm form the explants cultured on MS with 35.52 µM BA and 2.69 µM NAA (Figure 5A and 6A).

After 4 weeks of culture on MS with PGR, the regenerated shoots were separated into single shoots and transferred to MS medium without PGR for further 6 weeks. The shoot regeneration rates and average shoot lengths increased in all treatments. The maximum shoot regeneration rate increased more than two times upto 26.85±5.06 shoots/response explant with average shoot length of 3.49±0.23 cm obtained when the explants were cultured on MS with 35.52 µM BA. For the combination treatments, the best regeneration rates of 17.18±2.74 shoots/response explant (3.19±0.24 cm shoot length) and 17.13±1.12 shoots/response explant (2.64±0.15 cm shoot length) obtained from MS with BA (17.76 and 35.52 µM) in combination of 2.69 µM NAA, respectively (Figure 5B and 6B).

In the experiment 1.2, the duration in shoot induction media was increased from 4 weeks to 6 weeks. The frequency of explant responding to shoot formation was also 100%. The maximum shoot regeneration rate revealed from MS with 35.52 µM BA was 25.50±4.03 shoots/response explant with average shoot length of 0.81±0.07 cm. In the combination treatments, the shoot regeneration rate of 26.60±4.13 shoots/response explant was obtained from the explant cultured on 17.76 µM BA and 2.69 µM NAA, whereas, the average shoot length was 1.40±0.12 cm (Figure 7A and 8A).

After 6 weeks of culture on MS with PGR, the regenerated shoots were separated into single shoots and transferred to MS medium without PGR for further 4 weeks. All treatments, the multiple shoots and shoot length increased. MS with BA 35.52 µM was the optimum medium revealed the highest shoot regeneration rate of 52.80±10.00 shoots/response explant with 2.34±0.17 cm/shoot. For the combination treatments, the shoot multiplication rates of 38.70±10.96 shoots/ response explant (2.63±0.22 cm/shoot) were obtained at high concentration of 35.52 µM BA combination with 2.69 µM NAA (Figure 7B and 8B).

No rooting was observed in all treatments, therefore, the regenerated shoots were cut into single shoots and inoculated on MS medium without PGR. The rooting was observed after 2 weeks of culture.

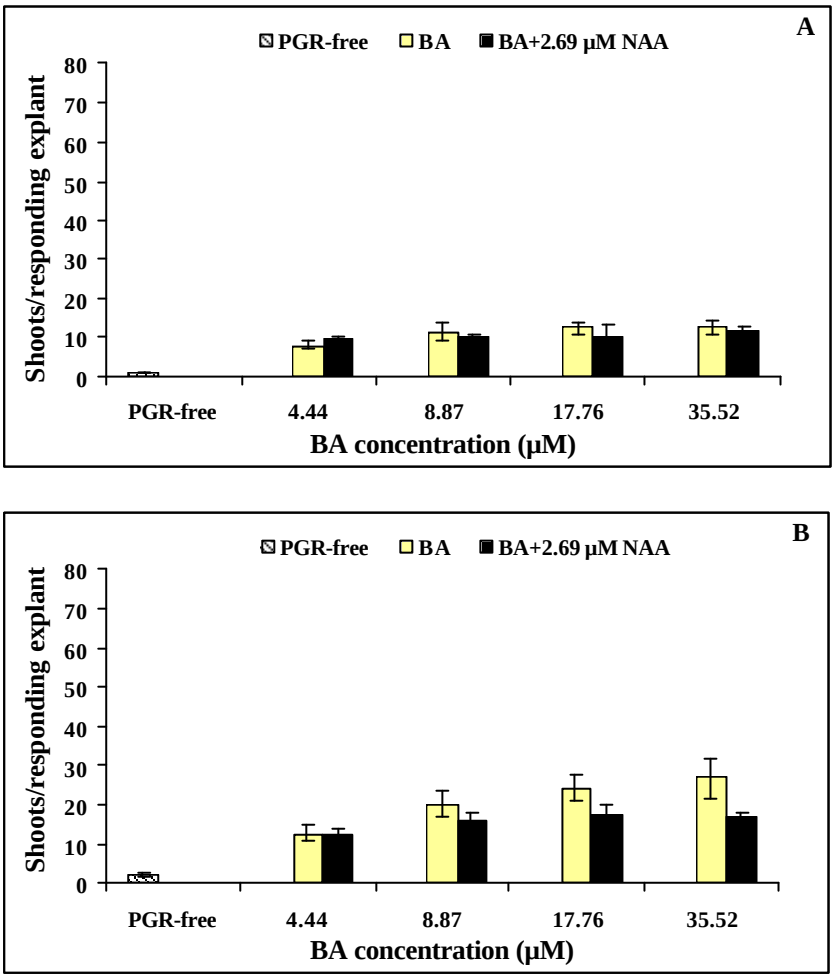

Figure 5 Effect of BA concentrations (4.44-35.52 µM) and 2.69 µM NAA on shoot induction of *in vivo G. procumbens* node explants. (A) Four weeks after inoculation on MS with PGR, (B) Six weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

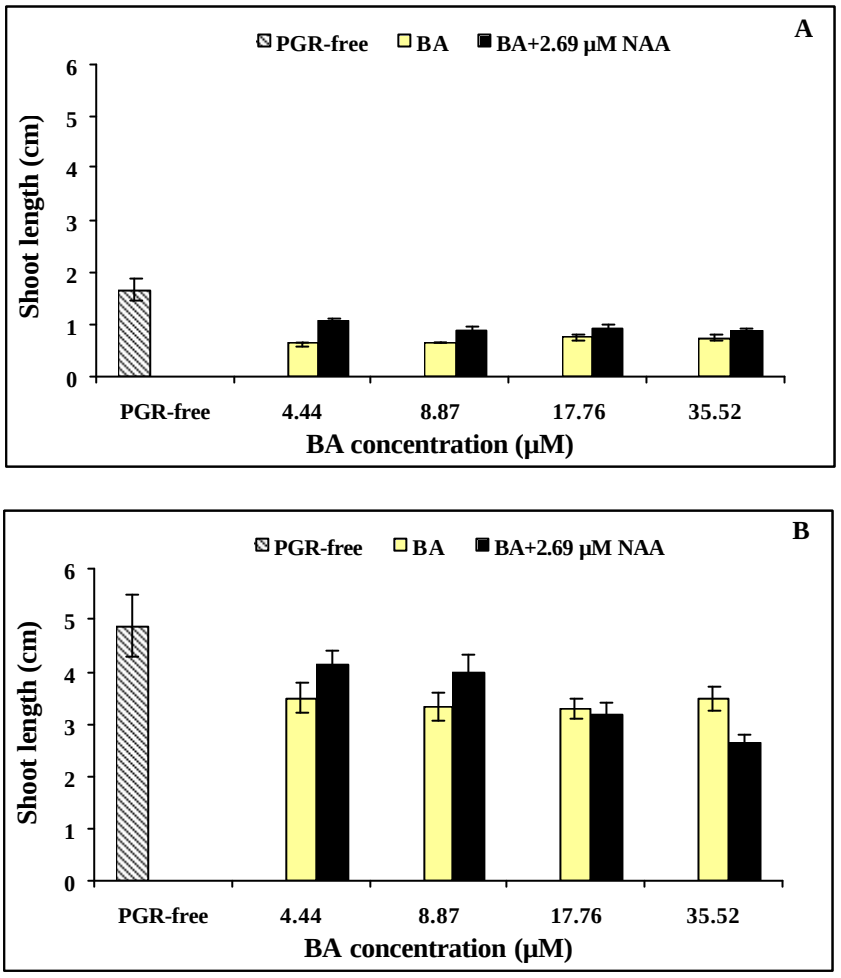

Figure 6 Effect of BA concentrations (4.44-35.52 µM) and 2.69 µM NAA on shoot elongation of regenerated shoots from *in vivo G. procumbens* node explants. (A) Four weeks after inoculation on MS with PGR, (B) Six weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

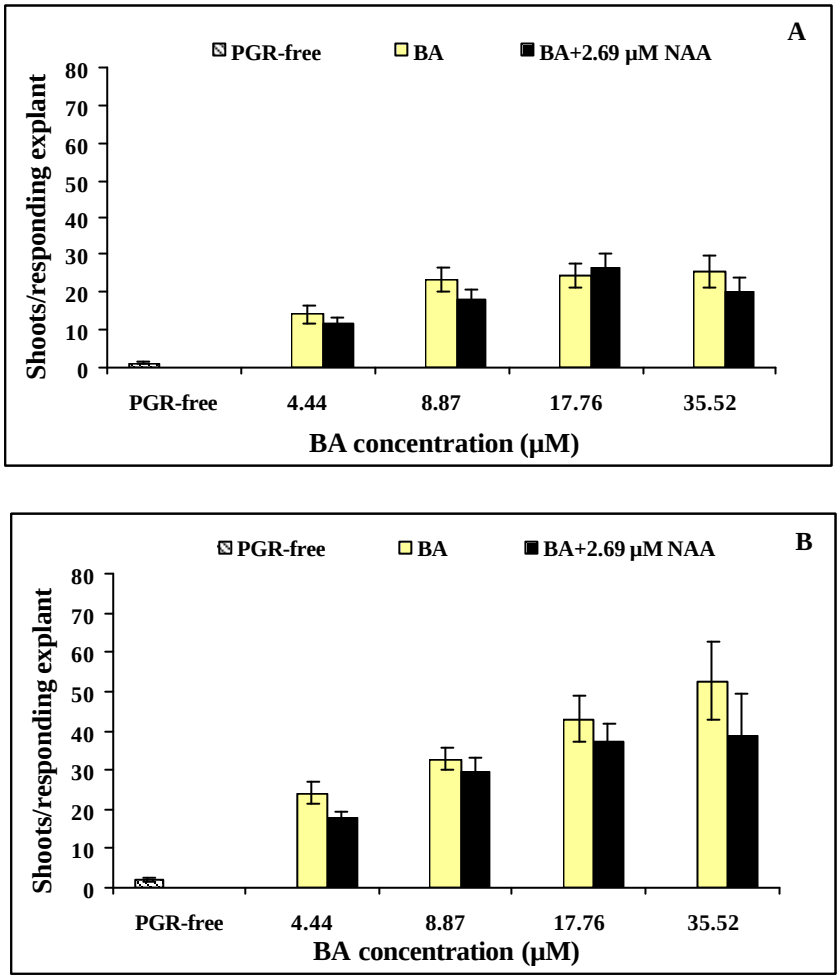

Figure 7 Effect of BA concentrations (4.44-35.52 µM) and 2.69 µM NAA on shoot induction of *in vivo G. procumbens* node explants. (A) Six weeks after inoculation on MS with PGR, (B) Four weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

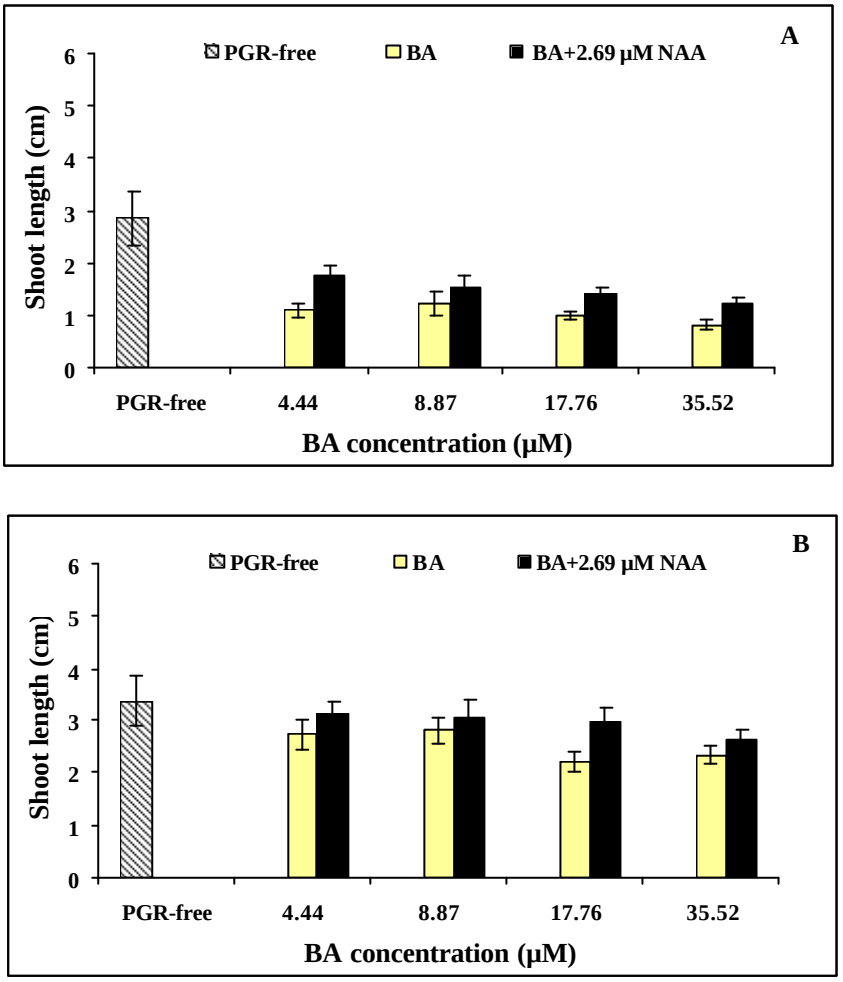

Figure 8 Effect of BA concentrations (4.44-35.52 µM) and 2.69 µM NAA on shoot elongation of regenerated shoots from *in vivo G. procumbens* node explants. (A) Six weeks after inoculation on MS with PGR, (B) Four weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

From the experiment 1, the incubation period of 6-weeks PGR and 4-weeks PGR-free incubation revealed a higher regeneration rate than the incubation period of 4-weeks PGR and 6-weeks PGR-free incubation. The shoot regeneration rates increased gradually when the BA concentrations elevated (Figure 5). The highest shoot regeneration rate were obtained from the explants incubated on MS medium supplemented with high concentration of BA (35.52µM) alone or in combination with 2.69 µM NAA for 6 weeks (52.80±10.00 and 38.70±10.96 shoots/response explant, respectively). Thus, experiments 2 was performed using the incubation protocol of experiment 1.2 and a high concentration of BA (71.04 µM) was additionally tested. Moreover, experiment 3 was performed using TDZ (4.54-72.64 µM) alone and in combination with 2.69 µM NAA.

In experiment 2, *in vitro G. procumbens* node explants were inoculated on MS media supplemented with BA (4.44-71.04 µM) and combination with 2.69 µM NAA for 6 weeks. The frequencies of explants responding to shoot formation were between 23.08 to 100% (Figure 9A), while no responding to rooting was found in all treatments except in MS medium without PGR (53.84%). The maximum shoot regeneration rate was obtained from explants cultured on MS with 35.52 µM BA (8.84±1.44 shoots/explant with average shoot length of 0.68±0.11 cm). Among the BA and NAA treatments, the combination of 71.04 µM BA and 2.69 µM NAA revealed the regeneration rate of 6.20±1.56 shoots/response explant with average shoot length of 0.59±0.04 cm (Figure 10A, 11A, and 12A).

After 6-weeks cultures in PGR treatment, the clusters of regenerated shoots were transferred to MS medium without PGR for 4 weeks. Then, the regenerated shoots were separated into single shoots and transferred to MS medium without PGR for 4 weeks. The frequency of shoot regeneration and rooting were 100% (Figure 9B). The shoot regeneration rate and shoot length increased. The highest shoot regeneration rate in BA alone treatment was obtained at concentration of 35.52 µM (115.54±19.56 shoots/explant, 1.37±0.10 cm shoot length). The optimum shoot regeneration rate of 70.96 µM BA combination with 2.69 µM NAA was 30.67±11.05 shoots/explant with 1.39±0.22 cm shoot length (Figure 10B, 11B, and 12B).

