Enhanced in vitro production of diosgenin in shoot cultures of Dioscorea deltoidea by elicitation and precursor feeding

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Abstract

Background Dioscorea deltoidea (Family: Dioscoreaceae) is a critically endangered medicinal plant widely used in traditional medicine and pharmaceutical industries for the preparation of appropriate drugs. The present study was conducted to investigate the effect of different elicitors such as salicylic acid (SA) and methyl jasmonate (MeJa) on the synthesis of diosgenin production in D . deltoidea shoot cultures. In addition, the effect of different precursors (squalene, β-sitosterol, and cholesterol) was also demonstrated.

Results Results showed that precursors significantly influenced diosgenin production as compared to elicitors. Application of SA (200 µM) showed highest diosgenin production (0.912% DW) at 4 h incubation time, whereas MeJA (100 µM) exhibited 0.814% DW of diosgenin content at 8 h of incubation time. Among precursors, β-sitosterol at 200 µM produced maximum diosgenin content (1.006% DW) followed by 100 µM squalene (0.947% DW) harvested after 5 th day of culture. Interestingly, cholesterol showed low diosgenin production, but significant than control cultures.

Conclusions The results revealed that exposure to different elicitors and precursors have a promising application in accumulation of diosgenin in D.deltoidea shoot cultures.

Background

Plants synthesize a diversity of structurally complex bioactive compounds commonly known as secondary metabolites. Bioactive compounds do have an important role in bio pesticides, food, cosmetics and pharmaceutical industries [1]. The different species of Dioscoreacea family have acquired significant economic values, because of their therapeutic value and pharmacological efficacies. Globally, they are also considered as the rich sources of important bioactive compounds used in traditional medicine and pharmaceutical industry [2]. *Dioscorea deltoidea* Wall (Family: Dioscoreaceae) is an important therapeutic plant, grows in tropical and sub-tropical regions of world, and north-western Himalayas of India [3]. It has a long and rich history in traditional as well as advance medicinal fields. This plant species contain many bioactive compounds such as diosgenin, stigmasterol, β-sitosterol and campasterol [4, 5]. Past few decades, diosgenin has been of commercial interest and utilised as a precursor for the preparation of sex hormones, contraceptives and other steroidal hormones [6]. In the past few years, diosgenin has also revealed various therapeutic and preventive properties against cardiovascular ailments, allergic diseases, skin aging, neurodegenerative syndromes and menopausal symptoms [7, 8]. It attributes to anti-cancerous, anti-diabetic, anti-fungal, anti-microbial, anti-thrombosis and anti-coagulation effects [9 – 12]. Due to its high usage and illegal harvesting, this species is leads to the reduction of its wild population and genetic diversity. Furthermore, variation in climate and environment conditions also manipulates the chemical profile of *D. deltoidea* especially wild ones. Therefore, Plant tissue culture (PTC) techniques have transformed the conservation scenario of natural genetic resources and is considered as favourable choice for the production of such valuable bioactive compounds and fulfil its demand on pharmaceutical basis throughout the year without seasonal

restrictions [13 – 19]. This technique is significantly vital for endangered and rare species as it stimulates biomass production without deleterious effect on natural populations [20, 21]. *In vitro* production of bioactive compounds could be improve by using different strategies like biotic/abiotic elicitation, precursor feeding, manipulation of plant growth regulators (PGRs) and media type, concentration, carbon and nitrogen source [13, 22, 23]. Among these strategies, elicitation and precursor feeding, are the innovative approaches for the synthesis and production of secondary metabolites when given in suitable amounts to *in vitro* cultures [24, 25]. Various factors like concentration and selectivity of elicitor and precursor, exposure time, culture age are important parameters affecting the successful and significant production of secondary metabolites [26]. Elicitors like benzoic acid, methyl jasmonate (MeJa), salicylic acid (SA), chitosan, jasmonic acid (JA) and precursors (progesterone, cholesterol, squalene, alanine, phenylalanine, mevalonic acid, shikimic acid) were used to increase the amount of phenolics, flavonoids, triterpenoids, alkaloids, anthocyanins in callus cultures, cell suspension culture as well as in organ cultures of many plant families [27 – 30]. In cell or organ cultures, SA and MeJa have found effective for initiation of secondary metabolites. It is considered that MeJa takes part in the pathway of signal transduction that makes specific enzymes to catalyse biochemical reactions for the formation of compounds with low molecular weight, like polyphenols, polypeptides, terpenoids, alkaloids and quinones [31]. SA considered as key-signaling molecule and is responsible in the stimulation of defence responses in plants [32]. In *D. deltoidea*, diosgenin is biosynthesized by mevalonate pathway and squalene, β-sitosterol, cholesterol are intermediate precursors. Cell suspensions or organ cultures precursor feeding in a metabolic pathway has produced large amount of bioactive compounds [33].

