2015-06-05

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Scientific Research Publishing Inc.

http://dx.doi.org/10.4236/ajps.2015.69132

American Journal of Plant Sciences, 2015, 6, 1329-1340
Published Online June 2015 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2015.69132

In Vitro Callus Induction and Shoot Regeneration from Leaf Explants of *Glinus lotoides* (L.)—An Important Medicinal Plant

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Received 26 January 2015; accepted 1 June 2015; published 5 June 2015

Abstract

G. lotoides* L. is a threatened plant that is frequently harvested for medicinal purpose. However, its distribution in the world is limited because of short period of seed viability and poor seed germination. The objective of this study was to develop *in vitro* propagation protocol for *G. lotoides* through callus induction. For callus induction, different concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (*α*-naphthalene acetic acid) and BAP (6-benzyl amino purine) were used. Seeds were sown on growth regulator-free MS medium and shoots from the *in vitro* germinated seedlings were excised and cultured on MS medium containing 0.5 mg/l BAP. Young leaves from these shoots were used as explant for callus induction and shoot regeneration. Maximum callus induction (100%) was observed on medium containing 2,4-D (0.5, 2.0, 3.5 mg/l) or NAA (2.0, 2.5 mg/l) in combination with 0.5 mg/l BAP. However, 2,4-D was the best in overall callus induction. The highest regeneration (20%) frequency was achieved on the medium containing 0.5 mg/l BAP. Highest number of shoot (2.83 ± 1.22) and shoot length (2.16 ± 0.87 cm) per explant were obtained in the presence of 0.25 mg/l BAP + 0.5 mg/l KIN (Kinetin). In shoot multiplication media, maximum mean (6.43 ± 0.87) of shoot was observed on MS medium containing 0.5 mg/l BAP. The best shoot length (1.70 ± 0.14 cm) was recorded on control medium. The highest (95%), maximum root number (14.10 ± 1.47) and root length (1.01 ± 0.10 cm) were obtained on a medium supplemented with 1.5 mg/l Indole-3-butyric acid (IBA). All the plants (100%) were survived after acclimatization in greenhouse. The present study can be useful for callus induction and indirect shoot regeneration form *G. lotoides.

Keywords

***Glinus lotoides*, Growth Regulator, Kinetin, Medicinal Plant, Regeneration**

How to cite this paper: Teshome, S. and Feyissa, T. (2015) *In Vitro* Callus Induction and Shoot Regeneration from Leaf Explants of *Glinus lotoides* (L.)—An Important Medicinal Plant. *American Journal of Plant Sciences*, **6**, 1329-1340.
http://dx.doi.org/10.4236/ajps.2015.69132

1. Introduction

Glinus lotoides L. locally known as "Mettere" is a popular medicinal plant. It is herb, whose stems are prostrate or semi-erect, and belongs to Molluginaceae family [1]. This plant is native to Eurasia and Africa and has become widespread in tropical, subtropical and warm-temperate areas worldwide [1]. In Ethiopia it grows at altitudes between 530 - 1650 m.a.s.l. [2].

G. lotoides has diverse medicinal properties in the folk medicinal systems. In Ethiopia, the seeds of *G. lotoides* are traditionally used as anti-helminthic for the prevalent tapeworm infestation [3] [4], to treat *Oestrus ovis* [5], *Moniezia* and *Thysaniezia* spp. [6]. The seeds of this plant have anticancer activity and nutrition value [7]. In south India, its dried part was used for treatment of purgative, cure for boils, bilious attack, wounds and pains, and tender shoots are cooked and eaten with normal diet twice a day for 5 - 7 days to get relief from urinary disorders [8] [9]. In Pakistan, poultice made up of leaves is applied over wounds and inflammation. The plant is used to treat syphilis and intestinal worms [10] [11]. Its anti-helminthic and taenicidal activity has been attributed to their saponins and flavonoids contents [4] [12]-[15].