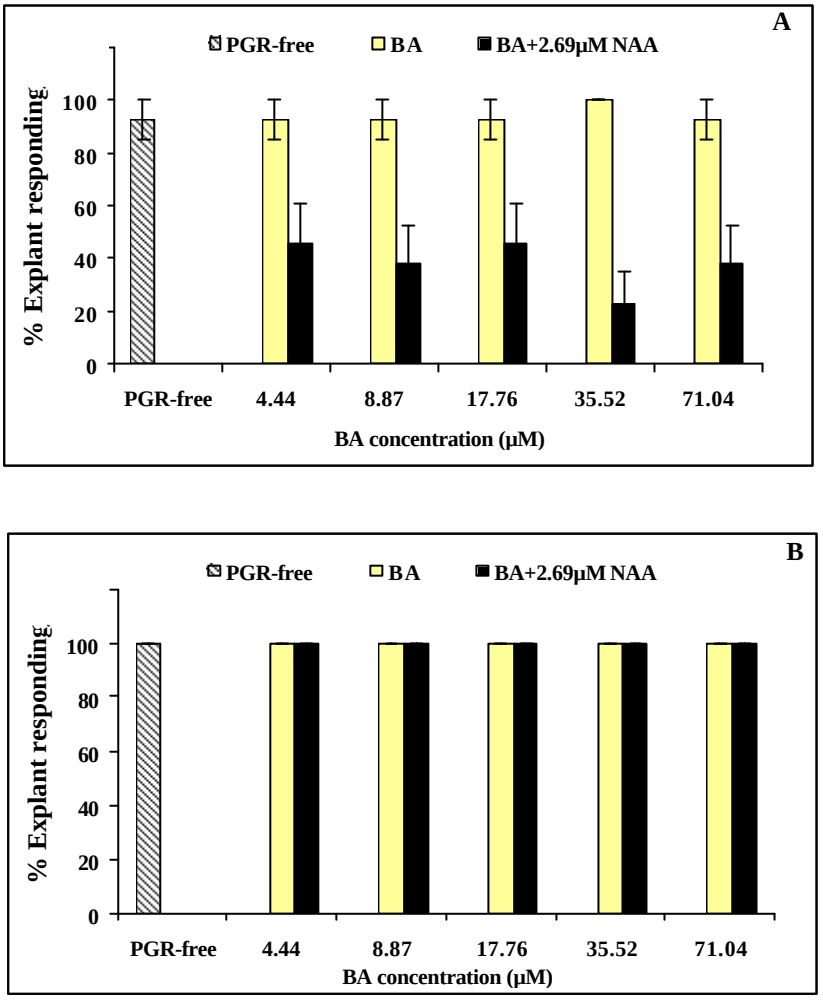

Figure 9 Effect of BA concentrations (4.44-71.04 µM) and 2.69 µM NAA on frequency of shoot responding of *in vitro G. procumbens* node explants. (A) Six weeks after inoculation on MS with PGR, (B) Eight weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

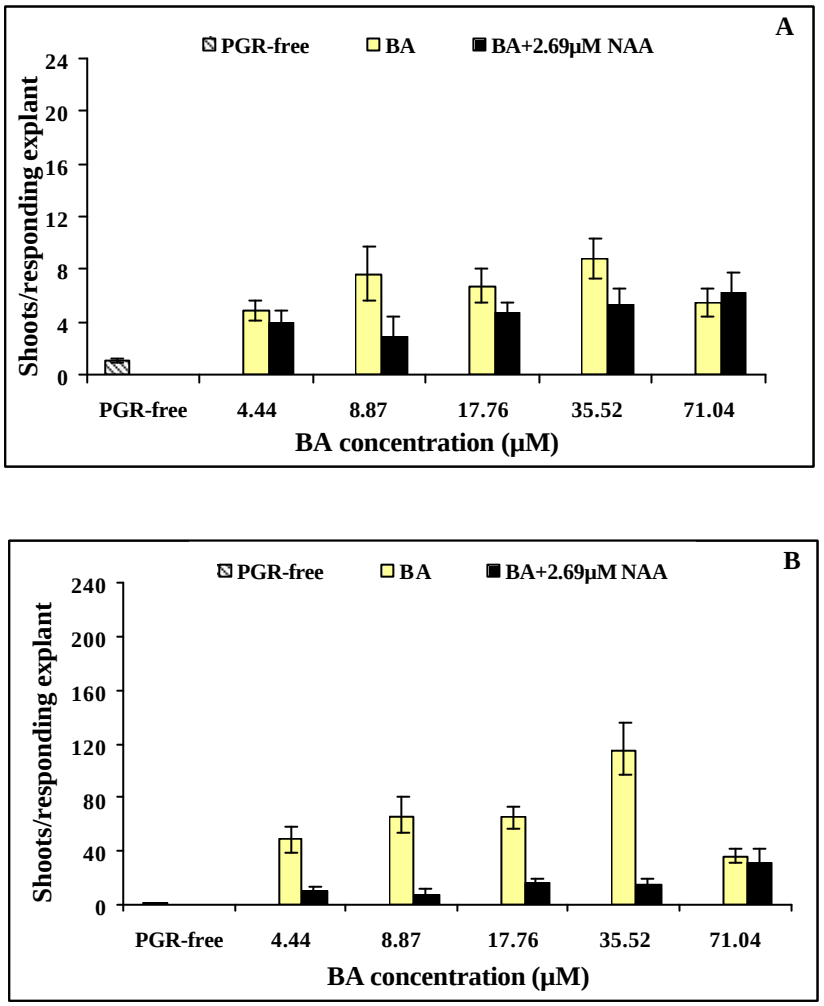

Figure 10 Effect of BA concentrations (4.44-71.04 µM) and 2.69 µM NAA on shoot induction of *in vitro G. procumbens* node explants. (A) Six weeks after inoculation on MS with PGR, (B) Eight weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

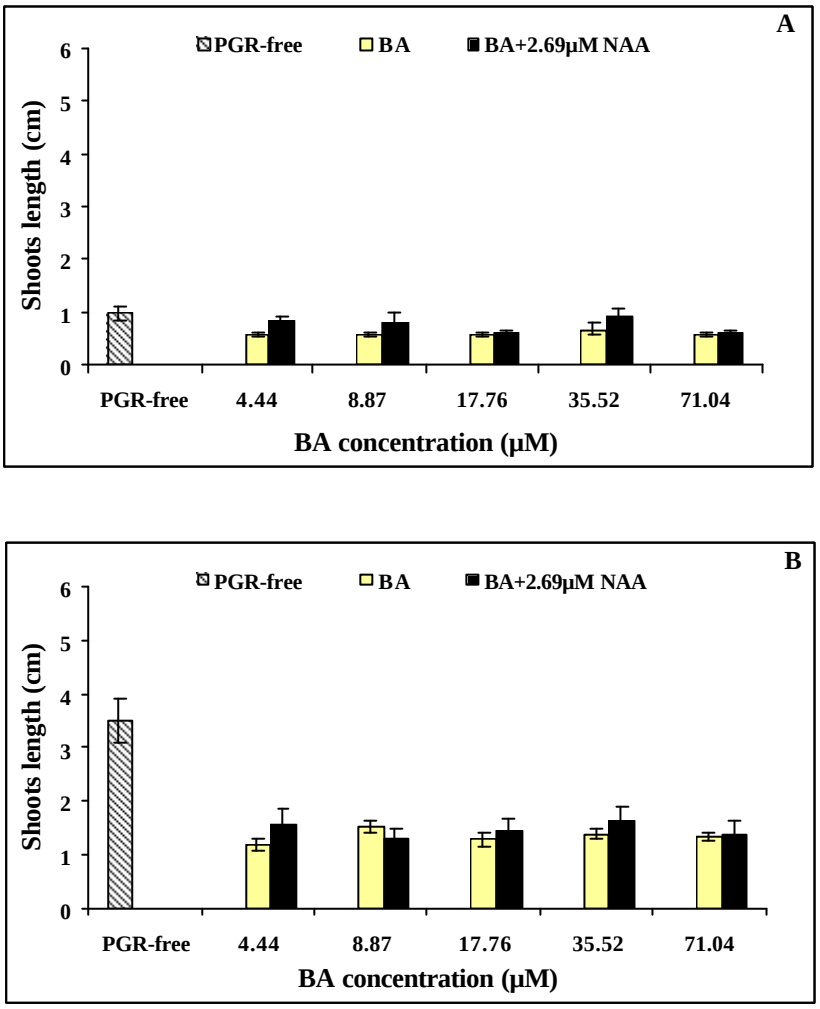

Figure 11 Effect of BA concentrations (4.44-71.04 µM) and 2.69 µM NAA on shoot elongation of regenerated shoots from *in vitro G. procumbens* node explants. (A) Six weeks after inoculation on MS with PGR, (B) Eight weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

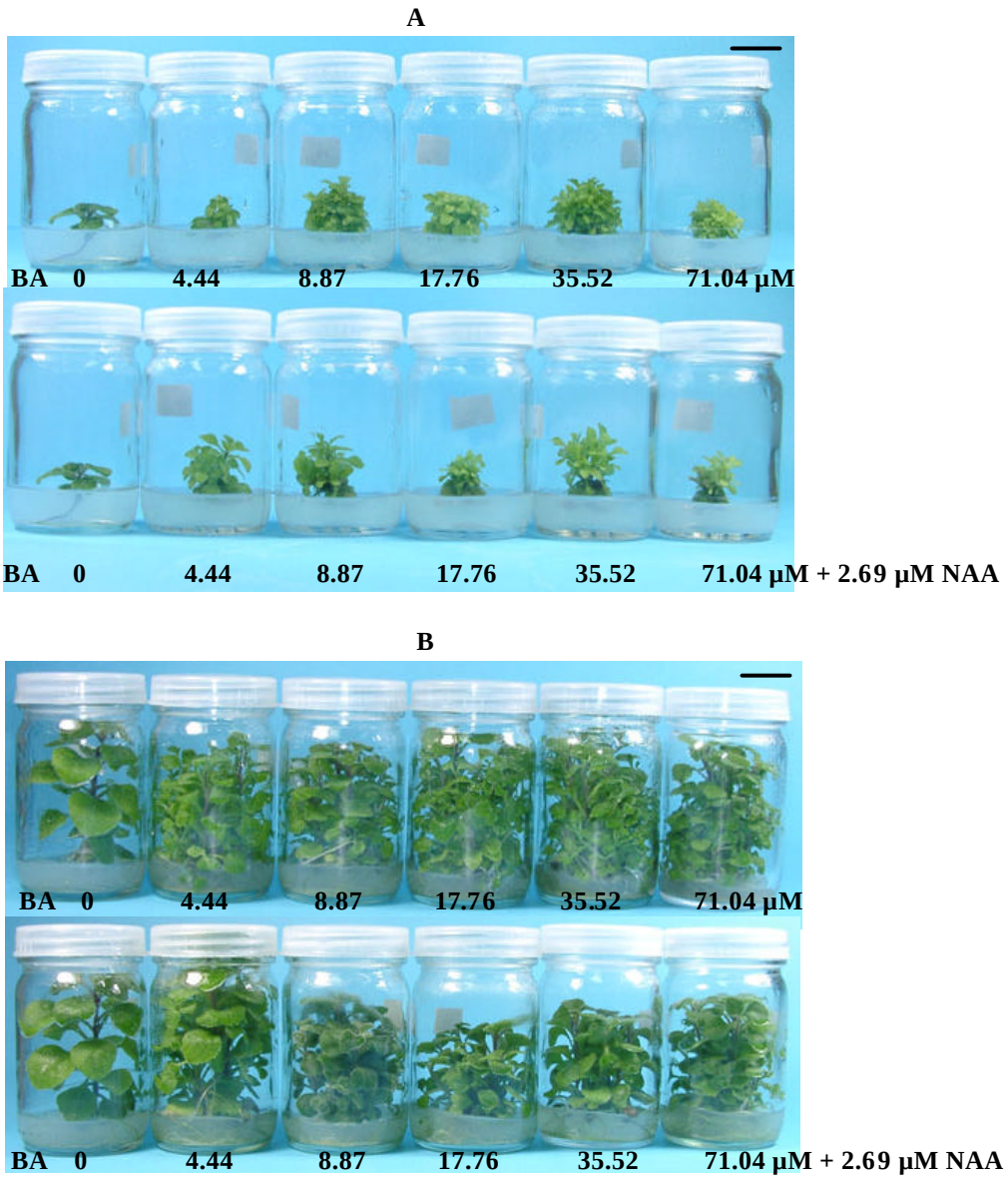

Figure 12 Regenerated shoots of *G. procumbens* cultured on BA and NAA treatments. (A) Six weeks on MS medium with BA alone and in combination with 2.69 µM NAA, (B) Eight weeks after transfer to culture on MS medium without plant growth regulator. Scale bar = 2 cm.

Experiment 3 demonstrated the effects of TDZ (4.54-72.64 µM) alone and in combination with 2.69 µM NAA on shoot regeneration of *G. procumbens* node explants. The frequencies of explants responding to shoot formation were between 23.08 to 92.31% (Figure 13A), when the explants were cultured on MS with PGRs for 6 weeks. Only the explants inoculated on MS medium without PGR (control) produced roots with the frequency of rooting of 76.92%. The maximum shoot regeneration rate was revealed at the concentration of 9.08 µM TDZ (9.67±2.97 shoots/response explant with an average shoot length of 0.63±0.06 cm). Whereas, TDZ and NAA treatments, the highest shoot regeneration rate was obtained from MS with 9.08 µM TDZ and 2.69 µM NAA (7.37±1.76 shoots/explant, with an average shoot length of 0.71±0.07 cm) (Figure 14A, 15A and 16A).

After 6-weeks cultures in PGR treatment, the clusters of regenerated shoots were transferred to MS medium without PGR for 4 weeks. Then, the regenerated shoots were separated into single shoots and transferred to MS medium without PGR for 4 weeks. The frequency of shoot regeneration was 100% (Figure 13B). The shoot regeneration rates and shoots lengths increased in all treatments. In TDZ treatments, the highest shoot regeneration rate of 202.50±20.75 shoots/response explant with 2.26±0.21 cm shoot length, was obtained from the explants transferred from MS with TDZ 9.08 µM. In TDZ and NAA treatments, the maximum shoot regeneration rate was 69.12±31.03 shoots/explant with 2.26±0.21 cm shoot length from MS with 9.08 µM TDZ combination with 2.69 µM NAA (Figure 14B, 15B and 16B). Rooting was spontaneous obtained.