In this study, influence of elicitors MeJa and SA, and precursors (squalene, β-sitosterol and cholesterol) were evaluated on diosgenin production from *in vitro* shoot cultures of *D. deltoidea*. To the best of our knowledge, current study is the first attempt on the influence of elicitors and precursors on diosgenin production in *D. deltoidea* shoot cultures.

Results And Discussion

Effect of elicitors on shoot biomass accumulation of *D.deltoidea*

The effect of SA and MeJa was determined on growth rates and has different influence on biomass accumulation in *D. deltoidea* shoot cultures. Different concentration of SA and MeJa (100, 200 µM) were added separately to the liquid medium at different incubation times (4-16 h) respectively on 5^{th} week of culture. Table 1 displays biomass production of shoot cultures treated with SA and MeJa after 10 days. Shoots elicited with MeJa showed reduced biomass production at 4 and 16 h of incubation time but increased at 8 h. MeJa (200 µM) reduced biomass production as related to control cultures while cultures elicited with SA also showed similar pattern of biomass reduction. The results were inconsistent with *Hypericum hirsutum* and *Hypericum maculatum* shoot cultures, in which jasmonic acid (JA) at higher concentration significantly inhibited biomass production and SA slightly effected biomass production relative to control [34]. Recently, Jirakiattikul et al. [35] also indicated that, JA had low effect on *D. membranacea* shoot cultures, whereas SA had no effect. In an another study, it was found that MeJa

reduced the *Centella asiatica* culture growth at concentration above 0.1 mM [36]. In *Andrographis paniculata*, biomass production was adversely effected by various concentrations of SA (10, 20, 50, 100 µM) [37]. Sivanandhan et al. [38] also reported that MeJa at 150-250 µM completely inhibited biomass production but SA showed insignificant reduction in biomass as compared to control. Elicitor retardation has been found in other plant cell cultures also such as *Salvia miltiorrhiza* [39], *Panax ginseng* [31] and *Rubia cordifolia* [40]. These results suggest that growth response to different elicitors may vary among different plant species.

