

# Micropropagation of *Glinus Lotoides* L.: An Endangered Medicinal Plant

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## Abstract

*Glinus lotoides* L., an endangered medicinal herb, is valued for its dietary vegetable and medicinal properties. It is propagated by seed. However, the seeds remain viable for very short period of time resulting in scarce distribution throughout the world. Hence, the objective of this study was to enhance germination of the seeds and to develop a micropropagation protocol for this plant. For seed germination experiment, Plant Growth Regulator (PGR) free MS (Murashige and Skoog) medium, filter paper and potted soil were used. Freshly harvested seeds from green house were sowed in PGR free MS medium to serve as explants. MS media containing different concentrations of 6-Benzyl Amino Purine (BAP) and Kinetin (KIN) were used for culture initiation and multiplication. Root induction was done on half strength MS medium containing different concentrations of Indole-3-Butyric Acid (IBA). Maximum *ex vitro* germination (73.77%) was obtained from fresh (0- day- old) seeds. The highest germination response (73.55%) was obtained from fresh (0- day- old) seeds sowed on MS medium. Highest shoot initiation (98.11%) was observed on medium containing 0.5 mg/l BAP. Best shoot multiplication (8.82 shoots per explant) was achieved on MS medium containing 0.5 mg/l BAP. Maximum shoot height (2.20 cm) was attained on PGR-free MS medium. Highest root number per shoot (6.86) with 96.67% rooting response was obtained in the presence of 0.5 mg/l IBA. Maximum root length (4.06 cm) was also achieved on this medium. The plantlets were transferred to sand-red soil-compost in 2:1:1 ratio and 87% of the plantlets survived after acclimatization. Thus, this study is useful for micropropagation of this medicinally important species.

**Keywords:** Acclimatization, Rooting, Seed germination, Shoot multiplication

## Abbreviations

BAP	6-Benzylaminopurine
GA <sub>3</sub>	Gibberellic Acid
IBA	Indole-3-butyric acid
IBC	Institute of Biodiversity Conservation
KIN	Kinetin
MS	Murashige and Skoog 1962
PGRs	Plant growth regulators
UNEP	United Nations Environment Program

## 1. INTRODUCTION

Herbal medicines in most developing countries of Asia, Africa and Latin America have played a central role in health-care (Vasisht and Kumar 2002). The WHO estimates that more than 80% of the world population in developing countries depends primarily on herbal medicine for basic healthcare needs and these is also growing in developed countries (Vines, 2004). In Ethiopia, 70-90% of the population rely on traditional medicine mainly from medicinal plant and these will not diminish in the near future because modern health-care service is both limited and expensive (Vasisht and Kumar 2002; Lehoux and Chakib 2012).

*G. lotoides* L. is a medicinal plant species that belongs to the Molluginaceae family and widespread in tropical, subtropical and warm-temperate areas worldwide (Dequan and Hartmann 2003). It is locally known as "Mettere". In Ethiopia it grows at altitudes between 530-1650 meters above sea level (Gilbert, 2000). Its seeds contain 10% crude saponin (Abebe et al., 1998) and the total fat content was found to be 14% (Biftu et al., 1979; Abebe et al., 2004). It also serves as source of endophytic microorganisms for production of enzymes (El-Zayat, 2008). The tablet formulations from crude extract of its seeds were reported (Abebe, 2005; Abebe et al., 2008).

Its traditional uses are various in different countries. In Ethiopia, the seeds of *G. lotoides* are traditionally used as anti-helminthic for the prevalent tapeworm infestation (Kloos, 1976; Mulatu, 1978) and to treat *Oestrus ovis* without addition of any ingredients (Gidey et al., 2011). Its effectiveness against *Moniezia* and *Thysaniezia* spp. has been evaluated (Mesfin and Obsa 1994). Seeds of this plant have anticancer activity and nutrition value (Abebe and Youan 2010). In south India, its dried part was used for treatment of purgative, cure for boils, bilious attack, wounds, pains, and urinary disorders (Kumar et al., 2011; Shanmugam et al., 2012). In

Pakistan, poultice made up of leaves is applied over wounds and inflammation. It is a major cur for syphilis and intestinal worms (Qureshi and Bhatti 2008; Qureshi et al., 2010). Its anti-helminthic and taenicial activity has been attributed to their saponins and flavonoids contents (Chopra et al., 1956; Mulatu, 1978; Berhanu and Berhane 1980; Abebe et al., 1997; Kavimani et al., 1999).