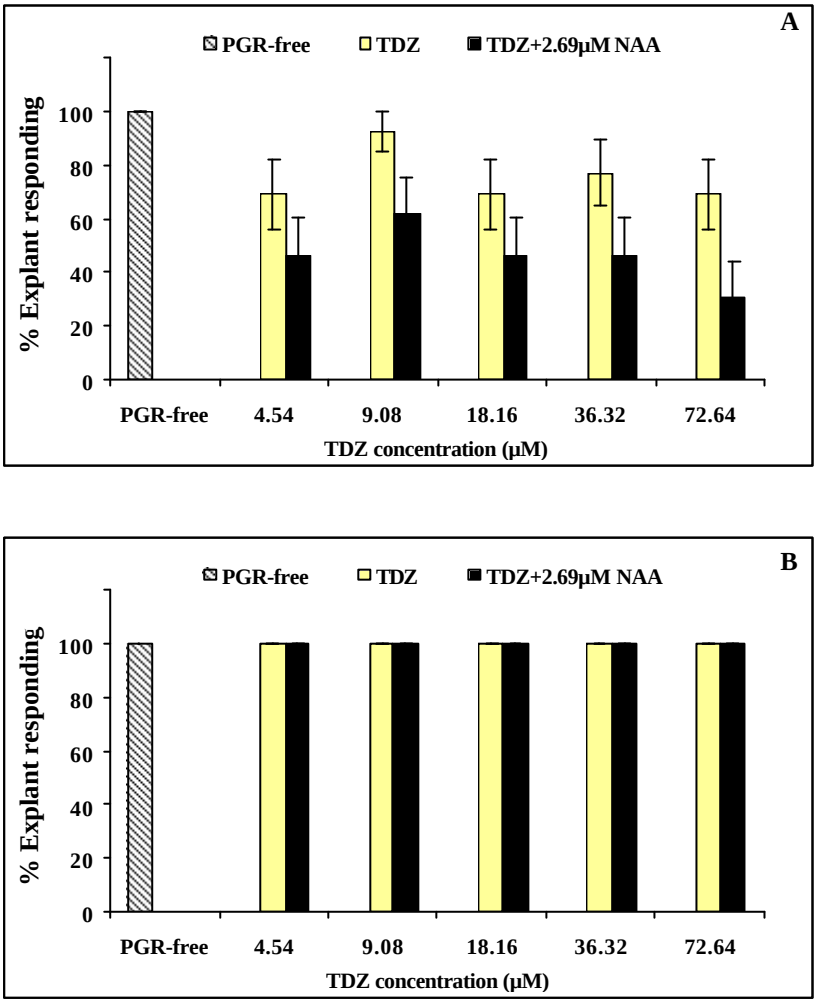

Figure 13 Effect of TDZ concentrations (4.54-72.64 µM) and 2.69 µM NAA on frequency of shoot responding of *in vitro G. procumbens* node explant. (A) Six weeks after inoculation on MS with PGR, (B) Eight weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

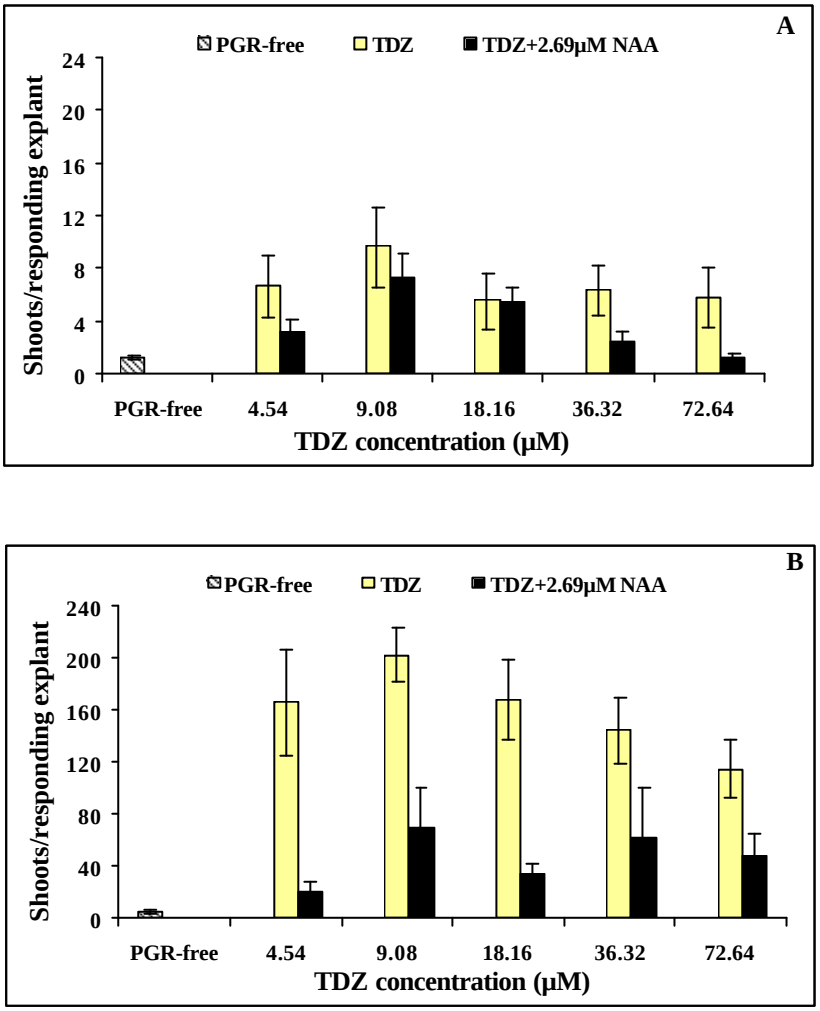

Figure 14 Effect of TDZ concentrations (4.54-72.64 µM) and 2.69 µM NAA on shoot induction of *in vitro G. procumbens* node explant. (A) Six weeks after inoculation on MS with PGR, (B) Eight weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

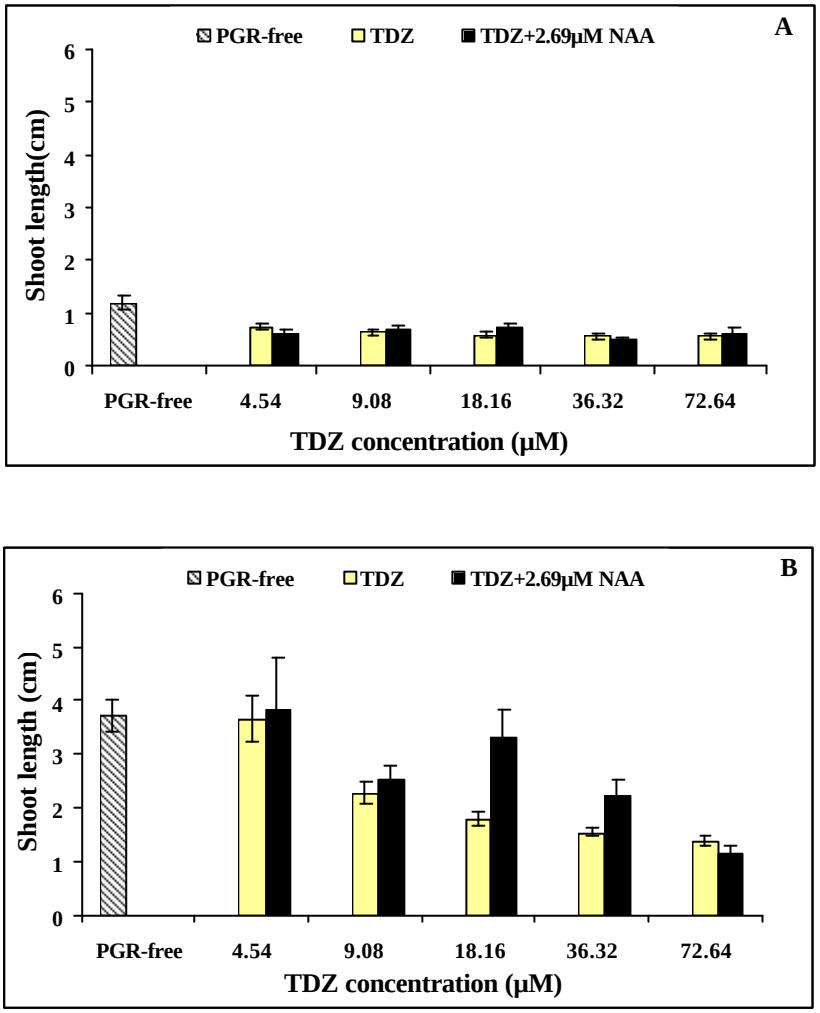

Figure 15 Effect of TDZ concentrations (4.54-72.64 µM) and 2.69 µM NAA on shoot elongation of regenerated shoots from *in vitro G. procumbens* node explant. (A) Six weeks after inoculation on MS with PGR, (B) Eight weeks after transfer to culture on PGR-free MS medium. Vertical lines indicate SE.

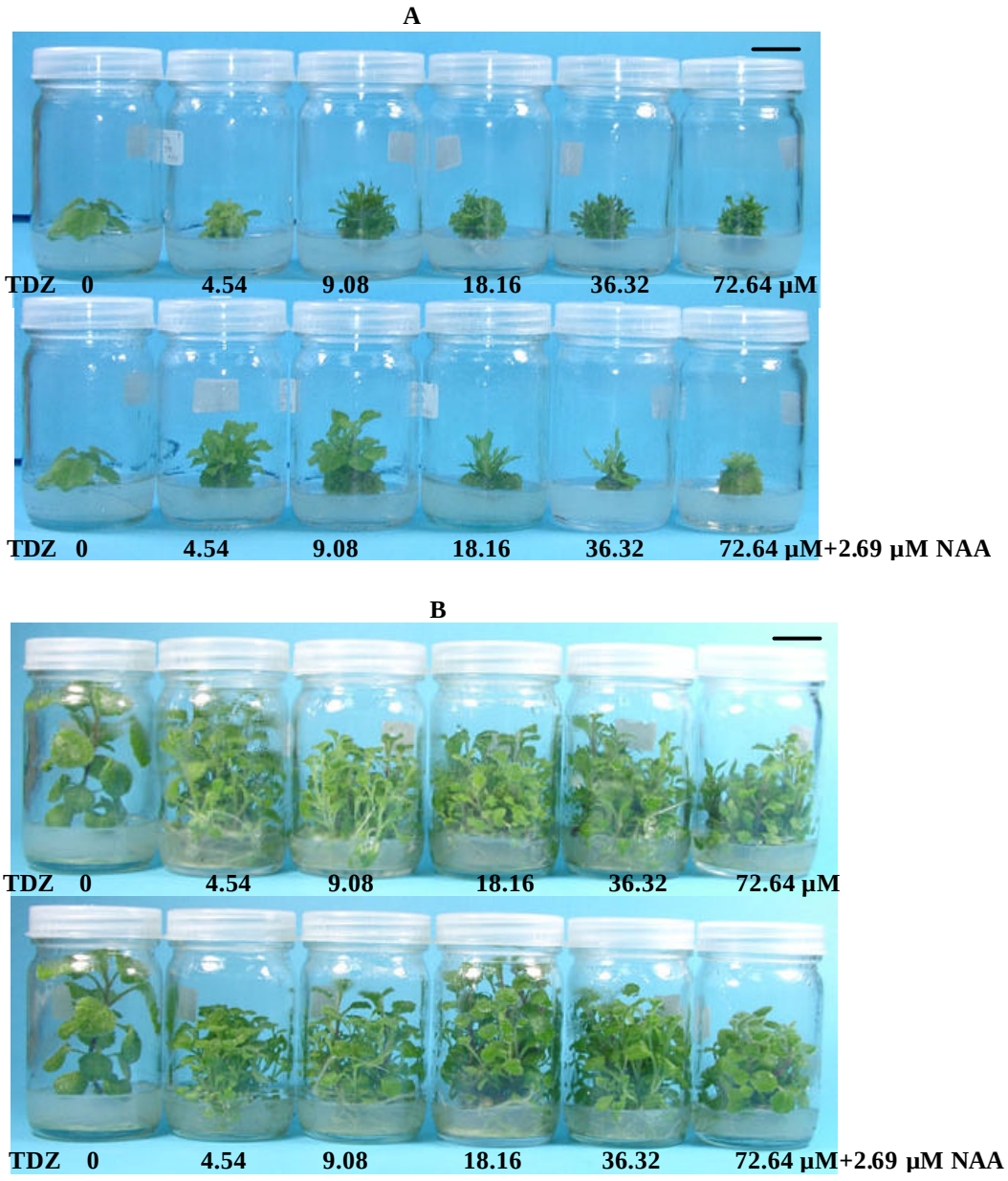

Figure 16 Regenerated shoots of *G. procumbens* cultured on TDZ and NAA treatments. (A) Six weeks on MS medium with TDZ alone and in combination with 2.69 µM NAA, (B) Eight weeks after transfer to culture on MS medium without plant growth regulator. Scale bar = 2 cm.

1.3 Indirect shoot regeneration

Experiment 4 demonstrated indirect shoot induction of *G. procumbens via* node, internode, and leaf explants. The experiment was performed using three media including callus induction media (MS medium supplemented with BA (4.44 and 8.87 µM) and 2, 4-D (0.45-9.05 µM)) for 8 weeks, indirect shoot proliferation medium I (MS medium supplemented with 4.44 µM BA), and indirect shoot proliferation medium II (MS medium without PGR).

After 4 weeks of culture on the callus induction media (MS with BA and 2,4-D), callus was induced from the cut margin of explants. The frequency of explants responding to callus formation was 100% in all treatment except the explants cultured on MS medium without PGR (control). The calli were compact with white, light green to green in color.

The callus formation was 1.06-3.00, 1.62-3.00 and 1.31-3.00 on node, internode, and leaf explants, respectively, at the week 4^{th} of culture. After 8 weeks of PGR treatments, callus induction was increased in all treatments. The callus formation was 2.50-3.00, 2.10-3.00 and 1.62-3.00 on node, internode, and leaf explants, respectively. Since the induced callus intensity of every explant type reached the maximum scale after the first four weeks of culture, therefore, the optimum of callus induction medium was MS medium with 8.87 µM BA and 2.26 µM 2,4-D (Table 8-10 and Figure 17). All three explant types formed callus with almost the same rate, 8 weeks after inoculation (Table 8-10).

During the incubation on callus induction medium (MS with BA and 2,4-D), the shoot formation were found only in node explants cultured on MS medium supplemented with 4.44 µM BA in combination with 0.45 and 2.26 µM 2,4-D and 8.87 µM BA in combination with 0.45, 2.26 and 4.52 µM 2,4-D. After eight weeks of cultures, the frequency of explants responding to shoot formation was 12.50% to 87.50%, while on MS without PGR was 100%. In 4.44 µM BA in combination with 0.45 and 2.26 µM 2,4-D treatments, the shoots regeneration rates were 4.43±0.90 shoots/explant with 1.00±0.09 cm shoot length and 1.50 shoots/response explant with 0.50 cm shoot length, respectively. Whereas, in 8.87 µM BA in combination with 2,4-D treatments, the maximum shoot regeneration rate was obtained on 8.87 µM BA and

0.45 µM 2,4-D (10.00±1.21 shoots/response explant with 0.73±0.09 cm shoot length). In control treatment, the shoot regeneration rate was 1.00 shoots/response explant with 1.62 cm shoot length (Table 8-10 and Figure 18A).

Then, the compact green calli of node explants were transferred to the indirect shoot proliferation medium I (MS medium supplement with 4.44 µM BA) for shoot proliferation. After 4 weeks, the regenerated shoots were observed only in node explants (87.50-100%). The maximum shoot induction rate of 10.75±1.31 shoots/response explant with 1.24±0.08 cm shoot length was obtained on callus transferred from MS with 8.87 µM BA and 0.45 µM 2,4-D, while callus from MS with 4.44 µM BA and 0.45 µM 2,4-D revealed a shoot induction rate of 9.71±0.92 shoots/respond explant with 2.31±0.30 cm shoot length (Table 11 and Figure 18B).

After 4 weeks of culture on the indirect shoot proliferation medium I, the regenerated shoots were separated into single shoots and transferred to the indirect shoot proliferation medium II (MS medium without PGR) for further shoot multiplication and elongation. After 4 weeks on the indirect shoot proliferation medium II, the shoot regeneration rate and shoot length increased. All regenerated shoots formed roots. The highest shoots induction rate of 71.28±18.11 shoots/response explant with an average shoot length of 2.72±0.16 cm was revealed from the callus derived from MS with 8.87 µM BA in combination with 0.45 µM 2,4-D (Table 11 and Figure 18C).

Table 8 Effect of BA and 2, 4-D on indirect shoot induction of *G. procumbens* node explants, 4 and 8 weeks of culture on callus induction media.