Despite its economic and medicinal importance, only few studies have been carried out on *G. lotoides* and almost all researches have focused on its medicinal properties. Seed germination (*ex vitro* and *in vitro*) was reported [16]. Its seeds contain 10% of crude saponin [17] and 14% of fat [18] [19]. It also serves as source of endophytic microorganism for production of enzymes [20]. The tablet formulations from crude extract of its seeds were reported [21] [22]. The *in vivo* and *in vitro* anthelminthic activities on *Hymenolopis nana* and *Taenia saginata* worms were reported [4] [14] [17]. Its antitumour activity was evaluated [15]. Toxicological study of its extract was conducted on rats through oral administrations as single and repeat dose on both sexes [23]. The potential effects of this herb on the feeding behavior and survival of nymphs of desert locust grasshopper have been reported [24]. Extraction, isolation, quantification and structural elucidation of five hopane-type saponins (glinusides F, G, H, I, and succulentoside B) and two flavonoids (vicenin-2 andvitexin-2"-*O*-glucoside) from its seeds were described [21]. Three new hopanesaponins (A-C) and four oleananesaponins (D-G) were also isolated from its root [25].

Glinus lotoides propagates by seed but poor seed germination percentage is the limiting factor to large-scale cultivation of this species and in these days this plant is threatened [26]. Hence, to overcome all these obstacles, micropropagation is used to propagate plants within short period of time and for *in vitro* conservation. Callus cultures are used to study cell development, to obtain primary and secondary metabolites through cell suspension cultures. As *G. lotoides* contains important phytochemicals such as hopane-type saponins, oleananesaponins and flavonoids [21] [25], for pharmaceutical applications, using callus to extract these chemicals is very important. Moreover, different novel products can be obtained through bio-transformation by using suspension cultures. Developing regeneration protocol through callus has also enormous applications in using this plant to produce novel products through genetic transformation. There is no report on shoot regeneration using through callus in this plant species. Hence, the objective of the present study is to develop *in vitro* regeneration protocol through callus induction from leaf explants.

2. Materials and Methods

2.1. Callus Induction

The matured seeds of *G. lotoides* were purchased from the local market in Addis Ababa and received from Institute of Biodiversity Conservation (IBC), Addis Ababa, Ethiopia. Seeds were surface disinfected with 70% (v/v) alcohol for 3 min, followed by 10% (v/v) sodium hypochlorite solutions containing two drops of Tween-20 for 5 min and subsequently rinsed five times thoroughly with sterile distilled water. Then the seeds were sown on growth regulator-free MS [27] medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. Shoots excised from *in vitro* germinated seedlings were transferred to MS medium containing 0.5 mg/l BAP for shoot multiplication.

Young leaves from *in vitro* multiplied shoots were cultured on callus induction MS medium with adaxial (upper) surface in direct contact with the medium. Leaves were wounded perpendicular to its midrib. Culture medium was supplemented with 30 g/l sucrose and different concentrations of 2,4-D (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l) and NAA (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l) in combination with BAP (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l). The pH of the medium was adjusted to 5.8 before addition of 8 g/l agar. The medium was autoclaved at 121˚C for 15 min at 105 Kpa. After autoclaving, 25 ml of the medium was dispensed

into Petri dishes (90 mm diameter). A total of 10 leaves per Petri dish with three replications were used. Growth regulator-free MS medium was used as control. The number of shoots that induced callus was recorded after six weeks of culture.

2.2. Shoot Regeneration

Six-week-old calli were transferred to shoot regeneration medium containing various concentrations of BAP (0.0, 0.25, 0.5, 1.0, 1.5 mg/l), KIN (0.0, 0.25, 0.5, 1.0, 1.5 mg/l) alone or in combinations with 0.20 mg/l NAA. Culture was maintained under dark conditions at 25˚C ± 2˚C for a month. When shoots emerge, the cultures were placed under dim light for a week followed by transfer to full light of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 16 h photoperiod and number of calli that regenerated shoots and number of shoots per explant was recorded.

2.3. Shoot Multiplication

Regenerated micro-shoots were cultured on shoot multiplication medium containing different concentrations of BAP (0.0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 mg/L) or Kinetin (0.0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 mg/l). After autoclaving at 121˚C for 15 min at a pressure of 105 Kpa, 50 ml of the medium was dispensed into Magenta GA-7 culture vessels. A total of 30 shoots per treatmentwith10 shoots per culture vessel in 3 replications were used. All subsequent subcultures were done at 4 weeks intervals to fresh medium of the same composition. The number of shoots per explant was recorded at each subculture stage.