The *in vitro* taenicial activity of plant on *Taenia saginata* worms were investigated (Mulatu, 1978). The *in vitro* and *in vivo* anti-helminthic activity in Albino mice infested with *Hymenolepis nana* worms were reported (Abebe et al., 1997; Abebe et al., 1998). The antitumour activity against Dalton's ascitic lymphoma (DAL) in Swiss albino mice of this plant has been evaluated (Kavimani et al., 1999). Toxicological study of its extract was conducted on rats' through oral administrations as single and repeat dose on both sexes (Jemal et al., 2007). The potential effect of this herb on the feeding behavior and survival of nymphs of desert locust grasshopper have been reported (Ould et al., 2001). Different hopane-type saponins and flavonoids were isolated from its seed and root (Abebe, 2005; Hamed et al., 2005)

Only a single report was available on vegetative and seed (*ex vitro* and *in vitro*) based propagation of *G. lotoides* (Balcha, 2009). It is critically endangered and there was no report on cultivation of the species (UNEP, 2012). Poor seed germination percentage and difficulty to propagate by usual vegetative propagation methods are the limiting factors for large scale cultivation of this species. There is no report on micropropagation study of *G. lotoides*. Therefore, it is important to develop an efficient micropropagation technique for *G. lotoides* to overcome all these obstacles and for mass propagation of genetically identical plants. This study is the first in its kind to investigate seed germination rate of *G. lotoides* using long term stored and freshly harvested seeds by treating with exogenous GA<sub>3</sub> and also develop micropropagation protocol.

## 2. MATERIALS AND METHODS

### 2.1. Seed Germination

#### 2.1.1. Seed collection and surface disinfection

Seeds of *G. lotoides* were harvested from stock plant in Addis Ababa university green house, and two accessions (8500 & 242326) were obtained from Institute of Biodiversity Conservation (IBC). Seeds were categorized based on their ages, fresh = 0-day-old seeds, 8500=4-year-old seeds & 242326= 10-year-old seeds. For *in vitro* germination, each seed category was tightly sealed in cotton cloth and washed with detergent under running tap water. Then, surface disinfected with 70% (v/v) alcohol for 3 min and rinsed three times with sterile distilled water, followed by 10% (w/v) sodium hypochlorite solution containing two drops of Tween-20 for 5 min and subsequently rinsed three to five times thoroughly with sterile distilled water.

#### 2.1.2. Seed germination

For *ex vitro* seed germination, seeds were sown in 20 cm diameter plastic pots containing sand, red soil and compost in a ratio of 2:1:1 respectively and kept in greenhouse under natural light condition. For *in vitro* seed germination, all seeds were soaked in different concentrations of GA<sub>3</sub> (0, 12.5, 25, 40 mg/l) for 16 h before sowing on filter paper and culturing on growth regulators-free MS medium (Murashige and Skoog 1962). MS medium was supplemented with 3% (w/v) sucrose and pH was adjusted to 5.8 before addition of 0.8% (w/v) agar. The medium was dispensed into culture vessels (baby food jars of 6 cm diameter) before autoclaving at 121°C with a pressure of 105 Kpa for 15 min. The culture was maintained in growth room under light intensity of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 16 h photoperiod provided by cool-white fluorescent lamps with a temperature of 27 $\pm$ 2 °C.

All germination experiments were conducted using three replications with a total of 900, 450 and 60 seeds on soil, MS medium and filter paper respectively. Three hundred seeds were sown per pot in soil for *ex vitro* germination whereas 150 seeds were sown per culture vessel and 20 seeds on filter paper. The seeds were monitored for germination every day and the number of germinated seeds was recorded.

## 2.2. Micropropagation

### 2.2.1. Culture initiation

Seedlings obtained from culturing of seeds on MS medium were used for culture initiation. Roots were removed from the seedlings and immediately cultured on the MS medium in Magenta GA-7 culture vessel containing 50 ml medium and sealed with Parafilm. The medium contained BAP (0.5, 1.0 mg/l) in combination with 0.5 mg/l kinetin (KIN). Four explants per culture vessel with thirteen replications per treatment were used. All cultures were maintained in culture room under light intensity of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 16 h photoperiod provided by cool-white fluorescent lamps at a temperature of 27 $\pm$ 2 °C.

### 2.2.2. Shoot multiplication

After shoot initiation, young and healthy micro-shoots were cultured on shoot multiplication medium containing different concentrations of BAP (0.0, 0.5, 1.0, 1.5, 2.0 mg/l) in combination with Kinetin (0.0, 0.5, 1.0, 1.5, 2.0 mg/l). A total of 35 shoots per treatment and seven shoots per culture vessel (Magenta GA-7) with five replications were used. Cultures were maintained in growth room with the same culture condition as for shoot

initiation. All subsequent subcultures were done to fresh medium of the same composition at 5 weeks intervals.