PGR (µM)		% Explant responding to form callus (S.E.)		Callus intensity (S.E.)		% Explant responding to form shoots (S.E.)		Shoots/response explant (S.E.)		Shoot length (S.E.)	
BA	2,4-D	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
0	0	0	0	0	0	100 (0)	100 (0)	1.00 (0)	1.00 (0)	0.94 (0.15)	1.62 (0.36)
4.44	0.45	100 (0)	100 (0)	2.50 (0.27)	3.00 (0)	87.50 (12.50)	87.50 (12.50)	3.71 (0.61)	4.43 (0.90)	0.76 (0.04)	1.00 (0.09)
4.44	2.26	100 (0)	100 (0)	2.50 (0.27)	3.00 (0)	50.00 (18.90)	50.00 (18.90)	1.25 (0.25)	1.25 (0)	0.50 (0)	0.50 (0)
4.44	4.52	100 (0)	100 (0)	1.63 (0.26)	2.63 (0.18)	0	0	0	0	0	0
4.44	9.05	100 (0)	100 (0)	1.38 (0.26)	2.50 (0.33)	0	0	0	0	0	0
8.87	0.45	100 (0)	100 (0)	1.06 (0.29)	2.50 (0)	87.50 (12.50)	87.50 (12.50)	8.75 (1.07)	10.00 (1.21)	0.61 (0.05)	0.73 (0.09)
8.87	2.26	100 (0)	100 (0)	3.00 (0)	3.00 (0)	62.50 (12.50)	87.50 (12.50)	2.40 (0.60)	3.00 (0.95)	0.72 (0.08)	0.64 (0.07)
8.87	4.52	100 (0)	100 (0)	2.63 (0.26)	2.62 (0.26)	12.50 (12.50)	12.50 (12.50)	1.00 (0)	1.00 (0)	0.50 (0)	0.50 (0)
8.87	9.05	100 (0)	100 (0)	2.69 (0.31)	2.75 (0.25)	0	0	0	0	0	0

Table 9 Effect of BA and 2, 4-D on indirect shoot induction of *G. procumbens* internode explants, 4 and 8 weeks of culture on callus induction media.

PGR (µM)		% Explant responding to form callus (S.E.)		Callus intensity (S.E.)		% Explant responding to form shoots (S.E.)		Shoots/response explant (S.E.)		Shoot length (S.E.)	
BA	2,4-D	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
0	0	0	0	0	0	0	0	0	0	0	0
4.44	0.45	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
4.44	2.26	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
4.44	4.52	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
4.44	9.05	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
8.87	0.45	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
8.87	2.26	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
8.87	4.52	100 (0)	100 (0)	2.25 (0.16)	2.25 (0.16)	0	0	0	0	0	0
8.87	9.05	100 (0)	100 (0)	1.62 (0.26)	2.12 (0.12)	0	0	0	0	0	0

Table 10 Effect of BA and 2, 4-D on indirect shoot induction of *G. procumbens* leaf explants, 4 and 8 weeks of culture on callus induction media.

PGR (µM)		% Explant responding to form callus (S.E.)		Callus intensity (S.E.)		% Explant responding to form shoots (S.E.)		Shoots/response explant (S.E.)		Shoot length (S.E.)	
BA	2,4-D	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
0	0	0	0	0	0	0	0	0	0	0	0
4.44	0.45	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
4.44	2.26	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
4.44	4.52	100 (0)	100 (0)	2.25 (0.16)	3.00 (0)	0	0	0	0	0	0
4.44	9.05	100 (0)	100 (0)	2.13 (0.13)	3.00 (0)	0	0	0	0	0	0
8.87	0.45	100 (0)	100 (0)	2.50 (0.27)	3.00 (0)	0	0	0	0	0	0
8.87	2.26	100 (0)	100 (0)	3.00 (0)	3.00 (0)	0	0	0	0	0	0
8.87	4.52	100 (0)	100 (0)	2.50 (0.19)	3.00 (0)	0	0	0	0	0	0
8.87	9.05	100 (0)	100 (0)	1.31 (0.28)	1.62 (0.32)	0	0	0	0	0	0

Table 11 Indirect shoot induction of *G. procumbens* node explants after 8 weeks of culture on MS with BA and 2,4-D and transfer to shoot proliferation medium I and II.

Callus induction medium		Indirect Shoot Proliferation Medium I*				Indirect Shoot Proliferation Medium II**			
BA (µM)	2,4-D (µM)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)
0	0	100 (0)	1.25 (0.12)	2.25 (0.60)	0	100 (0)	1.25 (0.12)	2.25 (0.60)	0
4.44	0.45	100 (0)	9.71 (0.92)	2.31 (0.30)	0	100 (0)	32.37 (5.51)	3.33 (0.22)	100 (0)
4.44	2.26	100 (0)	1.83 (0.40)	0.50 (0)	0	100 (0)	4.67 (1.76)	3.31 (1.25)	100 (0)
4.44	4.52	0	0	0	0	0	0	0	0
4.44	9.05	0	0	0	0	0	0	0	0
8.87	0.45	100 (12.50)	10.75 (1.31)	1.24 (0.08)	0	100 (12.50)	71.28 (18.11)	2.72 (0.16)	100 (0)
8.87	2.26	87.50 (12.50)	3.86 (1.39)	0.84 (0.12)	0	87.50 (12.50)	15.33 (4.98)	1.71 (0.25)	100 (0)
8.87	4.52	12.50 (12.50)	1.00 (0)	1.00 (0)	0	12.50 (12.50)	1.00 (0)	3.00 (0)	100 (0)
8.87	9.05	0	0	0	0	0	0	0	0

* Indirect shoot proliferation medium I: MS with 4.44 µM BA; duration of culture: 4 weeks

** Indirect shoot proliferation medium II: MS without PGR; duration of culture: 4 weeks

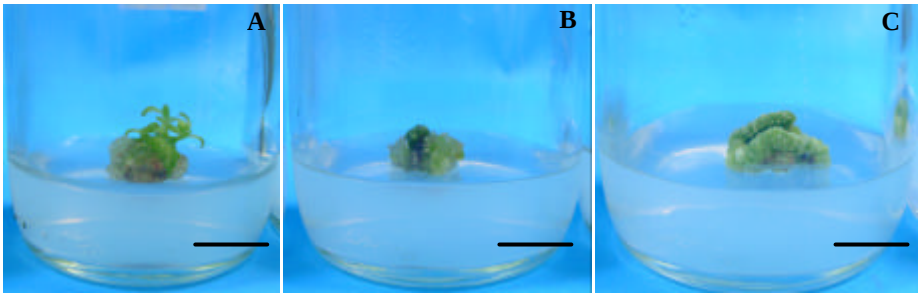

Figure 17 Callus formation of *G. procumbens* explants on MS medium with BA and 2,4-D at 4 weeks. (A) node explant with regenerated shoots, (B) internode explant, (C) leaf explant. Scale bars = 1 cm

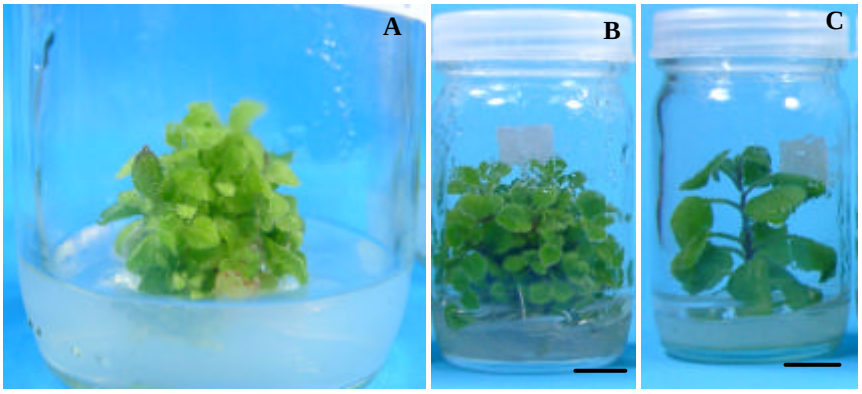

Figure 18 Indirect shoot regeneration of *G. procumbens* node explant on MS medium with BA and 2, 4-D (A) After 8 weeks on MS medium with 8.87 µM BA and 0.45 µM 2,4-D, (B) Cluster of regenerated shoots after transfer to MS medium with 4.44 µM BA for 4 weeks, (C) Rooted plant on MS without PGR for 4 weeks. Scale bars = 1 cm

In experiment 5, callus induction of *G. procumbens* internode and leaf explants was performed using MS medium supplemented with 4.44-71.04 µM BA alone and in combination with 2.69 µM NAA. The duration of callus induction was adjusted to 4 weeks in stead of 8 weeks in the experiment 4. Calli were compact with white and light green to green. The frequencies of explants responding to callus formation on MS with BA combination with 2.69 µM NAA were almost 100%. In BA treatments, only internode explants responded to form callus at the frequency of 8.33%, whereas no response was observed in leaf explants. Callus induction rate of internode explant was higher than leaf explant on BA alone, while leaf explant was callus higher than internode explant on BA combination with 2.69 µM NAA. For leaf explants, every tested media of BA in combination with NAA gave 100% responding rate and maximum callus intensity after 4 weeks of culture. Internode explants incubated on MS with BA alone also formed callus with intensities of 0.5-1. (Table 12-13 and Figure 19A1-2, 19B1-2). However, the browning rate of internode and leaf explants on MS medium supplement with BA alone was higher than BA combination with 2.69 µM NAA (data not show).

After 4 weeks on callus induction media, only callus from BA combination NAA treatments were transferred to the indirect shoot proliferation medium I (MS with 4.44 µM BA) for shoot proliferation. After 4 weeks of culture, the frequencies of callus forming shoots of leaf-derived callus was higher than those of internode-derived callus. In internode explant, the callus only from MS with 8.87 µM BA in combination with 2.69 µM NAA and 35.52 µM BA in combination with 2.69 µM NAA regenerated to shoots. The optimum media for shoot proliferation of internode-derived callus was MS with 35.52 µM BA in combination with 2.69 µM NAA. The frequency of explant responding to form shoot was 16.67% with an induction rate was 4.50±2.52 shoots/response explant with 0.43±0.07 cm shoot length. After transfer to the indirect shoot proliferation medium II (MS without PGR), the induction rate increased to 9.33±3.84 shoots/response explant with an average shoot length of 0.93±0.07 cm (Table 12 and Figure 19A). In leaf explant, the callus from all combinations of MS with BA and NAA formed shoots. The optimum media for shoot proliferation of leaf-derived callus was also MS with 35.52 µM BA in combination with 2.69 µM NAA. The frequency of explant responding to form shoot was 25%

with an induction rate of 14.33±2.52 shoots/response explant with 0.89±0.03 cm shoot length. After transfer to the indirect shoot proliferation medium II (MS without PGR), the induction rate increased to 26.50±9.39 shoots/response explant with an average shoot length of 1.32±0.01 cm (Table 13 and Figure 19B).

Table 12 Indirect shoot induction of *G. procumbens* internode explants using BA and NAA.

Callus Induction Media*				Indirect Shoot Proliferation Medium I**				Indirect Shoot Proliferation Medium II***			
BA (µM)	NAA (µM)	% Explant responding to form callus (S.E.)	Callus intensity (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)
0	0	25.00 (13.06)	0.50 (0)	#	#	#	#	#	#	#	#
4.44	0	0	0	#	#	#	#	#	#	#	#
8.87	0	8.33 (8.33)	0.50 (0)	#	#	#	#	#	#	#	#
17.76	0	8.33 (8.33)	1.00 (0)	#	#	#	#	#	#	#	#
35.52	0	8.33 (8.33)	0.50 (0)	#	#	#	#	#	#	#	#
71.04	0	0	0	#	#	#	#	#	#	#	#
4.44	2.69	100 (0)	2.75 (0)	0	0	0	0	0	0	0	100 (0)
8.87	2.69	100 (0)	3.00 (0)	8.33 (8.33)	3.00 (0)	0.50 (0)	0	8.33 (8.33)	7.00 (0)	0.71 (0)	100 (0)
17.76	2.69	91.67 (8.33)	2.59 (0)	0	0	0	0	0	0	0	100 (0)
35.52	2.69	100 (0)	2.79 (0)	16.67 (16.67)	4.50 (2.50)	0.43 (0.07)	0	16.67 (16.67)	9.33 (3.84)	0.93 (0.07)	100 (0)
71.04	2.69	100 (0)	2.00 (0)	0	0	0	0	0	0	0	100 (0)

* Callus induction media: MS supplemented with 4.44 -71.04 44 µM BA alone or in combination with 2.6944 µM NAA; duration of culture: 4 weeks

** Indirect shoot proliferation medium I: MS with 4.44 µM BA; duration of culture: 4 weeks

*** Indirect shoot proliferation medium II: MS without PGR; duration of culture: 4 weeks

The explant were not transferred to the indirect shoot proliferation medium I and II.

Table 13 Indirect shoot induction of *G. procumbens* leaf explants using BA and NAA.

PGR (µM)		Callus Induction Media*		Indirect Shoot Proliferation Medium I**				Indirect Shoot Proliferation Medium II***			
BA	NAA	% Explant responding to form callus (S.E.)	Callus intensity (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)
0	0	0	0	#	#	#	#	#	#	#	#
4.44	0	0	0	#	#	#	#	#	#	#	#
8.87	0	0	0	#	#	#	#	#	#	#	#
17.76	0	0	0	#	#	#	#	#	#	#	#
35.52	0	0	0	#	#	#	#	#	#	#	#
71.04	0	0	0	#	#	#	#	#	#	#	#
4.44	2.69	100 (0)	3.00 (0)	41.67 (14.86)	5.00 (1.24)	1.13 (0.17)	0	41.67 (14.86)	5.00 (1.22)	0.69 (0.11)	100 (0)
8.87	2.69	100 (0)	3.00 (0)	8.33 (8.33)	5.00 (0.00)	1.20 (0)	0	8.33 (8.33)	10.00 (0)	1.40 (0)	100 (0)
17.76	2.69	100 (0)	3.00 (0)	25.00 (13.06)	11.67 (1.30)	0.91 (0.09)	0	25.00 (13.06)	24.67 (7.22)	1.44 (0.18)	100 (0)
35.52	2.69	100 (0)	3.00 (0)	25.00 (13.06)	14.33 (2.52)	0.89 (0.03)	0	25.00 (13.06)	26.50 (9.39)	1.32 (0.01)	100 (0)
71.04	2.69	100 (0)	3.00 (0)	8.33 (8.33)	11.00 (0.64)	0.63 (0)	0	8.33 (8.33)	14.25 (7.41)	1.21 (0.37)	75.00 (25.00)

* Callus induction media: MS supplemented with 4.44 -71.04 44 µM BA alone or in combination with 2.6944 µM NAA; duration of culture: 4 weeks

** Indirect shoot proliferation medium I: MS with 4.44 µM BA; duration of culture: 4 weeks

*** Indirect shoot proliferation medium II: MS without PGR; duration of culture: 4 weeks

The explant were not transferred to the indirect shoot proliferation medium I and II.