2.4. Rooting

Well developed microshoots were cultured on half strength MS medium containing 15 g/l sucrose and supplemented with different concentrations of IBA (0.0, 0.05, 0.1, 0.5, 1.5, 2.5 mg/l) or NAA (0.0, 0.05, 0.1, 0.5, 1.5, 2.5 mg/l). Number and length of roots were recorded after four weeks of culture.

2.5. Acclimatization

Plantlets were carefully removed from culture vessels and gently washed under running tap water followed by transfer to 20 cm diameter pots containing sand, red soil, and compost in 1:3:2 ratio and kept in greenhouse. The potted plantlets were covered with polyethylene bags. The number of survived plants was recorded after five weeks.

2.6. Culture Conditions

All cultures were maintained in culture room under light intensity of 40 $\mu mol/m^2/s$ and 16 h photoperiod provided by cool-white fluorescent lamp at 25˚C ± 2˚C. Cultures for callus induction were kept in dark room at 25˚C ± 2˚C.

2.7. Experimental Design and Data Analysis

In all experiments, Completely Randomized Design (CRD) was used. Data were subjected to one way ANOVA and least significant difference (LSD) test using statistical data analysis software SPSS version 20.0 at 5% probability level.

3. Results

3.1. Callus Induction

3.1.1. Effect of 2,4-D

Callus formation was observed after 15 days of culture. Callus formation on the explants occurred at wounding site of major veins, and then continues to growth until it covered the entire leaf section. The best responses (100%) callus induction was observed on the medium containing 0.5, 2.0, 3.5 mg/l 2,4-D (**Table 1**). Leaves on each medium containing 2,4-D in the range of 0.5 - 4.0 mg/l responded differently. At the highest concentration (4 mg/l) of 2,4-D, callus induction was declined. The formed calli were soft, compact with white, grey-white anddark-grey color (**Figure 1**).

Figure 1. Callus induction from leaf explants. (A) 2.0 mg/l 2,4-D; (B) 3.5 mg/l 2,4-D; (C) 1.0 mg/l 2,4-D + 0.5 mg/l BAP. Bars = 2 mm.

Table 1. Effect of 2,4-D on callus induction from leaf of *G. lotoides*.

2,4-D (mg·l^{-1})	Callus induction (%) (Mean ± SE)	Color	Texture
0.0	0 ± 0	-	-
0.5	100 ± 0	Grey-white	Soft
1.0	90 ± 10	Grey-white	Compact
1.5	36 ± 31.7	Grey-white	Soft
2.0	100 ± 0	Grey-white	Soft
2.5	96.6 ± 3.3	White	Soft
3.0	86.6 ± 13.3	White	Soft
3.5	100 ± 0	Dark-grey	Soft
4.0	86.6 ± 13.3	White	Compact

3.1.2. Synergistic Effect of 2,4-D and BAP

Callus formation primarily occurred at wounding site on leaf veins, and then continued to grow until it covered the entire leaf section. In the medium containing 1.0, 2.5, 4.0 mg/l 2,4-D combined with 0.5 mg/l BAP, the explants showed 96.6% of callus formation (**Table 2**). Noticeable callus response variation was observed in their morphology, color and degree of growth with changes in growth regulator combinations. The size of callus in this combination was larger than callus received from 2,4-D alone (**Figure 1**). Calli were less compact and grey-white color.

3.1.3. Synergistic Effect of NAA and BAP

Among various concentration tested, 2.0, 2.5 mg/l NAA combined with BAP (0.5 mg/l) resulted in a maximum percentage (100%) of fast callus formation (**Table 3**). Further increase inNAA concentration reduced callus production. Callus induction was not noticed in presence of 4.0 mg/l NAA and on control medium and the explants dried. In this experiment, calli were dark-grey with soft and less compact texture, fast growing and have well developed roots (**Figure 2**).

3.1.4. Synergistic Effect of BAP and NAA

After 4 weeks of culture, the most efficient callus induction percentage (93.3%) was observed in a medium supplemented with 3.0 and 4.0 mg/l BAP in combination with 0.5 mg/l NAA (**Table 4**). At lower concentration of BAP (0.5, 1.0, 1.5 mg/l) poor callus induction was observed. The calli obtained from various concentrations of BAP combined with 0.5 mg/l NAA were white and grey-white color with soft to compact texture. Under this growth regulators combination, callus has moderate growth rate and roots were developed (**Figure 2**).