### 2.2.3. Root induction

Shoots were cultured on half strength MS medium supplemented with different concentrations of IBA (0.01, 0.1, 0.5, 1.0, 1.5, 2.0 mg/l) and PGR free MS medium as control in test tubes (25 mm x 150 mm) each containing 25 ml medium. Each test tube was considered as unit of replication and there were a total of 30 replications for each treatment. The cultures were maintained in the growth room with the same culture condition as for culture initiation and shoot multiplication experiments. Number of roots and root length per shoot was recorded after 60 days.

### 2.2.4. Acclimatization

Sixty-day-old plantlets with well-developed roots were removed from the culture vessel and washed carefully and gently under running tap water. Plantlets were transferred to plastic pots (20 cm diameter) containing sand, red soil and compost in ratio of 2:1:1 respectively. The potted plantlets were covered with polyethylene bags and kept in greenhouse. After 30 days, the polyethylene bags were removed, but the pots were placed under shade in the greenhouse. The survived plantlets were recorded after 80 days of acclimatization.

### 2.2.5. Experimental design

A completely randomized design (CRD) was used for all experiments. Data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) test using statistical data analysis software SPSS version 17.0 at 5% probability level.

## 3. RESULTS

### 3.1. Ex vitro seed germination

The highest germination percentage (73.77%) was exhibited by fresh seeds but it decreased with increased storage time (Table 1, Figure 1). Study results revealed that germination was declined as low as 13.88% and 6.27% in accessions 8500 and 242326 seeds respectively. After 50 days, none of the seeds were able to germinate.

**Table 1.** Ex vitro seed germination of *G. lotoides*

Seeds	Germination %		
	Day 30	Day 40	Day 50
Fresh	73.77±0.61 <sup>a</sup>	48.82±2.77 <sup>a</sup>	0.00±0.00 <sup>a</sup>
8500	13.88±1.16 <sup>b</sup>	11.01±3.50 <sup>b</sup>	1.06±0.54 <sup>a</sup>
242326	0.99±0.19 <sup>c</sup>	6.27±0.72 <sup>b</sup>	0.72±0.55 <sup>a</sup>

Means followed by the same letter within column are not significantly different at  $p < 0.05$ . The values indicate mean  $\pm$  SE



**Figure 1.** Ex vitro seed germination of *G. lotoides* in stored seeds (8500 = 4-year-old and 242326 = 10-year-old) after 60 days and fresh seeds (0-day-old) after 30 days. A) Fresh, B) 8500, C) 242326. Bars represent 2 cm.

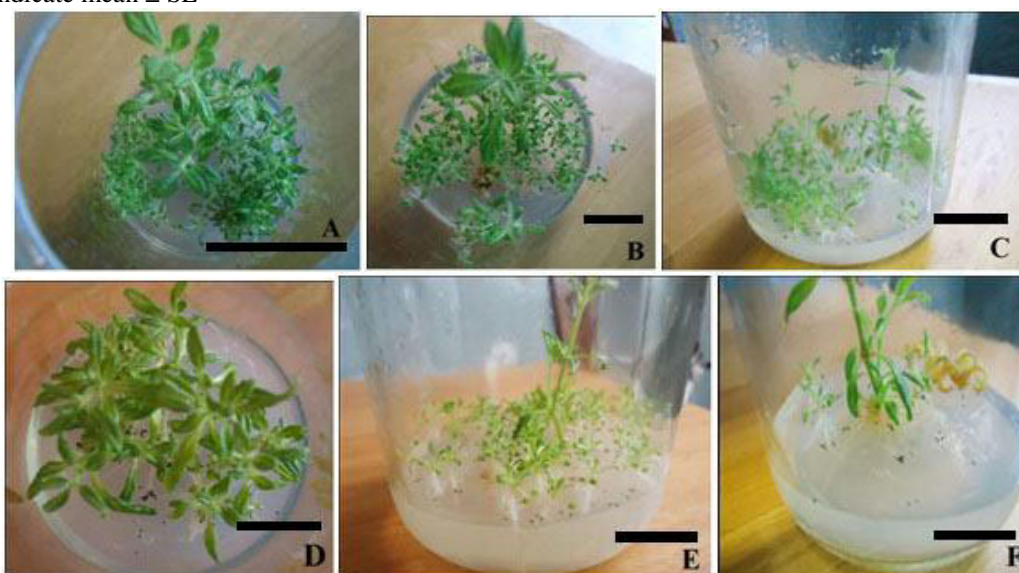
### 3.2. In vitro seed germination

A primary indicator of germination is appearance of radical. However, because of the size of *G. lotoides* seeds, germination was considered when very small green color seedlings appeared. The highest germination percentage was obtained in all seeds treated with 25 mg/l GA<sub>3</sub>. The maximum germination percentage (73.55%) was achieved from fresh seeds. The next highest germination responses (46.28% and 33.80%) were observed in accessions 8500 and 242326 seeds, respectively. The fresh seeds showed much more germination percentage than the stored seeds (Figure 2). Within four to five months, *in vitro* germinated seedlings were resulted in flower bud formation. On filter paper, the highest (3.3%) germination was recorded in the fresh seeds (data not shown). After 80 days, none of the seeds germinated (Table 2).