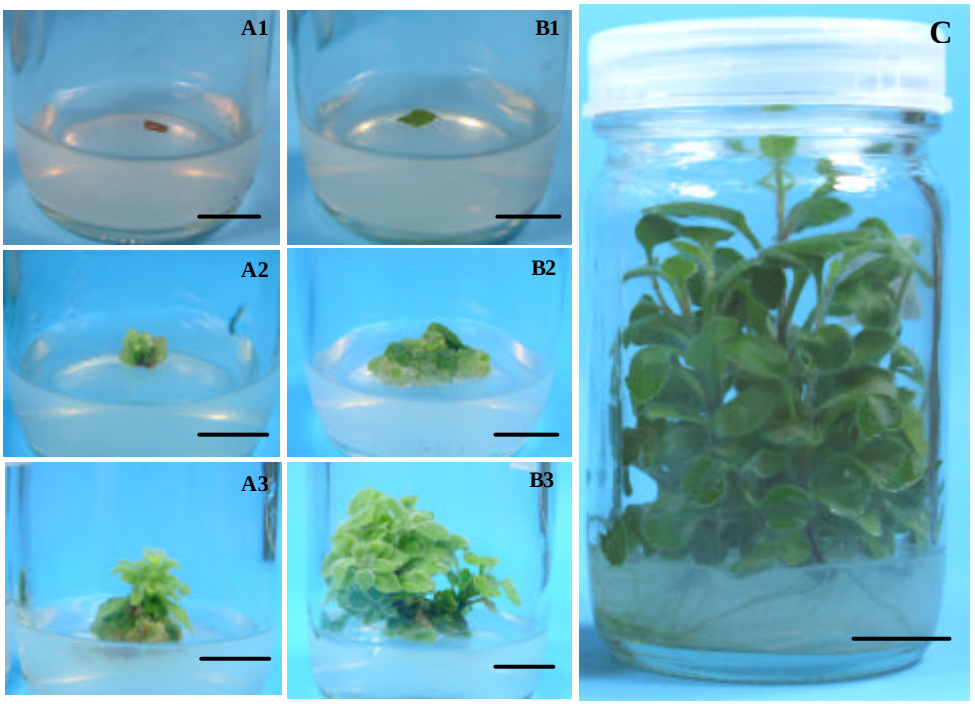

Figure 19 Indirect shoot regeneration of *G. procumbens* explants on MS medium with BA and 2.69 µM NAA. (A) Internode explant, (B) Leaf explant, (A2-B2) Callus formations, 4 weeks after inoculation, (A3-B3) Regenerated shoot after transfer to MS medium with 4.44 µM BA for 4 weeks, (C) Shoot elongation and rooting, after 4 weeks on MS (PGR-free) medium. Scale bars = 1 cm

Experiment 6 was performed to test the effect of TDZ (4.54-72.64 µM) alone and in combination with 2.69 µM NAA on callus induction of internode and leaf explants. Calli were compact with light green or green. After 4 weeks of cultures, the frequencies of explants responding to callus formation of the explants cultured on TDZ in combination with 2.69 µM NAA was higher than that of TDZ alone. The callus induction rates of leaf explants were higher than those of internode explants. In TDZ treatment, the frequencies of explants responding were 16.67-58.33% in two explant types, whereas in TDZ-NAA treatment, the frequencies increased to 66.67-100% and 100% in internode and leaf explants, respectively. The callus intensity of leaf explant reached the maximum scale in the explant cultured on MS medium supplement with TDZ (4.54-18.16µM) and 2.69 µM NAA. The callus intensity of internode explants were between 0.50-0.62 and 0.54-0.75 in TDZ alone and TDZ and NAA treatment. (Table 14-15 and Figure 20A1-2, 20B1-2). However, frequency of browning rate of internode and leaf explants on MS medium supplemented with TDZ alone was higher than those cultured on MS TDZ combination with 2.69 µM NAA (data not show).

After 4 weeks on callus induction media, only callus from TDZ and NAA treatments were transferred to the indirect shoot proliferation medium I (MS with 4.44 µM BA) for shoot proliferation. The calli turned to white, green to dark green and become compact.

In internode explant, the callus only from MS with 72.64 µM TDZ in combination with 2.69 µM NAA formed shoots. The frequency of explant responding to form shoot was 16.67% with an induction rate was 1.00 shoots/response explant with 0.50 cm shoot length. After transfer to the indirect shoot proliferation medium II (MS without PGR), the induction rate did not increase, but the shoot length increased to 1.50 cm. In leaf explant, the callus from all combinations of MS with TDZ and NAA formed shoots. The frequencies of explant responding to form shoot was 16.67-33.33% with an induction rate of 2.62-7.00 shoots/response explant. After transfer to the indirect shoot proliferation medium II (MS without PGR), the induction rate increased to 3.37-15.00 shoots/response explant. Although, MS with 72.64 µM TDZ and 2.69 µM NAA revealed the maximum induction rate (15.00 shoots/explant), but with a low responding rate of 16.67%. Therefore, the optimum media for callus

induction of leaf explant was MS with 9.08 µM TDZ and 2.69 µM NAA, which then transferred to indirect shoot proliferation medium I and II. This treatment revealed the responding frequency of 33.33% with an induction rate of 10.75±8.75 shoots/response explant which made the highest total number of regenerated shoots (Table 14-15 and Figure 20).

Table 14 Indirect shoot induction of *G. procumbens* internode explants using TDZ and NAA.

PGR (µM)		Callus Induction Media*		Indirect Shoot Proliferation Medium I**				Indirect Shoot Proliferation Medium II***			
TDZ	NAA	% Explant responding to form callus (S.E.)	Callus intensity (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)
0	0	8.33 (8.33)	0.50 (0)	#	#	#	#	#	#	#	#
4.54	0	25.00 (13.06)	0.50 (0)	#	#	#	#	#	#	#	#
9.08	0	58.33 (14.86)	0.50 (0)	#	#	#	#	#	#	#	#
18.16	0	33.33 (14.21)	0.50 (0)	#	#	#	#	#	#	#	#
36.32	0	33.33 (14.21)	0.62 (0.12)	#	#	#	#	#	#	#	#
72.64	0	16.67 (11.24)	0.50 (0)	#	#	#	#	#	#	#	#
4.54	2.69	100 (0)	0.75 (0.07)	0	0	0	0	0	0	0	0
9.08	2.69	100 (0)	0.67 (0.07)	0	0	0	0	0	0	0	0
18.16	2.69	66.67 (14.21)	0.62 (0.08)	0	0	0	0	0	0	0	0
36.32	2.69	75.00 (13.06)	0.56 (0.06)	0	0	0	0	0	0	0	0
72.64	2.69	100 (0)	0.54 (0.04)	16.67 (16.67)	1.00 (0)	0.50 (0)	0	8.33 (8.33)	1.00 (0)	1.50 (0)	100 (0)

* Callus induction media: MS supplemented with 4.44 -71.04 44 µM BA alone or in combination with 2.6944 µM NAA; duration of culture: 4 weeks

** Indirect shoot proliferation medium I: MS with 4.44 µM BA; duration of culture: 4 weeks

*** Indirect shoot proliferation medium II: MS without PGR; duration of culture: 4 weeks

The explant were not transferred to the indirect shoot proliferation medium I and II.

Table 15 Indirect shoot induction of *G. procumbens* leaf explants using TDZ and NAA.

PGR (µM)		Callus Induction Medium*		Indirect Shoot Proliferation Medium I**				Indirect Shoot Proliferation Medium II***			
TDZ	NAA	% Explant responding to form callus (S.E.)	Callus intensity (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)	%Explant responding to form shoots (S.E.)	Shoots/response explant (S.E.)	Shoot length (S.E.)	%Rooting (S.E.)
0	0	0	0	#	#	#	#	#	#	#	#
4.54	0	50.00 (15.08)	0.50 (0)	#	#	#	#	#	#	#	#
9.08	0	33.33 (14.21)	0.50 (0)	#	#	#	#	#	#	#	#
18.16	0	58.33 (14.86)	0.50 (0)	#	#	#	#	#	#	#	#
36.32	0	41.67 (14.86)	0.62 (0)	#	#	#	#	#	#	#	#
72.64	0	16.67 (11.24)	0.50 (0)	#	#	#	#	#	#	#	#
4.54	2.69	100 (0)	3.00 (0)	66.67 (14.21)	2.62 (1.19)	1.01 (0.20)	0	66.67 (14.21)	3.37 (1.19)	1.19 (0.28)	75.00 (16.37)
9.08	2.69	100 (0)	3.00 (0)	33.33 (14.21)	5.00 (3.67)	1.06 (0.21)	0	33.33 (14.21)	10.75 (8.75)	1.25 (0.43)	75.00 (16.37)
18.16	2.69	100 (0)	3.00 (0.17)	25.00 (12.50)	4.33 (1.76)	0.99 (0.25)	0	25.00 (12.50)	12.00 (4.00)	1.76 (0.36)	100 (0)
36.32	2.69	100 (0)	2.83 (0.08)	16.67 (11.24)	7.00 (4.00)	1.35 (0.01)	0	16.67 (11.24)	14.50 (10.50)	1.14 (0.11)	100 (0)
72.64	2.69	100 (0)	2.92 (0)	16.67 (11.24)	7.00 (6.00)	0.83 (0.33)	0	16.67 (11.24)	15.00 (14.00)	1.47 (0.97)	100 (0)

* Callus induction media: MS supplemented with 4.44 -71.04 44 µM BA alone or in combination with 2.6944 µM NAA; duration of culture: 4 weeks

** Indirect shoot proliferation medium I: MS with 4.44 µM BA; duration of culture: 4 weeks

*** Indirect shoot proliferation medium II: MS without PGR; duration of culture: 4 weeks

The explant were not transferred to the indirect shoot proliferation medium I and II.

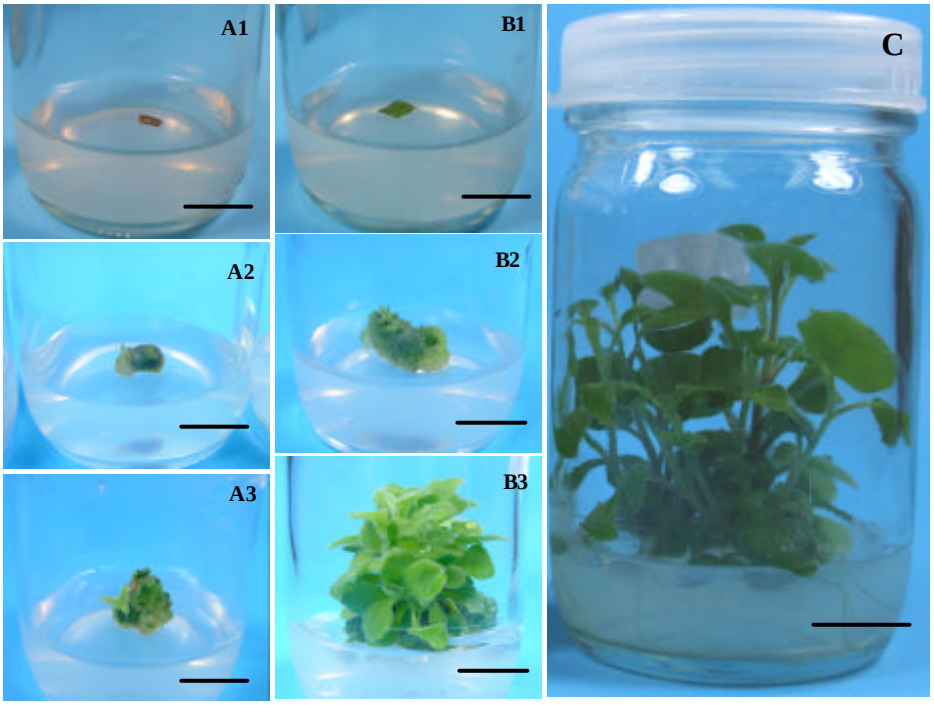

Figure 20 Indirect shoot regeneration of *G. procumbens* explants on MS medium with TDZ and 2.69 µM NAA. (A) Internode explant, (B) Leaf explant, (A2-B2) Callus formations, 4 weeks after inoculation, (A3-B3) Regenerated shoot after transfer to MS medium with 4.44 µM BA for 4 weeks, (C) Shoot elongation and rooting, after 4 weeks on MS (PGR-free) medium. Scale bars = 1 cm

1.4 Acclimatization

The vessels of regenerated plants were placed at room temperature for 3-5 days. They were carefully taken out of the medium and washed to remove all of medium. After that, they were placed into the cultivation trays containing sand and rice shell ash (1:1) and acclimatized in a saran tent under greenhouse conditions. Water was sprayed about 4-5 times a day for 2 weeks to control the humidity, and then adjust to 1-2 times a day for another 2 weeks. After 4 weeks, the rooted plantlets were successfully achieved with 95% survival rate (Figure 21).

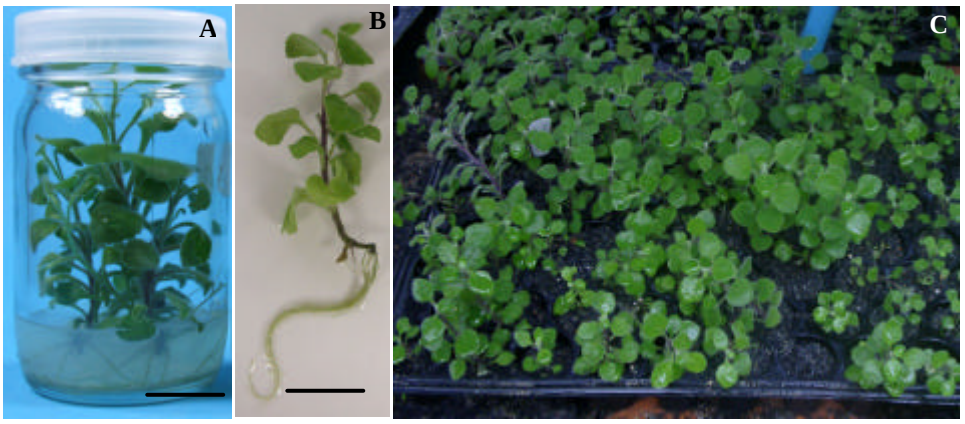

Figure 21 Acclimatization of *G. procumbens* regenerated plants. (A) *In vitro* regenerated plants, (B) Rooted plantlets, (C) Regenerated plantlets transplanted to a tray containing sand and rice shell ash (1:1). Scale bar = 2 cm.

2. Field cultivation and harvesting

After the regenerated plants were transplanted to an experimental field, the plants normally grew under the field conditions. The flowers forming and blooming showed in 2-4 months of growth. Each month, ten plants were harvested. Yields of plant material at 1-2 months-old were lower than those of at 3-5 months-old plants. High numbers of leaves at 3-5 months-old plant material were 351.12±47.73, 430.00±62.01 and 436.50±55.87, respectively. Averages of plant height were 15.00–64.37 cm. Averages of plant fresh weight and dried weight were 5.65-526.61 g/plant and 0.38-50.61 g/plant. Data of plant height, leaf size, number of leaves and flowers, fresh weight, and dry weight showed in table 16. The plants under field conditions and plant materials harvesting showed in figure 22.

Table 16 Yield of *G. procumbens* regenerated plants at different growth stage under field conditions (mean ± S.E).

Yield	Ages of plant (months)				
	1	2	3	4	5
Plant height (cm)	15.00 ± 0.82	30.86 ± 1.92	55.25 ± 2.58	64.37 ± 1.77	52.62 ± 3.02
Diameter (cm)	0.36 ± 0.03	0.41 ± 0.02	0.61± 0.03	0.68 ± 0.02	0.71 ± 0.06
Leaf size (cm)					
- width	2.52 ± 0.05	4.32 ± 0.14	4.35 ± 0.19	3.70 ± 0.17	3.37 ± 0.07
- length	3.34 ± 0.05	6.11 ± 0.23	6.10 ± 0.38	6.63 ± 0.28	5.99 ± 0.10
Number of leaves	17.75 ± 0.98	61.43 ± 19.11	351.12 ± 47.73	430.00 ± 62.01	436.50 ± 55.87
Number of inflorescences	0	0	9.50 ± 2.02	19.57 ± 3.53	0
Fresh weight (g/plant)	5.65 ± 0.46	72.34 ± 15.84	400.91 ± 59.32	526.61 ± 84.73	467.62 ± 52.06
Dry weight (g/plant)	0.38 ± 0.03	3.71 ± 0.90	29.83 ± 4.43	50.61 ± 7.46	44.65 ± 5.34

Figure 22 *G. procumbens* from the field experiment. (A) Micropropagated *G. procumbens* grown under field conditions, (B1-4) Plant materials harvested after 2, 3, 4, and 5 months of cultivation.