3.2. Shoot Regeneration

The calli induced on MS medium containing NAA (1.0, 2.0, 2.5 mg/l) in combination with BAP (0.5 mg/l) and 2,4-D (0.5, 1.0, 2.5 mg/l) showed the highest response in shoot regeneration media. However, calli induced on

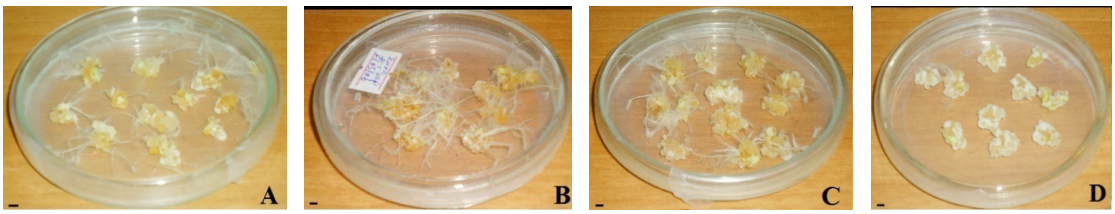

Figure 2. Callus induction from leaf explants. (A) 1.0 mg/lNAA + 0.5 mg/lBAP; (B) 2.0 mg/lNAA + 0.5 mg/lBAP; (C) 2.5 mg/lNAA + 0.5 mg/lBAP; (D) 4.0 mg/lBAP + 0.5 mg/lNAA. Bars = 2 mm.

Table 2. Effect of 2,4-D and BAP on callus induction from leaf explants.

2,4-D + BAP (mg·l^{-1})	Callus induction (%) (Mean ± SE)	Color	Texture
0.0	0 ± 0	-	-
0.5 + 0.5	83.3 ± 16.6	White	Compact
1.0 + 0.5	96.6 ± 3.3	Grey-white	Compact
1.5 + 0.5	20 ± 20	Grey-white	Compact
2.0 + 0.5	73.3 ± 26.6	Grey-white	Compact
2.5 + 0.5	96.6 ± 3.3	Grey-white	Compact
3.0 + 0.5	93.3 ± 6.6	Grey-white	Compact
3.5 + 0.5	86.6 ± 13.3	Grey-white	Compact
4.0 + 0.5	96.6 ± 3.3	Grey-white	Compact

Table 3. Effect of NAA and BAP on callus induction from leaf explants.

NAA + BAP (mg·l^{-1})	Callus induction (%) (Mean ± SE)	Color	Texture
0.0	0 ± 0.00	-	-
0.5 + 0.5	30 ± 30	Dark-grey	Soft
1.0 + 0.5	80 ± 20	Dark-grey	Soft
1.5 + 0.5	10 ± 10	Dark-grey	Soft
2.0 + 0.5	100 ± 0	Dark-grey	Soft
2.5 + 0.5	100 ± 0	Dark-grey	Soft
3.0 + 0.5	6.6 ± 6.6	Dark-grey	Compact
3.5 + 0.5	6.6 ± 6.6	Dark-grey	Compact
4.0 + 0.5	0 ± 0.00	-	-

Table 4. Effect of BAP and NAA on callus induction from leaf explants.

BAP + NAA (mg·l^{-1})	Response (%) (Mean ± SE)	Color	Texture
0.0	0 ± 0.00	-	-
0.5 + 0.5	30 ± 30	Grey-white	Soft
1.0 + 0.5	16.6 ± 16.6	Grey-white	Soft
1.5 + 0.5	16.6 ± 16.6	Grey-white	Soft
2.0 + 0.5	60 ± 30.5	Grey-white	Soft
2.5 + 0.5	73.3 ± 26.6	White	Soft
3.0 + 0.5	93.3 ± 6.6	White	Compact
3.5 + 0.5	73.3 ± 26.6	Grey-white	Soft
4.0 + 0.5	93.3 ± 6.6	Grey-white	Compact

medium containing NAA (1.0, 2.0, 2.5 mg/l) in combination with BAP (0.5 mg/l) were the most responsive in shoot regeneration. The maximum shoot regeneration percentage (20%) with 2.2 ± 0.65 mean number of shoot per callus was achieved on a medium containing 0.5 mg/l BAP (**Table 5**). Maximum mean number of shoots (2.83 ± 1.22) and mean shoot length (2.16 ± 0.87) were obtained on medium supplemented with the combination of 0.25 mg/l BAP and 0.5 mg/l KIN (**Figure 3**). Results in **Table 5** show that most calli did not result in shoot regeneration.