**Table 2.** *In vitro* seed germination percentage of *G. lotoides*

GA <sub>3</sub> (mg/l)	Seeds	Germination %				
		Day 10	Day 20	Day 40	Day 60	Day 80
0	Fresh	0.44±0.22 <sup>b</sup>	1.33±0.38 <sup>cd</sup>	0.22±0.22 <sup>c</sup>	2.34±1.31 <sup>e</sup>	0.00±0.00 <sup>c</sup>
0	8500	0.00±0.00 <sup>c</sup>	1.32±0.66 <sup>cd</sup>	0.44±0.44 <sup>c</sup>	0.00±0.00 <sup>c</sup>	0.00±0.00 <sup>c</sup>
0	242326	0.00±0.00 <sup>c</sup>	1.33±0.38 <sup>cd</sup>	0.90±0.59 <sup>c</sup>	0.68±0.38 <sup>e</sup>	0.00±0.00 <sup>c</sup>
12.5	Fresh	0.88±0.44 <sup>b</sup>	0.44±0.22 <sup>d</sup>	11.55±5.78 <sup>b</sup>	31.52±5.94 <sup>b</sup>	43.45±14.43 <sup>b</sup>
25	Fresh	2.88±0.38 <sup>a</sup>	8.01±1.23 <sup>a</sup>	23.57±5.21 <sup>a</sup>	45.53±7.57 <sup>a</sup>	73.55±8.38 <sup>a</sup>
40	Fresh	0.44±0.44 <sup>b</sup>	0.66±0.00 <sup>cd</sup>	2.24±0.45 <sup>c</sup>	0.00±0.00 <sup>c</sup>	0.22±0.22 <sup>c</sup>
12.5	8500	0.44±0.44 <sup>b</sup>	0.88±0.22 <sup>cd</sup>	3.37±0.38 <sup>c</sup>	4.89±1.21 <sup>d</sup>	5.93±1.90 <sup>c</sup>
25	8500	0.22±0.22 <sup>b</sup>	1.33±0.00 <sup>cd</sup>	0.90±0.45 <sup>c</sup>	21.10±6.62 <sup>c</sup>	46.28±15.35 <sup>b</sup>
40	8500	0.00±0.00 <sup>c</sup>	0.44±0.22 <sup>d</sup>	0.89±0.59 <sup>c</sup>	0.00±0.00 <sup>c</sup>	0.00±0.00 <sup>c</sup>
12.5	242326	0.44±0.44 <sup>b</sup>	1.33±0.38 <sup>cd</sup>	0.00±0.00 <sup>c</sup>	6.55±0.22 <sup>de</sup>	5.31±2.92 <sup>c</sup>
25	242326	0.22±0.22 <sup>b</sup>	2.00±0.76 <sup>cd</sup>	0.00±0.00 <sup>c</sup>	13.37±2.48 <sup>cd</sup>	33.80±7.55 <sup>b</sup>
40	242326	0.00±0.00 <sup>c</sup>	2.88±0.44 <sup>bc</sup>	0.68±0.68 <sup>c</sup>	0.22±0.22 <sup>e</sup>	1.85±1.00 <sup>c</sup>

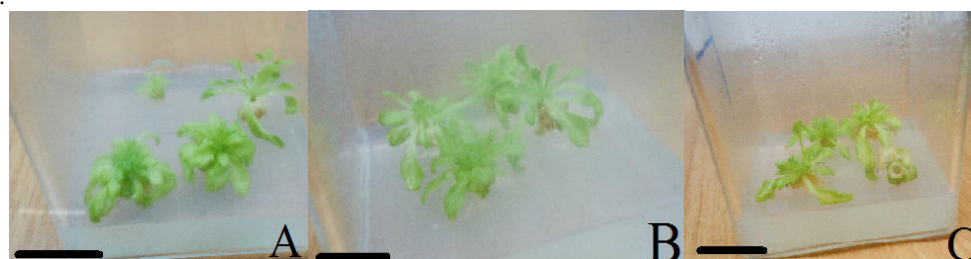
Means followed by the same letter (lower case) within column are not significantly different at  $p < 0.05$ . The values indicate mean  $\pm$  SE



**Figure 2.** *In vitro* seed germination of *G. lotoides* of stored and fresh seeds. A) Fresh in 25 mg/l GA<sub>3</sub>, B) Fresh in 12.5 mg/l GA<sub>3</sub>, C) 8500 in 25 mg/l GA<sub>3</sub>, D) 8500 in 12.5 mg/l GA<sub>3</sub>, E) 242326 in 25 mg/l GA<sub>3</sub>, F) 242326 in 12.5 mg/l GA<sub>3</sub>. Bars represent 2 cm.