3. Chemical analysis of micropropagated *Gynura procumbens*

The calibration curve of stigmasteryl-3-*O*-ß-D-glucopyranoside peak area showed a linear regression with correlation coefficient (R^2) of 0.9996, when detected at 210 nm UV absorbance (Figure 23). This calibration curve was used for quantitative determination of Ga content in plant material.

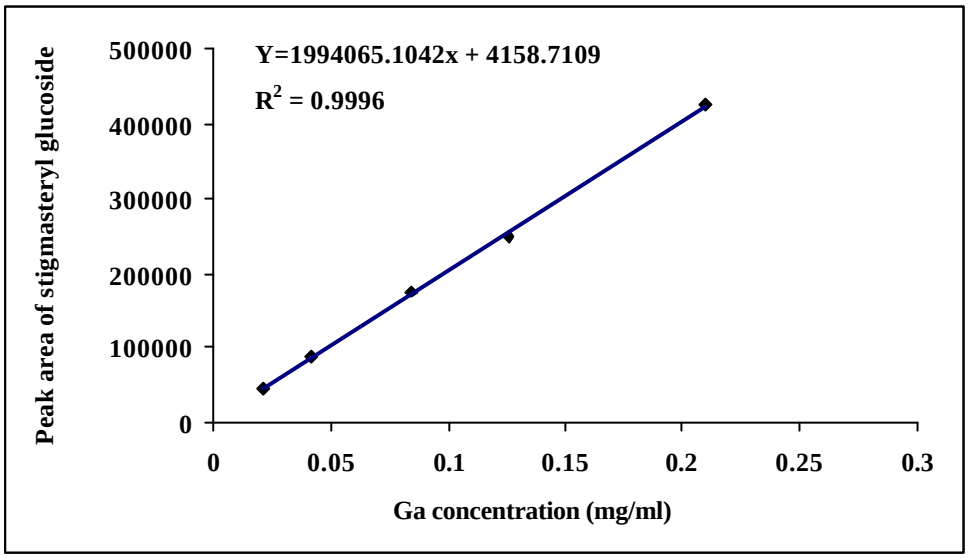

Figure 23 Calibration curve of a mixture of stigmasteryl and sitosteryl-3-*O*-ß-D-glucopyranosides (Ga) when detected at 210 nm UV absorbance.

Figure 24 and 25 showed the HPLC chromatogram of a mixture of stigmasteryl and sitosteryl-3-*O*-ß-D-glucopyranosides (Ga) and a regenerated plant extract, respectively. The amounts of Ga in the leaves and stems from the regenerated plants at 2-5 months-old plant material were between 0.42 to 0.74 mg/g dried weight (the calculation based on stigmasteryl glucoside). The highest amounts of Ga were 0.74 mg/g dried weight at three months-old plants indicated an optimum harvesting time of the plant (Figure 26). Table 17 demonstrated the dry leaves and stems (g) per plant and amount of Ga (mg) per plant harvested from 2 to 5 months. The dry phytomasses and amounts of Ga per plant were significant different ($p<0.001$), whereas, the amounts of Ga per gram extract was not significant different ($p<0.064$). The dry leaves and stems increased from 29.83 g/plant at the 3rd month to 50.64 and 44.65 g/plant at the 4th and 5th month, respectively. Therefore, the Ga per plant were 22.69, 21.18, and 24.23 mg/plant, when the plant was harvested at the 3rd, 4th, and 5th month, respectively.

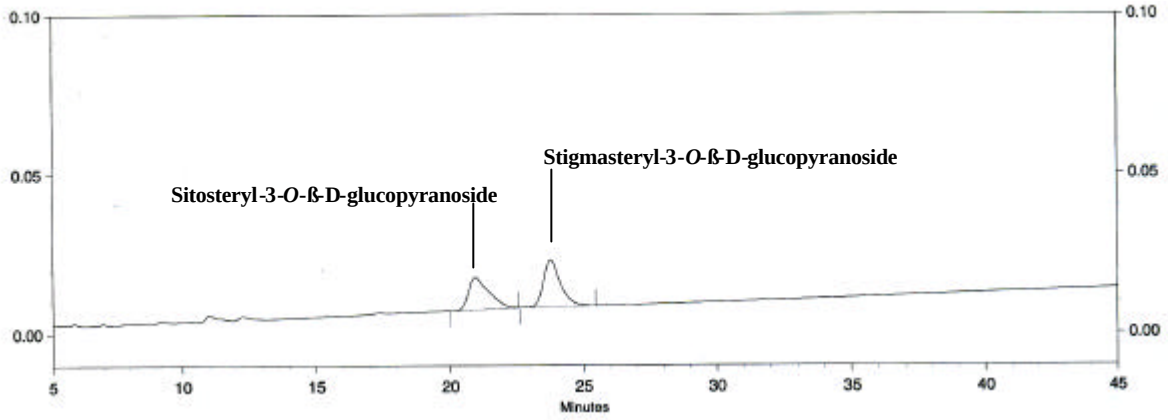

Figure 24 HPLC chromatogram of a mixture of stigmasteryl and sitosteryl-3-*O*-ß-D-glucopyranosides (Ga)

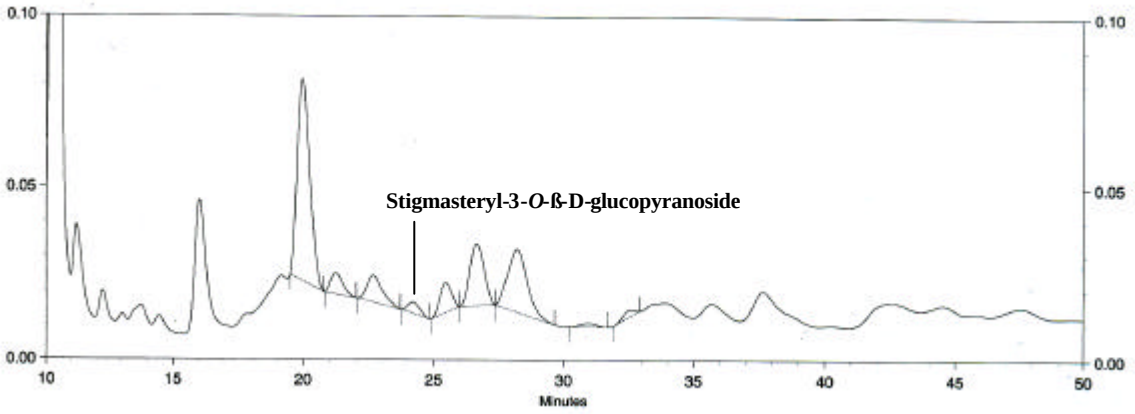

Figure 25 HPLC chromatogram of *a G. procumbens* regenerated plant extract.

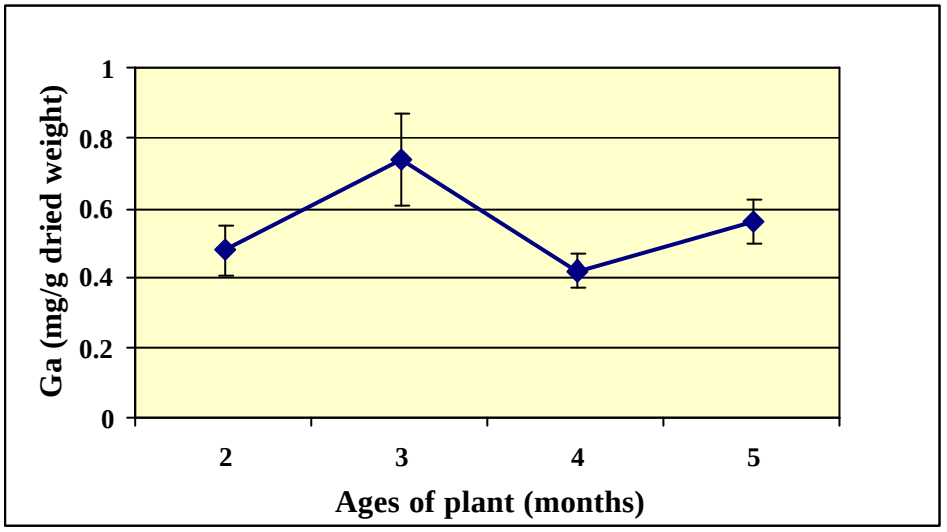

Figure 26 Content of a mixture of stigmasteryl and sitosteryl-3-*O*-ß-D-glucopyranosides (Ga) in micropropagated *G. procumbens* at different growth stages under field conditions. Vertical lines indicate SE.

Table 17 Dry weight and Ga contents of *G. procumbens* regenerated plant at different growth stages (mean ± S.D.)

Sample	2 months			3 months			4 months			5 months		
	DW/plant (g)	Ga (mg/g)	Ga/plant (mg)	DW/plant (g)	Ga (mg/g)	Ga/plant (mg)	DW/plant (g)	Ga (mg/g)	Ga/plant (mg)	DW/plant (g)	Ga (mg/g)	Ga/plant (mg)
1	6.02	0.75	4.52	21.43	1.41	30.22	85.40	0.29	24.77	30.54	0.42	12.83
2	9.18	0.82	7.53	53.37	1.04	55.50	53.92	0.68	36.67	33.16	0.82	27.19
3	3.56	0.44	1.57	20.66	0.53	10.95	70.74	0.47	33.37	56.15	0.82	46.04
4	3.86	0.49	1.89	37.57	0.61	22.92	53.54	0.49	26.23	71.10	0.31	22.04
5	2.22	0.25	0.56	19.50	0.39	7.61	46.75	0.45	21.04	38.55	0.44	16.96
6	3.72	0.56	2.08	24.49	1.12	27.43	47.56	0.15	7.13	37.45	0.65	24.34
7	2.08	0.19	0.40	40.62	0.46	18.69	21.62	0.45	9.73	31.94	0.48	15.33
8	1.38	0.33	0.46	21.00	0.39	8.19	25.60	0.41	10.49	58.30	0.50	29.15
Mean (S.D.)	**4.00* (2.37)**	**0.48 (0.21)**	**2.38* (2.32)**	**29.83* (11.72)**	**0.74 (0.36)**	**22.69* (14.78)**	**50.64* (19.76)**	**0.42 (0.14)**	**21.18* (10.43)**	**44.65* (14.14)**	**0.55 (0.18)**	**24.23* (9.84)**

*Significant difference at $p<0.001$

CHAPTER VI
DISCUSSIONS

1. *In vitro* propagation of *Gynura procumbens*

1.1 Direct shoots regeneration

1.1.1 Two-step induction

A two-step multiplication was used in this thesis. At first the node explants were maintained on MS media supplemented with plant growth regulators, and then transferred to cultured on MS without plant growth regulator. After a series of 6-week PGR treatment and 8-week culture on MS without PGR, the maximum shoot induction rate of 202.50±20.75 shoots/responding explant was revealed, when using 9.08 μM TDZ as a plant growth regulator. Although, this technique was also applied for the micopropagation of *Eclipta alba* (28), *Cichorium intybus* (12), *Petasites hybridus* (16), and *Spilanthes acmella* (20, 29). Those reports used two different media with PGR for shoot multiplication, but in this investigation, MS with PGR was used only in the first step of induction and PGR-free MS medium for the step of shoot proliferation. The results indicate that an optimum exposure time of explants in PGR-supplemented medium followed by the withdrawal of PGR effectively triggered shoot multiplication in *G. procumbens*. The PGR may be needed for initiating the multiplication of bud meristems. Subsequently, incubation on PGR- free MS medium led the explants to elongation of regenerated shoots as well as induction of more shoot initiations. These results agree with those found in Zingiberaceous medicinal plants, i.e. *Curcuma longa* L. (41, 42), *Gagnepainia godefroyi* K. Schum., *Gagnepainia thoreliana* (Baill.) K. Schum. (43), *Kaempferia parviflora* Wall. ex Baker (44), and *Zingiber petiolatum* (Holttum) I.Theilade (45).

1.1.2 Duration of the explant incubation on PGR-supplemented MS media and PGR-free MS medium

In experiment 1.1, the duration the explant incubation on the PGR-supplemented MS media was 4 weeks and adjusted to 6 weeks in experiment 1.2. The shoot regeneration rates increased in all treatments almost 2 times in both experiments after PGR treatment (7.93-12.69 and 12.10-26.60 shoots/responding explant for 1.1 and 1.2) and incubation on PGR-free MS medium (12.79-26.58 and 18.00-52.80 shoots/responding explant for 1.1 and 1.2). The result indicated that the duration of PGR-treatment affected the shoot proliferation of *G. procumbens*. Therefore, the length of the incubation period on PGR-supplemented MS media was adjusted to 6 weeks in experiments 2 and 3.

After the culture on MS without PGR, the average shoot lengths of the regenerated shoots obtained from experiment 1.2 (2.64-4.16 cm) were slightly shorter than those of experiment 1.1 (2.20-3.11cm). Thus, the length of the incubation period on MS without PGR was extended to 8 weeks.

1.1.3 Effect of plant growth regulators

Figures 10 and 14 indicate that TDZ is a more effective cytokinin than BA for the induction of multiple shoots from *G. procumbens* node explants. The highest shoot regeneration rate revealed at the concentration of 9.08 μM TDZ (202.50±20.75 shoots/responding explant), while the high level of TDZ (18.16-72.64 μM) inhibited shoot regeneration. Whereas, the maximum shoot regeneration rate obtained from BA treatment was 115.54±14.75 shoots/responding explant obtained at the concentration of 35.52 μM BA. The success of direct shoot regeneration indicated TDZ is a more effective cytkinin than BA for the induction of multiple shoots of *G. procumbens* explants. TDZ, a non-purine cytokinin-like compound, has been shown to exhibit stronger effects than conventional cytokinins over a wide rang species. TDZ has been come an integral component in the tissue culture of woody as well as herbaceous crop species (46). Its mode of action may be attributed to its ability to induce cytokinin accumulation (47) an also enhance the accumulation and translocation of auxin within TDZ expose tissue (48). The higher stability of TDZ, due to its resistance to cytokinin oxidase (49), may be one reason for its potency.

Addition of NAA did not enhance shoot regeneration of *G. procumbens*. Whereas shoots length of regenerated shoot was longer than regenerated shoot in BA or TDZ treatments alone.

1.2 Indirect shoot regeneration

In this present study, when node, intenode, and leaf explants were compared using BA and 2,4-D as plant growth regulators, internode explants formed callus with the highest rate, 4 weeks after inoculation (average callus intensity = 2.17, 2.73, 2.46, respectively, calculated across all PGR concentrations for each explant type). However, all explant types formed callus with almost the same intensity at week 8^{th} (average callus intensity = 2.75, 2.80, 2.83, respectively). Only node explant formed shoots during inoculation on the callus induction media (Tables 8-10). After transfer to culture on the indirect shoot proliferation medium I (MS with 4.44 μM BA) and II (PGR-free MS), only the calli derived from MS with PGR at the proportion of BA: 2,4-D not less than two could regenerate shoots (Table 11). The maximum shoot regeneration rate of 71.28±18.11 shoots/responding explant with a responding rate of 100% was obtained from the nodal callus induced by 8.87 μM BA and 0.45 μM NAA.