3.3. Shoot Multiplication

Multiplication of shoot showed the significant difference among the treatments (**Table 6**, **Figure 4**). Best number of shoot (6.43 ± 0.87) was observed on medium containing 0.5 mg/l BAP, while 4.60 ± 0.33 was obtained

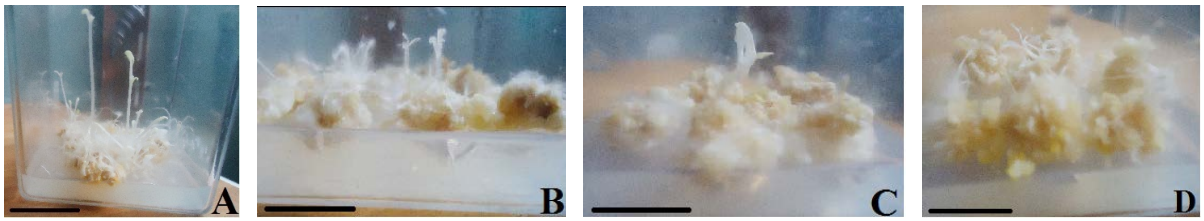

Figure 3. Shoot regeneration. (A) 0.25 mg/l BAP + 0.5 mg/l KIN; (B) 0.5 mg/l BAP; (C) 0.5 mg/l BAP + 0.2 mg/l NAA; (D) 1 mg/l BAP + 0.5 mg/l KIN. Bars = 2 cm.

Table 5. Effect of BAP and kinetin in combination with NAA on shoot regeneration from callus.

Treatments ($mg \cdot l^{-1}$)	Regeneration (%)	Mean no. of shoot per callus	Mean length of shoot
BAP			
0.0	-	-	-
0.25	6.7	0.67 ± 0.49^{c}	0.3 ± 0.19^{c}
0.5	20	2.2 ± 0.65^{b}	1.1 ± 0.08^{b}
1.0	-	-	-
1.5	6.7	0.6 ± 0.4^{c}	0.3 ± 0.2^{c}
BAP + 0.5KIN			
0.25 + 0.5	13.3	2.83 ± 1.22^{a}	2.16 ± 0.87^{a}
0.5 + 0.5	3.3	0.67 ± 0.66^{c}	0.08 ± 0.08^{c}
1.0 + 0.5	10	0.83 ± 0.47^{c}	0.29 ± 0.16^{c}
1.5 + 0.5	-	-	-
BAP + 0.2NAA			
0.25 + 0.20	-	-	-
0.5 + 0.20	3.3	0.2 ± 0.16^{d}	0.33 ± 0.33^{c}
1.0 + 0.20	-	-	-
1.5 + 0.20	-	-	-
KIN			
0.25	-	-	-
0.5	-	-	-
1.0	-	-	-
1.5	-	-	-

Means followed by the same letter(s) within column are not significantly different at $p < 0.05$.

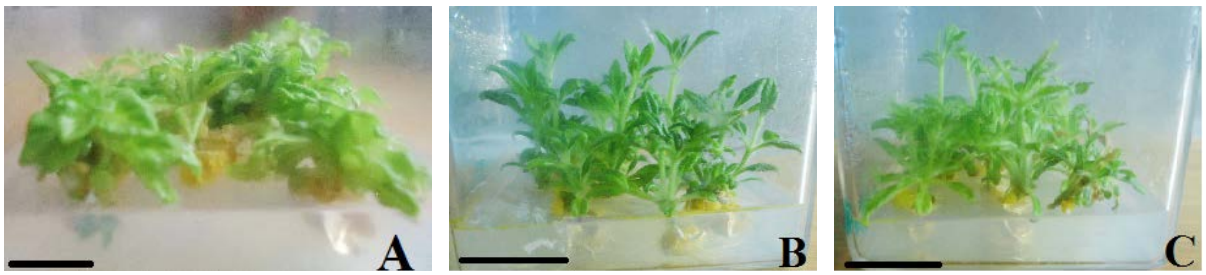

Figure 4. *In vitro* shoot multiplication on MS medium. (A) 0.5 mg/l BAP; (B) 0.5 mg/l KIN; (C) 1.0 mg/l KIN. Bars = 2 cm.