### 3.3. Culture initiation

Shoot tips started growing in MS medium within a week. The medium containing 0.5 mg/l BAP resulted in highest percentage (98.11%) of shoot initiation followed by medium containing 0.5 mg/l BAP in combination with 0.5 mg/l KIN which resulted in 94.33% shoot initiation. All cultures had good and normal appearance (Figure 3).



**Figure 3.** Shoot initiation from shoot tips derived from *in vitro* germinated seedlings of *G. lotoides* after 35 days. A) 0.5 mg/l BAP, B) 0.5 mg/l BAP in combination with 0.5 mg/l KIN, C) 1.0 mg/l BAP. Bars represent 2 cm.

### 3.4. Shoot multiplication

After 15 days all cultures were responded on multiplication media. The maximum mean number of shoots (8.82) per explant was obtained on medium supplemented with 0.5 mg/l BAP. Further increase in shoot number did not

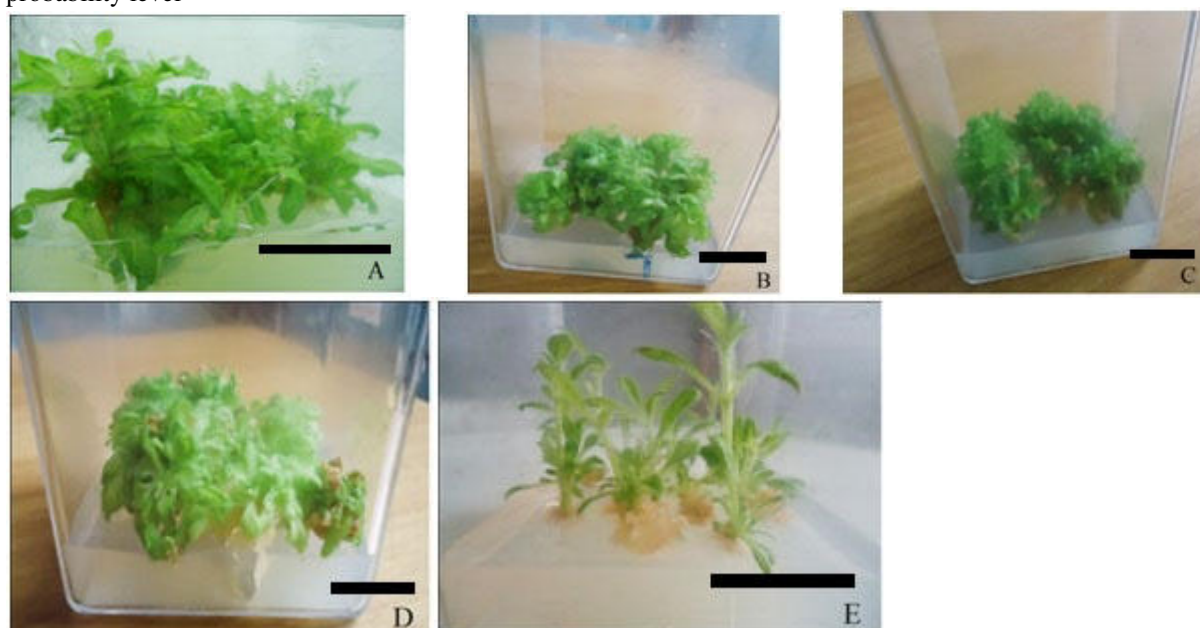
occur by increasing BAP concentrations higher than 0.5 mg/l and BAP combination with KIN except in a medium containing 1.0 mg/l BAP in combination with 1.0 mg/l KIN, which produced mean number of 5.82 shoots per explant (Table 3). With increasing concentration of the plant growth regulators, mean height of shoots decreased. The highest mean shoot length (2.20 cm) was obtained on PGR free medium.

Although BAP alone or in combination with KIN resulted in higher shoot proliferation rate, the shoots were bushy, dwarf and crowded with several leaves (Figure 4). However, each explant produced an average of 2 shoots per explants (Figure 5). Shoots that were cultured on medium containing 0.5 mg/l BAP and growth regulators free resulted in high shoot quality.