Using BA and NAA for callus induction, the combination of BA and NAA gave a good result on callus induction compared to BA alone in both explant type. Leaf explants formed callus with a higher rate than that of internode explant (average callus intensity = 3.00 and 2.63 respectively, calculated across all BA-NAA treatments for each explant type). After transfer to culture on the shoot proliferation medium I and II, the calli derived from leaf showed better shoot organogenesis. The maximum shoot regeneration rate of 26.50±9.39 shoots/responding explant with a responding rate of 25% was obtained from the leaf-derived callus induced by 35.52 μM BA and 2.69 μM NAA. For internode-derived callus, the maximum shoot regeneration rate of 9.33±3.84 shoots/responding explant with a responding rate of 16.67% was obtained from the callus induced by 35.52 μM BA and 2.69 μM NAA (Tables 12-13).

Using TDZ and NAA for callus induction, the combination of TDZ and NAA gave a good result on callus induction compared to TDZ alone in both explant

type. Only leaf explants gave a good response for both callus and shoot formation. The maximum total shoots was obtained from leaf-derived callus from the treatment of 9.08 μM TDZ and 2.69 μM NAA. This treatment revealed the responding frequency of 33.33% with an induction rate of 10.75±8.75 shoots/response explant which made the highest total number of regenerated shoots (Tables 14-15).

Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation, both of which are known to be affected by not only exogenous plant growth regulators but also endogenous content of the hormones. Different tissues may have different levels of endogenous hormones and, therefore, the type of explant source would have a critical impact on the regeneration success.

2. Field cultivation and chemical analysis of micropropagated *Gynura procumbens*

After the regenerated plants were transplanted to an experimental field, the plants normally grew under the field conditions. The plants were in bloom during the 3rd – 4th months. Yield of 1 month-old plant was very small, therefore the analysis could not be performed. The amounts of a stigmasteryl and sitosteryl-3-*O*-β-D-glucopyranosides (Ga) in the leaves and stems from 2-5 months-old plant materials were between 0.42 to 0.74 mg/g dried weight (the calculation based on stigmasteryl glucoside). The result was relatively the same as the amounts of Ga obtained from normally cultivated plants using the same extraction method, the amount of 0.36 to 0.62 mg/g dried weight were reported (unpublished data). However, the amounts of Ga of our report were less than those of Jiratchariyakul and Ruangwises (24). They used one-month maceration for extracting the plant, the amount of Ga were 3.50 to 5.50 mg/g dried weight from 10 *G. procumbens* Thai accessions.

The highest amount of Ga was 0.74 mg/g dried weight, three months after transplantation indicating an optimum harvesting time of the plant. This result demonstrated that the maximum amount of Ga was correlated with the blooming period of the plant.

CHAPTER VII
CONCLUSIONS

1. *In vitro* propagation of *Gynura procumbens*

1.1 Direct shoot regeneration

The results obtained from the described experiments demonstrated that:

- A two-step multiplication using MS media supplemented with plant growth regulators for shoot induction and PGR-free MS for shoot proliferation is well suited for the direct shoot induction of *G. procumbens*;
- Both *in vivo* and *in vitro* explants can be used for micropropagation of *G. procumbens*;
- The duration of PGR-incubation affected the shoot multiplication of *G. procumbens*;
- TDZ is more effective than BA for the induction of multiple shoots from *G. procumbens* node explants;
- Addition of NAA did not enhance shoot regeneration of *G. procumbens*;
- The efficient conditions for direct shoot induction of *G. procumbens* were culturing node explants on MS medium supplemented with 9.08 µM TDZ for 6 weeks prior to transfer to culture on MS medium without PGR for 8 weeks. After a total of 14-week cultures, the maximum shoot induction rates of 202.50±20.75 shoots/responding explant was obtained;
- Using BA as a plant growth regulator, the maximum shoot induction rate was obtained when using the same protocol with with 35.52 µM BA as a plant growth regulator (115.54±19.56 shoots/responding explant);
- Rooting was spontaneous achieved on MS without PGR;
- The regenerated plants were successfully transplanted to sand and rice shell ash (1:1) with survival rates of 95%.

1.2 Indirect shoot regeneration

The results obtained from the described experiments demonstrated that:

- Among all explant types, i.e. node, internode, and leaf explants, which responded to plant growth regulators at different levels of callus formation, the leaf explants showed the highest callus formation;
- Combinations of cytokinins (BA and TDZ) and auxins (2, 4-D and NAA) demonstrated higher callus induction effects than those of cytokinin alone;
- The efficient protocol for indirect shoot induction of *G. procumbens* was culturing node explants on MS medium supplemented with 8.87 µM BA and 0.45 µM 2,4-D for 8 weeks prior to transfer to culture on MS medium with 4.44 µM BA for 4 weeks and further culture on MS medium without PGR for 4 weeks. After a total of 16-week cultures, the maximum shoot induction rates of 71.28±18.11 shoots/responding explant with a responding rate of 100% was obtained;
- For leaf explants, the maximum shoot induction rate was obtained when culturing the explants on MS medium with 35.52 µM BA and 2.69 µM NAA for 4 weeks prior to transfer to culture on MS medium with 4.44 µM BA for 4 weeks and MS medium without PGR for 4 weeks. After a total of 12-week cultures, the maximum shoot induction rates of 26.50±9.39 shoots/responding explant with a responding rate of 25% was obtained;
- For internode explants, the maximum shoot induction rate was obtained when using the same protocol of leaf explant (9.33±3.84 shoots/responding explant with a responding rate of 16.67%);
- Rooting was spontaneous achieved on MS without PGR.

The complete protocols developed for the micropropagation of *Gynura procumbens via* direct and indirect shoot regeneration are described in figures 27 -29.

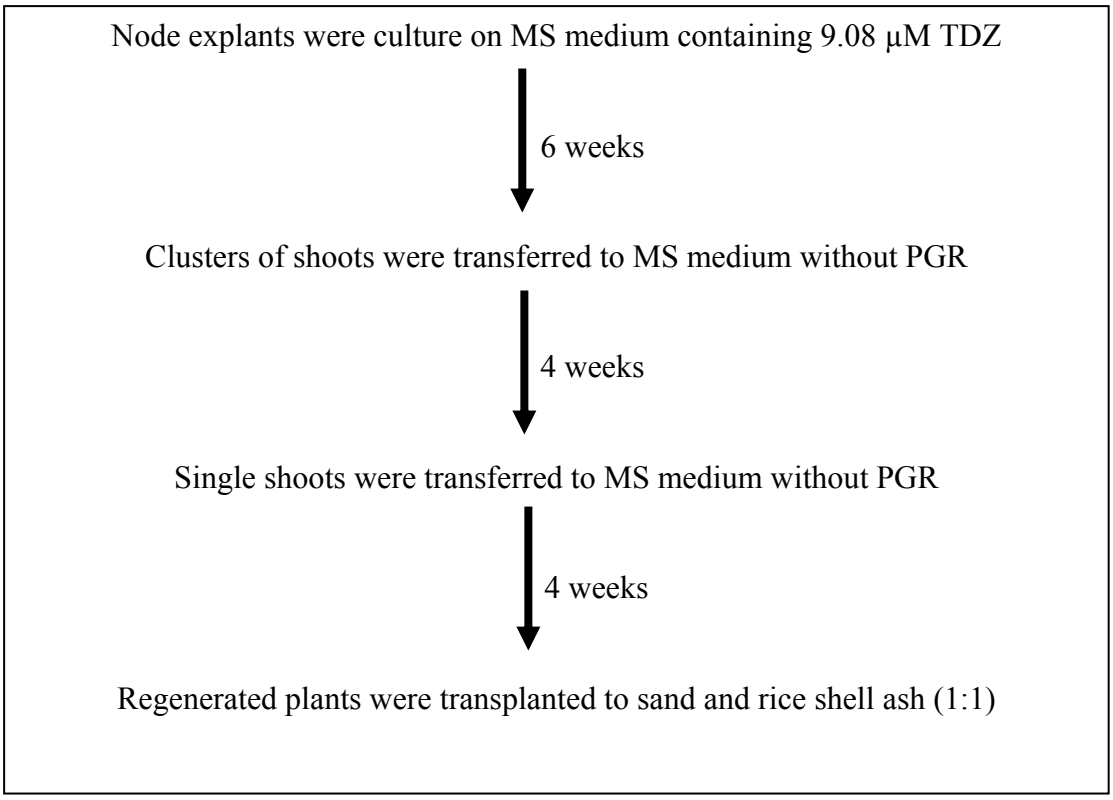

Figure 27 A schematic diagram of the *in vitro* propagation protocol for *G. procumbens* node explant *via* direct shoot regeneration

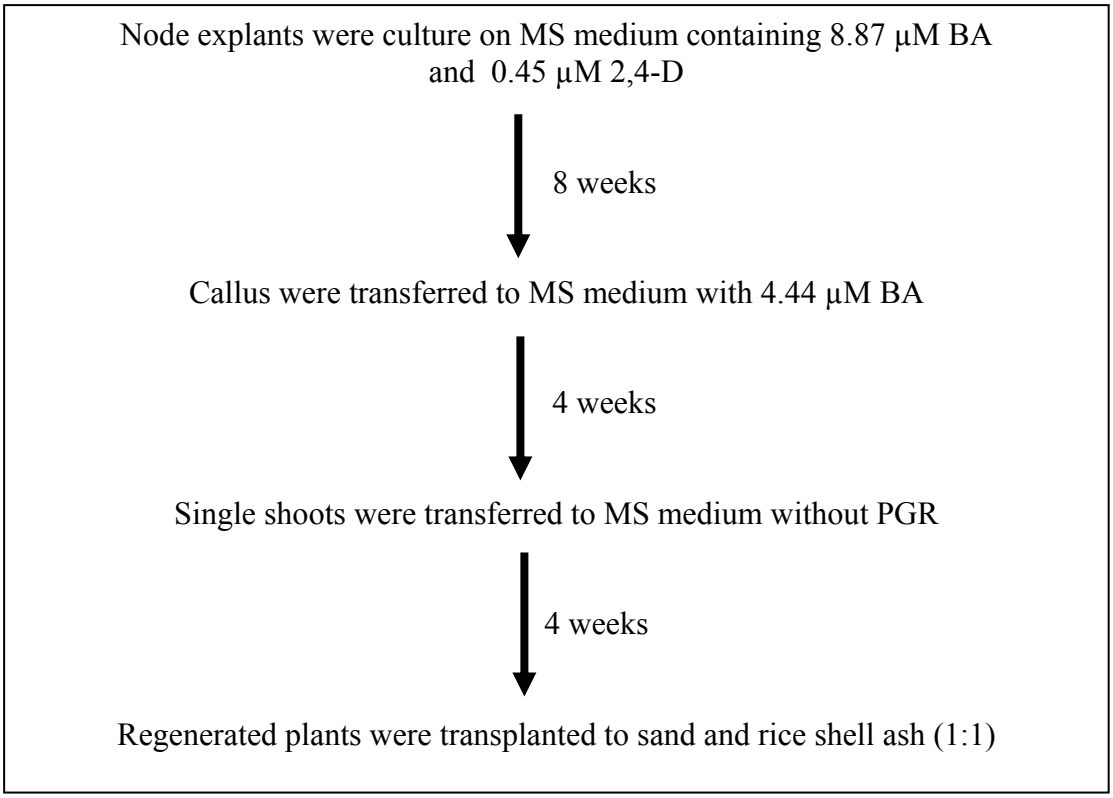

Figure 28 A schematic diagram of the *in vitro* propagation protocol for *G. procumbens* node explant *via* indirect shoot regeneration

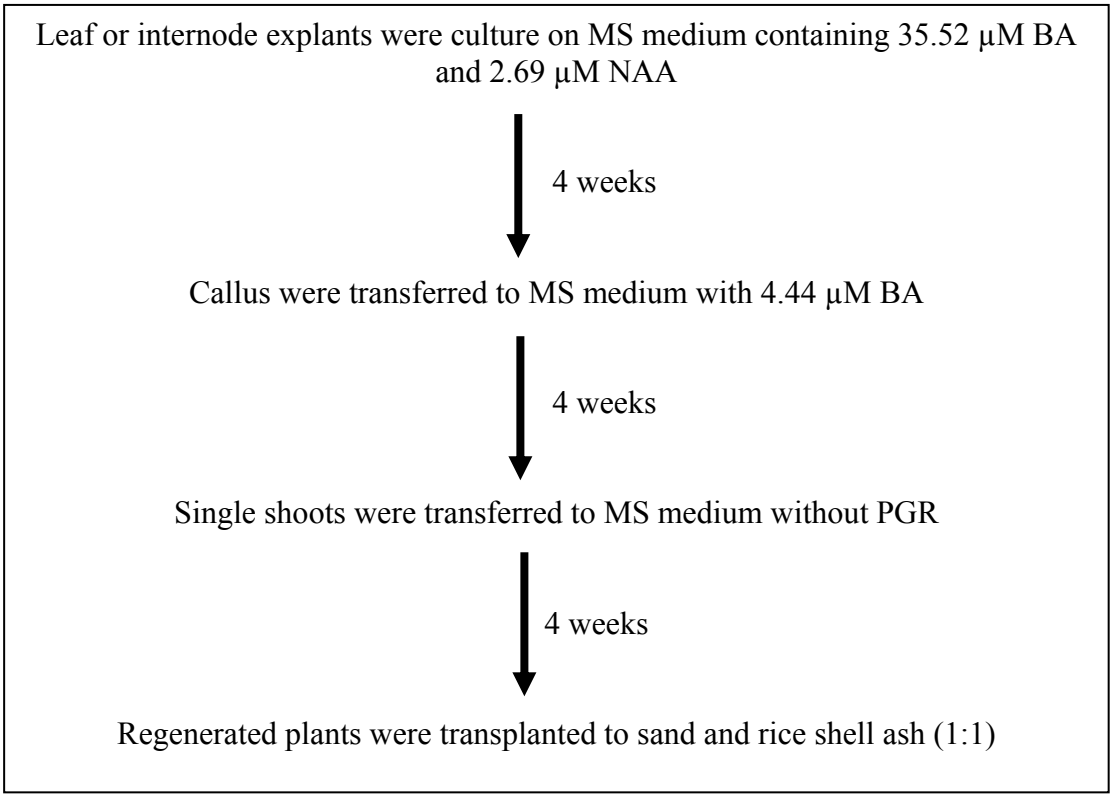

Figure 29 A schematic diagram of the *in vitro* propagation protocol for *G. procumbens* leaf and internode explant *via* indirect shoot regeneration

2. Field cultivation and chemical analysis of micropropagated *Gynura procumbens*

After the regenerated plants were transplanted to an experimental field, the plants successfully grew with normal characters. The plants were blooming during the 3^{rd} – 4^{th} months of cultivation. The amount of a stigmasteryl and sitosteryl-3-*O*-β-D-glucopyranosides (Ga) was 0.74 mg/g dried weight (calculation based on stigmasteryl-3-*O*-β-D-glucopyranosides) at the 3^{rd} month of plantation indicating an optimum harvesting time of the plant.

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APPENDIX
DATA FROM TISSUE CULTURE EXPERIMENTS

Table 18 Effect of BA concentrations and 2.69 µM NAA on the direct shoot regeneration of *in vivo G. procumbens* node explant for 4 weeks (Experiment 1.1: Figure 5A and 6A).