Table 6. Effect of BAP and KIN on shoot multiplication.

BAP (mg·l^{-1})	No. of shoot per explant mean ± SE	Shoot length (cm) mean ± SE
0.0	1.70 ± 0.11^{d}	1.70 ± 0.14^{a}
0.25	3.80 ± 0.34^{b}	0.93 ± 0.08^{b}
0.5	6.43 ± 0.87^{a}	0.84 ± 0.04^{bc}
0.75	2.73 ± 0.34^{cd}	0.71 ± 0.06^{cd}
1.0	2.83 ± 0.23^{bc}	0.78 ± 0.05^{bcd}
1.25	2.53 ± 0.21^{cd}	0.62 ± 0.05^{d}
1.5	3.86 ± 0.45^{b}	0.74 ± 0.04^{bcd}
1.75	3.70 ± 0.33^{b}	0.72 ± 0.04^{cd}
2.0	2.46 ± 0.17^{cd}	0.63 ± 0.04^{d}
KIN		
0.0	1.70 ± 0.11^{d}	1.70 ± 0.14^{a}
0.25	2.30 ± 0.15^{cd}	1.23 ± 0.09^{c}
0.5	3.60 ± 0.35^{b}	1.51 ± 0.08^{ab}
0.75	3.23 ± 0.32^{bc}	0.88 ± 0.07^{d}
1.0	3.66 ± 0.27^{b}	1.40 ± 0.07^{b}
1.25	2.76 ± 0.37^{c}	1.28 ± 0.08^{bc}
1.5	4.60 ± 0.33^{a}	1.03 ± 0.08^{cd}
1.75	3.70 ± 0.25^{b}	0.88 ± 0.08^{d}
2.0	2.80 ± 0.15^{c}	0.79 ± 0.05^{d}

Means followed by the same letter(s) within column are not significantly different at $p < 0.05$.

on MS medium containing 1.5 mg/l KIN. Maximum shoot length (1.70 ± 0.14 cm) was achieved on growth regulator free medium.

3.4. Rooting

The highest rooting percentage (95%), root number (14.10 ± 1.47) and root length (1.01 ± 0.10 cm) were obtained on a medium supplemented with 1.5 mg/l IBA (**Table 7**). In case of NAA, best mean number of roots (7.75 ± 1.66) and root length (0.76 ± 0.13 cm) were obtained in the presence of 0.1 mg/l. At the base of plantlets, callus was observed in the presence of 2.5 mg/l IBA and 1.5, 2.5 mg/l NAA. All plantlets were healthy and grown vigorously (**Figure 5**).

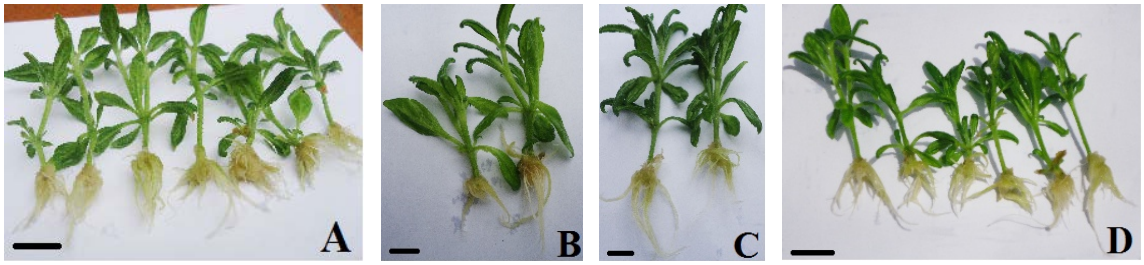

Figure 5. *In vitro* rooting of *G. lotoides* on 1/2 MS medium. (A) 1.5 mg/l IBA; (B) 0.05 mg/l IBA; (C) 0.1 mg/l NAA; (D) 0.05 mg/l NAA. Bars = 2 cm.