**Table 3.** The number and height of shoots of *G. lotoides* on MS medium containing different concentrations of BAP and KIN

PGRs (mg/l)		Number of shoots per explants (mean ±SE)	Length of shoots (cm) (mean± SE)
BAP	KIN		
0.0	0.0	2.11±0.17 <sup>gh</sup>	2.20±0.17 <sup>a</sup>
0.5	0.0	8.82±0.74 <sup>a</sup>	1.27±0.08 <sup>b</sup>
1.0	0.0	3.34±0.49 <sup>c</sup>	0.63±0.57 <sup>d</sup>
1.5	0.0	2.31±0.16 <sup>gh</sup>	0.66±0.04 <sup>d</sup>
2.0	0.0	4.05±0.35 <sup>cf</sup>	0.74±0.05 <sup>cd</sup>
0.5	0.5	3.02±0.21 <sup>c</sup>	0.64±0.03 <sup>d</sup>
0.5	1.0	4.48±0.26 <sup>cd</sup>	0.79±0.06 <sup>cd</sup>
0.5	1.5	3.40±0.25 <sup>de</sup>	0.70±0.04 <sup>cd</sup>
0.5	2.0	2.57±0.22 <sup>fgh</sup>	0.67±0.06 <sup>d</sup>
1.0	0.5	3.34±0.38 <sup>c</sup>	0.65±0.05 <sup>d</sup>
1.0	1.0	5.82±0.53 <sup>b</sup>	0.89±0.06 <sup>c</sup>
1.0	1.5	2.31±0.19 <sup>gh</sup>	0.71±0.06 <sup>cd</sup>
1.0	2.0	1.82±0.18 <sup>h</sup>	0.54±0.04 <sup>de</sup>
1.5	0.5	2.94±0.26 <sup>eg</sup>	0.70±0.05 <sup>cd</sup>
1.5	1.0	4.14±0.58 <sup>cf</sup>	0.77±0.62 <sup>cd</sup>
1.5	1.5	2.25±0.26 <sup>gh</sup>	0.72±0.06 <sup>cd</sup>
1.5	2.0	3.40±0.40 <sup>de</sup>	0.62±0.05 <sup>d</sup>
2.0	0.5	4.71±0.52 <sup>c</sup>	0.82±0.07 <sup>cd</sup>
2.0	1.0	4.00±0.46 <sup>ce</sup>	0.82±0.07 <sup>cd</sup>
2.0	1.5	2.25±0.23 <sup>gh</sup>	0.40±0.04 <sup>e</sup>
2.0	2.0	3.57±0.63 <sup>def</sup>	0.39±0.04 <sup>e</sup>

Data indicate mean ± SE and means followed by the same letter in a column are not significantly different at 5% probability level



**Figure 4.** Shoot proliferation from shoot tips explants of *G. lotoides* on different shoot multiplication media after 35 days. A) 0.5 mg/l BAP, B) 1.0 mg/l BAP in combination with 1.0 mg/l KIN, C) 2.0 mg/l BAP, D) 2.0 mg/l BAP in combination with 1.0 mg/l KIN, E) Control. Bars represent 2 cm.



**Figure 5.** Number of shoots obtained from explant with bushy and dwarf appearance; in case of this explant, there were 22 shoots per explant. Bars represent 2 cm.

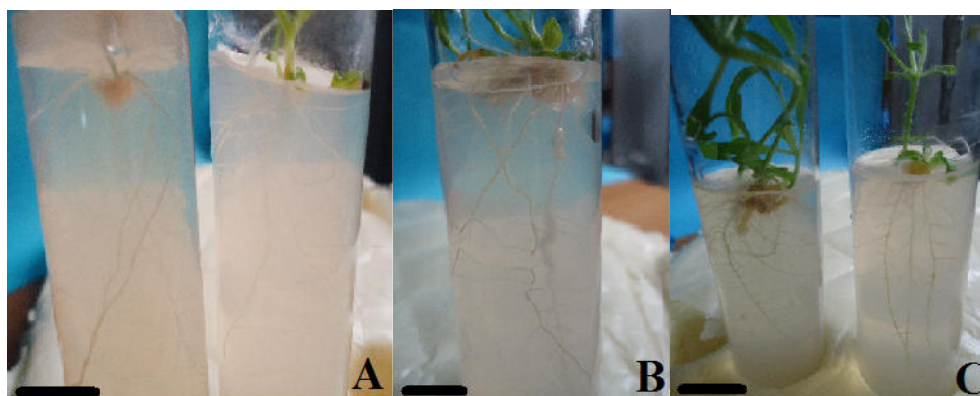
### 3.5. Rooting

Medium containing different concentrations of IBA significantly affected the root length and root number (Table 4). Rooting response was observed on medium after 15 days from the beginning of culture. A maximum mean numbers of roots (6.86) were obtained on MS medium containing 0.5 mg/l IBA. Maximum mean length (4.06 cm) was also achieved on the same medium. Lower or higher than 0.5 mg/l IBA concentrations did not increase the mean value of root numbers and root length. All cultures were healthy and had normal appearance (Figure 6).