BA (µM)	NAA (µM)	Shoots/response explant± S.E	Shoot length (cm) ± S.E.	% Shoot response ± S.E.	% Rooting ± S.E.
0	0	1.00 ± 0.00	1.67 ± 0.22	100 ± 0.00	0.00 ± 0.00
4.44	0	7.93 ± 1.12	0.62 ± 0.04	100 ± 0.00	0.00 ± 0.00
8.87	0	11.54 ± 2.31	0.64 ± 0.02	100 ± 0.00	0.00 ± 0.00
17.76	0	12.50 ± 1.58	0.76 ± 0.05	100 ± 0.00	0.00 ± 0.00
35.52	0	12.69 ± 1.83	0.73 ± 0.05	100 ± 0.00	0.00 ± 0.00
4.44	2.69	9.40 ± 0.71	1.07 ± 0.05	100 ± 0.00	0.00 ± 0.00
8.87	2.69	10.08 ± 0.98	0.90 ± 0.06	100 ± 0.00	0.00 ± 0.00
17.76	2.69	10.00 ± 1.00	0.93 ± 0.08	100 ± 0.00	0.00 ± 0.00
35.52	2.69	11.93 ± 0.66	0.86 ± 0.05	100 ± 0.00	0.00 ± 0.00

Table 19 Effect of BA concentrations and 2.69 µM NAA on the direct shoot regeneration of *in vivo G. procumbens* node explant and transferred on MS (PGR-free) medium for 6 weeks (Experiment 1.1: Figure 5B and 6B).

BA (µM)	NAA (µM)	Shoots/response explant± S.E.	Shoot length (cm) ± S.E.	% Shoot response ± S.E.	% Rooting ± S.E.
0	0	2.21 ± 0.35	4.88 ± 0.59	100 ± 0.00	100 ± 0.00
4.44	0	12.79 ± 1.97	3.51 ± 0.29	100 ± 0.00	100 ± 0.00
8.87	0	20.21 ± 3.28	3.33 ± 0.27	100 ± 0.00	100 ± 0.00
17.76	0	24.42 ± 3.46	3.30 ± 0.20	100 ± 0.00	100 ± 0.00
35.52	0	26.58 ± 5.06	3.49 ± 0.23	100 ± 0.00	100 ± 0.00
4.44	2.69	12.73 ± 1.33	4.16 ± 0.26	100 ± 0.00	100 ± 0.00
8.87	2.69	16.00 ± 1.62	3.99 ± 0.31	100 ± 0.00	100 ± 0.00
17.76	2.69	17.18 ± 2.74	3.19 ± 0.24	100 ± 0.00	100 ± 0.00
35.52	2.69	17.13 ± 1.12	2.64 ± 0.15	100 ± 0.00	100 ± 0.00

Table 20 Effect of BA concentrations and 2.69 µM NAA on the direct shoot regeneration of *in vivo G. procumbens* node explant for 6 weeks (Experiment 1.2: Figure 7A and 8A).

BA (µM)	NAA (µM)	Shoots/response explant± S.E.	Shoot length (cm) ± S.E.	% Shoot response ± S.E.	% Rooting ± S.E.
0	0	1.00 ± 0.15	1.65 ± 0.52	100 ± 0.00	0.00 ± 0.00
4.44	0	14.30 ± 2.24	1.08 ± 0.15	100 ± 0.00	0.00 ± 0.00
8.87	0	23.20 ± 3.29	1.23 ± 0.24	100 ± 0.00	0.00 ± 0.00
17.76	0	24.60 ± 3.25	0.98 ± 0.08	100 ± 0.00	0.00 ± 0.00
35.52	0	25.50 ± 4.03	0.81 ± 0.08	100 ± 0.00	0.00 ± 0.00
4.44	2.69	12.10 ± 0.98	1.76 ± 0.16	100 ± 0.00	0.00 ± 0.00
8.87	2.69	18.30 ± 2.57	1.54 ± 0.22	100 ± 0.00	0.00 ± 0.00
17.76	2.69	26.60 ± 4.13	1.40 ± 0.12	100 ± 0.00	0.00 ± 0.00
35.52	2.69	20.10 ± 4.06	1.21± 0.11	100 ± 0.00	0.00 ± 0.00

Table 21 Effect of BA concentrations and 2.69 µM NAA on the direct shoot regeneration of *in vivo G. procumbens* node explant and transferred on MS (PGR-free) medium for 4 weeks (Experiment 1.2: Figure 7B and 8B).

BA (µM)	NAA (µM)	Shoots/response explant± S.E.	Shoot length (cm) ± S.E.	% Shoot response ± S.E.	% Rooting ± S.E.
0	0	2.20 ± 0.62	3.37 ± 0.48	100 ± 0.00	100 ± 0.00
4.44	0	24.10 ± 3.00	2.74 ± 0.29	100 ± 0.00	100 ± 0.00
8.87	0	32.80 ± 3.01	2.83 ± 0.25	100 ± 0.00	100 ± 0.00
17.76	0	43.20 ± 5.87	2.20 ± 0.20	100 ± 0.00	100 ± 0.00
35.52	0	52.80 ± 10.00	2.34 ± 0.17	100 ± 0.00	100 ± 0.00
4.44	2.69	18.00 ± 1.73	3.11 ± 0.24	100 ± 0.00	100 ± 0.00
8.87	2.69	29.60 ± 3.25	3.05 ± 0.33	100 ± 0.00	100 ± 0.00
17.76	2.69	37.20 ± 4.68	2.97 ± 0.27	100 ± 0.00	100 ± 0.00
35.52	2.69	38.70 ± 10.96	2.62 ± 0.22	100 ± 0.00	100 ± 0.00

Table 22 Effect of BA concentrations and 2.69 µM NAA on the direct shoot regeneration of *in vitro* *G. procumbens* node explant for 6 weeks (Experiment 2: Figure 9A, 10A and 11A).

BA (µM)	NAA (µM)	Shoots/response explant± S.E.	Shoot length (cm) ± S.E.	% Shoot response ± S.E.	% Rooting ± S.E.
0	0	1.16 ± 0.11	0.98 ± 0.16	92.31 ± 7.69	53.84 ± 14.39
4.44	0	4.92 ± 0.80	0.57 ± 0.03	92.31 ± 7.69	0.00 ± 0.00
8.87	0	7.67 ± 2.01	0.58 ± 0.03	92.31 ± 7.69	0.00 ± 0.00
17.76	0	6.75 ± 1.32	0.58 ± 0.03	92.31 ± 7.69	0.00 ± 0.00
35.52	0	8.84 ± 1.44	0.68 ± 0.11	100 ± 0.00	0.00 ± 0.00
71.04	0	5.50 ± 1.00	0.57 ± 0.03	92.31 ± 7.69	0.00 ± 0.00
4.44	2.69	4.00 ± 0.89	0.82 ± 0.11	46.15 ± 14.39	0.00 ± 0.00
8.87	2.69	3.00 ± 1.52	0.81 ± 0.18	38.46 ± 14.04	0.00 ± 0.00
17.76	2.69	4.67 ± 0.84	0.62 ± 0.03	46.15 ± 14.39	0.00 ± 0.00
35.52	2.69	5.33 ± 1.20	0.91 ± 0.18	23.08 ± 12.16	0.00 ± 0.00
71.04	2.69	6.20 ± 1.56	0.60 ± 0.04	38.46 ± 14.04	0.00 ± 0.00

Table 23 Effect of BA and 2.69 µM NAA on the direct shoot regeneration of *in vitro* *G. procumbens* node explant and transferred on MS (PGR-free) medium for 8 weeks (Experiment 2: Figure 9B, 10B and 11B).

BA (µM)	NAA (µM)	Shoots/response explant± S.E.	Shoot length (cm) ± S.E.	% Shoot response ± S.E.	% Rooting ± S.E.
0	0	1.16 ± 0.11	3.50 ± 0.04	100 ± 0.00	100 ± 0.00
4.44	0	48.42 ± 9.29	1.19 ± 0.12	100 ± 0.00	100 ± 0.00
8.87	0	66.75 ± 12.63	1.53 ± 0.11	100 ± 0.00	100 ± 0.00
17.76	0	65.42 ± 8.79	1.29 ± 0.12	100 ± 0.00	100 ± 0.00
35.52	0	115.54 ± 14.75	1.37 ± 0.10	100 ± 0.00	100 ± 0.00
71.04	0	36.33 ± 4.64	1.33 ± 0.09	100 ± 0.00	100 ± 0.00
4.44	2.69	9.75± 3.91	1.57 ± 0.28	100 ± 0.00	100 ± 0.00
8.87	2.69	7.50 ± 3.51	1.31 ± 0.16	100 ± 0.00	100 ± 0.00
17.76	2.69	16.42 ± 3.11	1.45 ± 0.22	100 ± 0.00	100 ± 0.00
35.52	2.69	14.75 ± 5.12	1.61 ± 0.30	100 ± 0.00	100 ± 0.00
71.04	2.69	30.67 ± 11.05	1.39 ± 0.22	100 ± 0.00	100 ± 0.00

Table 24 Effect of TDZ and 2.69 µM NAA on the direct shoot regeneration of *in vitro G. procumbens* node explant for 6 weeks (Experiment 3: Figure 13A, 14A and 15A).

TDZ (µM)	NAA (µM)	Shoots/response explant± S.E.	Shoot length (cm) ± S.E.	% Shoot response ± S.E.	% Rooting ± S.E.
0	0	1.23 ± 0.12	1.19 ± 0.14	23.08 ± 12.16	76.92 ± 12.16
4.54	0	8.00 ± 2.70	0.66 ± 0.57	69.23 ± 13.32	0.00 ± 0.00
9.08	0	9.67 ± 2.97	0.63 ± 0.06	92.31 ± 7.69	0.00 ± 0.00
18.16	0	5.56 ± 2.10	0.59 ± 0.05	69.23 ± 13.32	0.00 ± 0.00
36.32	0	6.40 ± 1.89	0.56 ± 0.05	76.92 ± 12.16	0.00 ± 0.00
72.64	0	5.89 ± 2.32	0.56 ± 0.04	69.23 ± 13.32	0.00 ± 0.00
4.54	2.69	3.17 ± 1.08	0.62 ± 0.08	46.15 ± 14.39	0.00 ± 0.00
9.08	2.69	7.37 ± 1.76	0.71 ± 0.07	61.54 ± 14.04	0.00 ± 0.00
18.16	2.69	5.50 ± 1.20	0.73 ± 0.08	46.15 ± 14.39	0.00 ± 0.00
36.32	2.69	2.50 ± 0.67	0.52 ± 0.02	46.15 ± 14.39	0.00 ± 0.00
72.64	2.69	1.25 ± 0.25	0.62 ± 0.12	30.77 ± 13.32	0.00 ± 0.00

Table 25 Effect of TDZ and 2.69 µM NAA on the direct shoot regeneration of *in vitro G. procumbens* node explant and transferred on MS (PGR-free) medium for 8 weeks (Experiment 3: Figure 13B, 14B and 15B).

TDZ (µM)	NAA (µM)	Shoots/response explant± S.E.	Shoot length (cm) ± S.E	% Shoot response ± S.E.	% Rooting ± S.E.
0	0	5.15 ± 1.05	3.71 ± 0.30	100 ± 0.00	100 ± 0.00
4.54	0	166.00 ± 40.38	3.67± 0.40	100 ± 0.00	100 ± 0.00
9.08	0	202.50 ± 20.75	2.26 ± 0.21	100 ± 0.00	100 ± 0.00
18.16	0	168.27 ± 30.15	1.79 ± 0.14	100 ± 0.00	100 ± 0.00
36.32	0	144.82 ± 25.62	1.54 ± 0.07	100 ± 0.00	100 ± 0.00
72.64	0	115.40 ± 22.19	1.37 ± 0.10	100 ± 0.00	100 ± 0.00
4.54	2.69	21.00 ± 7.65	3.80 ± 0.99	100 ± 0.00	100 ± 0.00
9.08	2.69	69.12 ± 31.03	2.54 ± 0.23	100 ± 0.00	100 ± 0.00
18.16	2.69	33.14 ± 9.41	3.31± 0.57	100 ± 0.00	100 ± 0.00
36.32	2.69	62.17 ± 38.89	2.23 ± 0.28	100 ± 0.00	100 ± 0.00
72.64	2.69	48.20 ± 15.86	1.17 ± 0.12	100 ± 0.00	100 ± 0.00

APPENDIX
DATA FROM FIELD EXPERIMENT

Table 26 Yields of micropropagated *G. procumbens* at 1 month under field conditions.

Sample	Plant height (cm)	Diameter (cm)	Leaf size (cm)		Number of leaves	Number of inflorescent	Fresh weight (g)	Dry weight (g)
			width	length				
1	19	0.3	2.37	3.22	13	0	5.29	0.43
2	15	0.3	2.45	3.39	19	0	6.33	0.51
3	17	0.4	2.48	3.24	19	0	5.35	0.36
4	15	0.4	2.85	3.60	19	0	8.27	0.49
5	16	0.4	2.45	3.43	18	0	5.36	0.34
6	13	0.3	2.44	3.30	17	0	4.11	0.29
7	13	0.5	2.54	3.15	22	0	6.15	0.39
8	12	0.3	2.60	3.35	15	0	4.31	0.24

Table 27 Yields of micropropagated *G. procumbens* at 2 months under field conditions.

Sample	Plant height (cm)	Diameter (cm)	Leaf size (cm)		Number of leaves	Number of inflorescent	Fresh weight (g)	Dry weight (g)
			width	length				
1	35	0.42	4.55	6.30	159	0	129.29	6.02
2	37	0.46	4.79	6.60	161	0	162.19	9.18
3	34	0.47	4.10	6.70	49	0	66.88	3.56
4	30	0.45	4.63	6.85	60	0	78.48	3.86
5	35	0.3	4.10	5.45	35	0	44.69	2.22
6	33	0.50	4.80	6.50	47	0	76.48	3.72
7	21	0.38	4.05	5.35	47	0	43.32	2.08
8	26	0.30	3.80	5.30	31	0	34.45	1.38

Table 28 Yields of micropropagated *G. procumbens* at 3 months under field conditions.

Sample	Plant height (cm)	Diameter (cm)	Leaf size (cm)		Number of leaves	Number of inflorescent	Fresh weight (g)	Dry weight (g)
			width	length				
1	56	0.59	3.94	6.72	212	9	331.98	21.43
2	45	0.66	4.78	7.65	422	19	694.23	53.37
3	57	0.50	5.40	4.02	274	3	283.71	20.66
4	50	0.66	4.05	6.07	585	7	544.21	37.57
5	54	0.50	3.94	5.66	272	3	251.18	19.5
6	52	0.74	3.90	5.52	341	8	313.15	24.49
7	70	0.54	4.39	6.69	486	11	538.98	40.61
8	58	0.70	4.42	6.47	217	16	249.87	21.00

Table 29 Yields of micropropagated *G. procumbens* at 4 months under field conditions.

Sample	Plant height (cm)	Diameter (cm)	Leaf size (cm)		Number of leaves	Number of inflorescent	Fresh weight (g)	Dry weight (g)
			width	length				
1	57	0.74	3.59	6.65	582	20	895.85	85.40
2	65	0.71	4.01	7.50	516	34	591.77	53.92
3	63	0.69	3.77	5.98	629	22	790.08	70.74
4	65	0.74	3.47	5.75	388	14	498.93	53.54
5	51	0.65	4.31	8.20	486	8	521.61	46.75
6	65	0.70	4.20	7.30	379	30	473.90	47.56
7	58	0.53	2.86	5.11	226	9	198.91	21.62
8	70	0.66	3.43	6.55	171	0	241.86	25.60

Table 30 Yields of micropropagated *G. procumbens* at 5 months under field conditions.

Sample	Plant height (cm)	Diameter (cm)	Leaf size (cm)		Number of leaves	Number of inflorescent	Fresh weight (g)	Dry weight (g)
			width	length				
1	48	0.73	3.14	5.55	245	0	334.16	30.54
2	49	0.52	3.38	6.15	287	0	347.52	33.16
3	43	0.68	3.62	6.16	576	0	620.56	56.15
4	59	0.88	3.53	6.38	716	0	697.47	71.10
5	59	0.58	3.34	6.07	367	0	431.33	38.55
6	50	0.90	3.27	5.83	385	0	365.49	37.45
7	45	0.20	3.05	5.67	388	0	346.63	31.94
8	68	0.85	3.60	6.10	528	0	597.82	58.30

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