Table 7. Effect of IBA and NAA on rooting of *G. lotoides*.

IBA ($mg \cdot l^{-1}$)	Response (%)	Root no. per plantlet mean ± SE	Root length (cm) mean ± SE	Shoot no. per plantlet mean ± SE	Shoot length (cm) mean ± SE
0.0	40	3.95 ± 1.28^{bc}	0.50 ± 0.14^{b}	1.10 ± 0.06^{ab}	2.85 ± 0.39^{a}
0.05	75	7.05 ± 1.45^{b}	0.80 ± 0.11^{a}	1.30 ± 0.10^{a}	1.77 ± 0.12^{b}
0.1	85	6.40 ± 1.69^{b}	0.80 ± 0.08^{a}	1.20 ± 0.09^{ac}	2.92 ± 0.21^{a}
0.5	50	1.20 ± 0.09^{c}	0.50 ± 0.00^{b}	1.00 ± 0.00^{bc}	2.00 ± 0.00^{b}
1.5	95	14.10 ± 1.47^{a}	1.01 ± 0.10^{a}	1.25 ± 0.09^{a}	3.25 ± 0.25^{a}
2.5	80	5.54 ± 0.96^{b}	0.80 ± 0.11^{a}	1.00 ± 0.00^{b}	2.15 ± 0.20^{b}
NAA					
0.0	40	3.95 ± 1.28^{b}	0.50 ± 0.14^{ab}	1.10 ± 0.06^{ab}	2.85 ± 0.39^{a}
0.05	65	5.90 ± 1.23^{ab}	0.75 ± 0.13^{a}	1.20 ± 0.09^{a}	2.55 ± 0.26^{ab}
0.1	70	7.75 ± 1.66^{a}	0.76 ± 0.13^{a}	1.15 ± 0.08^{ab}	1.95 ± 0.19^{b}
0.5	70	6.00 ± 1.68^{ab}	0.70 ± 0.10^{a}	1.00 ± 0.00^{b}	1.30 ± 0.10^{bc}
1.5	25	2.05 ± 1.01^{b}	0.25 ± 0.09^{b}	1.00 ± 0.00^{b}	1.60 ± 0.11^{bc}
2.5	20	1.22 ± 0.52^{b}	0.44 ± 0.17^{ab}	1.00 ± 0.00^{b}	1.00 ± 0.00^{c}

Means followed by the same letter(s) within column are not significantly different at $p < 0.05$.

3.5. Acclimatization

All plants (100%) survived after acclimatization in greenhouse. Plantlets were grown vigorously and any morphological abnormality was not observed (**Figure 6**).

4. Discussion

4.1. Callus Induction

Plant tissue culture play a significant role in the conservation of endangered medicinal plant and enhance the production of secondary metabolites. *In vitro* production of secondary metabolite is used to reduce harvesting of plants from natural habitats. *In vitro* callus mass propagation and production of their secondary metabolites were reported on *Solanum trilobatum* [28], *Citrullus colocynthis* [29], and *Convolvulus alsinoides* [30].

Sole application of 2,4-Dwas more effective in callus induction than in combination with BAP. Similarly, callus induction frequency was higher in all media containing 2,4-D than the media with NAA and BAP. This indicates the efficiency of 2,4-D in cell division. Effectiveness of 2,4-D in callus formation was also reported in other medicinal herbs such as *Achyranthes aspera* [31], *Ocimum sanctum* [32], *Solanum trilobatum* [28], *Ionidium suffruticosum* [33].

The combination effect of 2,4-D and BAP played a significant role as plant growth regulator and has a noticeable effect on callus induction percentage than NAA in combination with BAP. The interaction effect of 2,4-D and BAP on callus induction of other medicinal plants were reported on *Tridax procumbens* [34], *Amsonia*

Figure 6. Acclimatization of *G. lotoides* after five weeks. Bar = 2 cm.

orientalis [35], *Swertia chirayita* [36], *Strobilanthus foliosus* [37], *Stevia rebaudiana* [38], *Scrophularia striata* [39].