**Table 4.** Number and length of roots of *G. lotoides* on half strength MS medium containing different concentrations of IBA

IBA (mg/l)	Number of roots per explants (mean $\pm$ SE)	Length of roots (cm) (mean $\pm$ SE)
0.0	1.75 $\pm$ 0.47 <sup>c</sup>	0.87 $\pm$ 0.12 <sup>c</sup>
0.01	1.13 $\pm$ 0.37 <sup>c</sup>	0.70 $\pm$ 0.20 <sup>c</sup>
0.1	1.46 $\pm$ 0.37 <sup>c</sup>	1.63 $\pm$ 0.50 <sup>b</sup>
0.5	6.86 $\pm$ 0.65 <sup>a</sup>	4.06 $\pm$ 0.57 <sup>a</sup>
1.0	4.70 $\pm$ 0.77 <sup>b</sup>	2.06 $\pm$ 0.32 <sup>b</sup>
1.5	2.43 $\pm$ 0.69 <sup>c</sup>	1.11 $\pm$ 0.35 <sup>b</sup>
2.0	4.13 $\pm$ 0.88 <sup>b</sup>	1.26 $\pm$ 0.25 <sup>b</sup>

Data indicate mean  $\pm$ SE and means followed by the same letter in a column are not significantly different at 5% probability level



**Figure 6.** *In vitro* rooting of shoots of *G. lotoides* on half-strength MS medium containing different concentrations of IBA. A. 0.5 mg/l IBA, B. 1.5 mg/l IBA, C. 2.0 mg/l IBA. Bars represent 0.5 cm.

### 3.6. Acclimatization

Among the plantlets planted in greenhouse for acclimatization, 87% survived after 80 days of acclimatization. Acclimatized plants were normal and no aberrant plants were observed (Figure 7).



**Figure 7.** Acclimatization. A) Plants covered in polyethylene, B) After polyethylene bags removed and 30-day-old, C) After 80 days. Bars represent 2 cm.

## 4. DISCUSSION

### 4.1. *Ex vitro* seed germination

The result revealed that the years of storage had significant effect on the percentage of germinated seeds. This is in agreement with work of (Chauhan and Nautiyal 2007) on *Nardostachys jatamansi*.

### 4.2. *In vitro* seed germination

The present experiments have shown that soaking in GA<sub>3</sub> for 16 h enhanced seed germination. Seeds treated with various GA<sub>3</sub> concentrations germinated efficiently but depends on age of seeds. Significant germination difference was observed between fresh and stored seeds. This result indicated that increase in storage time resulted in decrease of seed germination potentials. The same result was also obtained from previous work on *Nardostachys jatamansi* (Chauhan and Nautiyal 2007).

All concentrations of GA<sub>3</sub>, except the control and 40 mg/l, were effective for enhancing seed germination. This result shows that GA<sub>3</sub> has a potential to break dormancy. With this, GA<sub>3</sub> has been found to be effective in increasing germination in several species and to break dormancy in dormant seeds. It has been reported that germination could be induced by GA<sub>3</sub> in *Morus nigra* (Koyuncu, 2005), *Penstemon digitalis* (Mello et al., 2009), *Pedicularis olympica* (Kirmizi et al., 2010) and *Coscinium fenestratum* (Goveas et al., 2011).

*In vitro* flowering was observed from *in vitro* germinated plants. Previous finding showed that *in vitro* flowering from *in vitro* germinated seeds of *Orychophragmus violaceus* on MS medium containing zeatin and GA<sub>3</sub> (Luo et al., 2000).

### 4.3. Culture initiation

Shoot initiation response of 98.11% was obtained on MS medium containing 0.5 mg/l BAP. Highest percentage (100%) of shoot induction was observed on *Solanum nigrum* with (3 $\mu$ M) BAP (Rathore and Gupta 2013). Increasing BAP concentration resulted in the reduction of shoot induction. This could be due to supra-optimal application of exogenous growth regulators. The next maximum shoot response (79.24%) on the medium supplemented with 1.0 mg/l BAP was observed. Increase in BAP concentration led to decrease in number of shoots initiated per explants in many species such as *Psoralea corylifolia* (Pandey et al., 2013).

### 4.4. Shoot multiplication

*G. lotoides* is difficult to propagate by micropropagation because of the high amount of phenolic compounds exudates (Balcha, 2009). MS medium supplemented with different concentrations of BAP alone and BAP in combination with KIN were used for shoot proliferation. The highest mean number of shoots per explant (8.82 $\pm$ 0.74) was obtained on MS medium containing 0.5 mg/l BAP. This level (0.5 mg/l) of the cytokinin could possibly have highly stimulated 'branching' or bud formation more than the other concentrations. This result agrees with previous results using the same concentration (0.5 mg/l) BAP on *Hypericum retusum* (Naml et al., 2010), *Glinus lotoides* (Shiferaw and Tileye 2015), selected cassava varieties, 'Qulle' and 'Kello' (Dawit, 2009).