There was positive interaction result of NAAXBAP relatively in production of fast grown calli than 2,4-D alone or in combination with BAP. Callus production efficiency of NAA and BAP on *Solanum nigrum* [40] and *Orthosiphon aristatus* [41] were reported. However, in the present study, spontaneous root formation during callus formation on medium containing NAA and BAP was observed. In *Ferula assa-foetida* [42] and *Eryngium foetidum* [43] root formation from callus on medium containing NAA was reported. A significant reduction in the percentage of callus formation with increased NAA concentration while keeping a constant BAP concentration was observed. However, increased BAP concentration at constant NAA concentration led to increased callus induction percentage.

4.2. Shoot Regeneration

The plant growth regulator and the explants played a significant role in shoot regeneration. Overall, calli from NAA X BAP were excellent in shoot regeneration as compared to calli from 2,4-D alone or 2,4-D in combination with BAP. Maximum regeneration percentage (20%) was obtained on a medium supplemented with 0.5 mg/l BAP. However, interactive effect of BAP and KIN resulted inmaximum number of shoot per callus. This is in agreement with [38] on *Stevia rebaudiana* and [31] on *Achyranthes aspera.*

All concentrations of KIN did not produce any shoots from calli. This is in agreement with [36] *in Swertia chirayita.* Poor regeneration response was observed in the presence of BAP plus NAA. This is in contrast with the results obtained from [41] *Orthosiphon aristatus* and [31] *Achyranthes aspera.*

4.3. Shoot Multiplication

The present study revealed that BAP was the most effective cytokinin than KIN for shoot multiplication in this species. Earlier reports showed the efficiency of BAP in production of multiple shoots on *Portulaca grandiflora* [44], *Thymus satureioides* [45], *Origanum sipyleum* [46], and *Salvia guaranitica* [47]. However, increased concentration of BAP and KIN beyond the optimum level resulted in the reduction of shoot per explants. The earlier reports also showed this phenomenon in *Majorana hortensis* [48], *Vitex agnus-castus* [49] and *Prunella vulgaris* [50].

Shoots developed on MS medium containing various concentrations of KIN were taller than shoots obtained on medium containing BAP. This might be BAP reduced the apical dominance than KIN. Increasing the concentration of cytokinins resulted in the production of stunted shoots in *Psoralea corylifolia* [51].

4.4. Rooting

Microshoots were tested on 10 different concentrations of auxins, IBA and NAA, for root induction. IBA was found to be the superior over NAA for root induction in this taxon. Root induction efficiency of IBA was also reported in other medicinal plant species including *Psoralea corylifolia* [51], *Chlorophytum borivilianum* [52], *Stevia rebaudiana* [53], *Origanum sipyleum* [46], *Solanum nigrum* [54], *Cunila galioides* [55], *Achyranthes aspera* [31], *Majorana hortensis* [48], *Operculina turpethum* [56].

Although, root induction was observed on MS medium enriched with different concentrations of NAA, the overall analyzed parameters such as mean root number, root length, shoot number and shoot length were lower than in case of IBA. However, the best root induction competence of NAA was reported in *Clitoria ternatea* [57], *Solanum surattense* [58] and *Thymus satureioides* [45]. At higher concentrations of IBA and NAA, root development was declined.

All plantlets were survived after acclimatization. This might be due to the occurrence of somaclonal variation that different from those of parent plants, which made the plant resistance to environmental difficulty.

5. Conclusion

An efficient protocol was developed for indirect plant regeneration. MS medium containing 2,4-D (0.5, 2.0, 3.5 mg/l) or NAA (2.0, 2.5 mg/l) in combination with 0.5 mg/l BAP was the most effective for callus induction whereas 0.5 mg/l BAP was optimum for shoot regeneration. Maximum means of shoot and root were observed on MS medium containing 0.5 mg/l BAP and 1.5 mg/l IBA respectively. All the plants were survived after acclimatization in greenhouse. Callus production from rare medical plant is used to harvest secondary metabolites and reduce the over exploitation of plants from their *in situ* habitats. *G. lotoides* is the rare medicinal herb with different groups of phytochemicals. Therefore, this protocol is very important for mass propagation of the species and also opens a new way to facilitate secondary metabolites production and isolation of pharmaceuticals from callus rather than harvesting the plant itself.

Acknowledgements

The authors are grateful to Institute of Biodiversity Conservation (IBC) for providing seed of *Glinus lotoides*, Woliata Sodo University and Institute of Biotechnology of Addis Ababa University for unreserved support.

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