Increasing BAP concentrations higher than 0.5 mg/l and different concentrations of BAP and KIN combinations have exhibited negative effect on shoot proliferations and shoot length. Reductions in shoot number because of higher concentration of cytokinins were also reported in the earlier findings including *Adhatoda vasica* Nees (Khalekuzzaman et al., 2008), *Majorana hortensis* (Tejavathi and Padma 2012), *Prunella vulgaris* (Rasool et al., 2009), and *Glinus lotoides* (Shiferaw and Tileye 2015).

The shoots obtained from higher concentrations of BAP alone and combination of BAP and KIN morphologically looked stressed, bushy and dwarf. This feature was expressed because of high concentration of cytokinins. Reduction in shoot number as well as shoot length in *Adhatoda vasica* Nees at higher concentration of cytokinin was reported (Khalekuzzaman et al., 2008). The number of shoots produced and the condition of cultures, such as dwarfing on higher concentrations indicated an inhibitory level and that lower concentrations

could be more appropriate. High concentrations of growth regulators did not allow recovery of the explants in tissue cultures in becoming complete normal plants due to the habituation effect of regulators. Abnormality feature such as the presence of undifferentiated tissue were also observed in the present study. These agree with Naml et al. (2010) who observed many abnormality features on *Hypericum retusum* due to high concentrations of BAP.

Morphological abnormality of culture due to high concentrations of cytokinins was also observed in cassava varieties (Dawit, 2009). The next highest mean numbers of shoots per explant ( $5.82 \pm 0.53$ ) was achieved on the medium containing 1.0 mg/l BAP in combination with 1.0 mg/l KIN. Spontaneous rooting was observed on growth regulators-free shoot multiplication medium. Spontaneous root formation in cassava varieties was reported (Dawit, 2009).

#### 4.5. Root induction and acclimatization

In the present study, highest root number was recorded on medium containing 0.5 mg/l IBA which is similar to the results of (Rakkimuthu et al., 2011) and (Erdag and Emek 2009) who obtained best root development on half strength MS medium with 0.5 mg/l IBA of *Alpinia zerumbet* and *Anthemis xylopoda* respectively. Further raising or lowering IBA concentrations than 0.5 mg/l did not raise mean number of roots per shoot. However, higher number of roots was reported on *Curculigo orchoides* and *Glinus lotoides* with 1.0 mg/l IBA and 1.5 mg/l IBA (Nagesh, 2008) and (Shiferaw and Tileye 2015) respectively. In full strength MS medium, (Dawit, 2009) reported highest rooting response on selected cassava varieties 'Qulle' and 'Kello' using 0.5 mg/l IBA. Concentrations different from 0.5 mg/l also did not increase mean length of roots. Root length less than 1 cm was not recorded as it is detached with residual medium during washing under running tap water.

Rooted plantlets with less than 1 cm length that reserved on medium for long period of time (3-4 months) resulted in flower bud formation. *In vitro* flowering of *Anthemis xylopoda* on half and full strength MS medium containing various concentrations of IBA (0.5, 1.0, and 1.5 mg/l) were reported (Erdag and Emek 2009). This result could be due to an association between plant maturation and IBA stimulating *in vitro* flowering. However, this hypothesis requires further investigation.

The acclimatized plantlets with highest survival rate were grown normally and displayed a typical feature of species. This is in agreement with previous reports on *Alternanthera sessilis* with 83% survival rate (Gnanaraj et al., 2011).

#### Conclusion

In the present study, it was found out that seed-based propagation of *G. lotoides* was limited by poor seed germination percentage. Under the same culture conditions, germination was very poor in long term stored seeds than fresh seeds. Storage of this seed is not recommended. A protocol has been developed for quick and large scale micropropagation of the rare medicinal herb, *G. lotoides*. The PGRs concentration, 0.5 mg/l BAP and 0.5 mg/l IBA were the most effective in multiplication and rooting of *G. lotoides* respectively. Maximum shoot height (2.20 cm) was obtained on PGRs free medium. Maximum root length (4.06 cm) was also recorded on MS medium containing 0.5 mg/l IBA. Acclimatization of the plantlets was highly successful. Therefore, the tissue culture plantlets showed more advantages over seed based seedlings both in terms of time and number of plantlets obtained. The micropropagation method described ensures a regular supply of planting material of *G. lotoides*.

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