Effect of elicitors on diosgenin production in *D.deltoidea* shoot cultures

The effect of SA and MeJa on the diosgenin content of *D. deltoidea* shoots is shown in Table 1. In order to stimulate diosgenin production, shoot cultures were exposed to elicitor treatment, which is considered as one of the effective strategies to enhance the secondary metabolite production. The present study showed remarkable differences in diosgenin production in shoot cultures treated with elicitors (MeJa and SA) at different incubation times (Table 1). The diosgenin content was significantly increased in all the treated cultures as compared to control. It was found that the addition 100 µM MeJa with 8 h incubation time induced an increase in diosgenin production (0.814% DW). In this study, incubation time showed significant role in upregulating the diosgenin content in *D. deltoidea* shoot cultures; 8 h and 100 µM MeJa was observed to be optimum for the production of diosgenin while 4 h of incubation time for SA (200 µM) exhibited better yield under *in vitro* conditions. This suggested that elicitor used and its concentrations play an important role on the effectiveness of elicitation. In previous studies, it was reported that MeJa has increased furanocoumarin production in *Ruta graveleons* shoot cultures and asiaticoside production in *Centella asiatica* whole plant culture [41, 36]. Mendoza et al. [28] reported that elicitation with 3 µM MeJa increased phenolics and flavonoids content in *Thevetia peruviana* suspension cultures. The production of psoralen was enhanced when MeJa and SA at 100 µM was added to the suspension culture of *Psoralea corylifolia* [42]. Therefore, MeJa have been well established as key signal compounds and positively involved in the signal transduction pathway that leads to secondary metabolite production [43, 35]. Diosgenin production was also favoured by SA which is widely studied signaling molecule to trigger secondary metabolite production in plants [44]. SA is generally produced in plants and accumulated at pathogen attack sites, and then spreads to several plant parts triggering the biosynthetic pathway for accumulation of secondary metabolite production [45]. This is an agreement with Diwan and Malpathak [41] and Coste et al. [34] who reported that 200 µM SA increased the furanocoumarin production in *Rutagraveolens* shoot cultures and hypericin and pseudohypericin content in *Hypericum maculatum* shoot cultures. However, 50 µM SA significantly reduced the diosgenin accumulation in micro-tubers of *Chlorophytum borivilianum* [46]. In *Hypericum perforatum*, the hyperforin production was increased in shootlet meristem cultures when treated with 1mM of SA [47]. In *in vitro* shoot cultures of *Swertia paniculata* SA promoted amarogentin, swertiamarin and mangiferin yield at optimal concentrations [48]. In the present study, the diosgenin production attained through elicitation of *D. deltoidea* shoot cultures was higher than reported in field plants (0.197% DW). Considering the diosgenin accumulation observed in the elicited shoots, *D. deltoidea* is undoubtedly a potential industrial plant for the production of valuable bioactive compounds.

Effect of precursors on biomass accumulation in *D.deltoidea* shoot cultures

Precursors such as squalene, cholesterol and β-sitosterol correlated to the biosynthesis of diosgenin were supplemented in shoot cultures of *D.deltoidea* showed significant reduction in biomass accumulation (Table 2). The precursors treated shoots remained in range of 0.517 to 1.559 g of DW. Squalene showed insignificant biomass reduction when cultures were harvested after 5th day but biomass accumulation was significantly reduced in both concentrations when harvesting was done after 10th day. In case of cholesterol, there was biomass reduction at both concentrations. β-sitosterol at 100 μM concentration showed insignificant biomass reduction after 5th day of harvesting, whereas 200 μM concentration of β-sitosterol greatly affected biomass accumulation. Sivanandhan et al. [27] reported that squalene and cholesterol reduced biomass accumulation in *Withania somnifera* suspension cultures. In another report, it was found that biomass production was decreased due to the addition of squalene and cholesterol in shoot cultures of *Digitalis purpurea* [12].

Effect of precursors on diosgenin production in *D.deltoidea* shoot cultures

GC-MS analysis confirmed that β-sitosterol significantly influenced the diosgenin content when compared to other precursors. Our results indicated that 100 μM of squalene induced upregulation of diosgenin production (0.947% DW) for 5 days followed by 200 μM of squalene (0.636% DW) for 5 days as compared to control (0.319% DW). It was found that diosgenin content was significantly reduced with the increase of harvesting time. Squalene (200 μM) yielded lowest diosgenin content (0.412% DW) after 10 day of exposure time. In addition, β-sitosterol also greatly affected diosgenin content in *D.deltoidea* shoot cultures. Our results suggest that β-sitosterol (200 μM) was critical for diosgenin synthesis and maximum content (1.006% DW) was found on 5th day of exposure time followed by β-sitosterol 100 μM (0.782% DW). After 10 days of time diosgenin content was significantly reduced as exposure period play an important role in diosgenin production. Cholesterol showed less effect on diosgenin production as compared to squalene and β-sitosterol. Cholesterol (200 μM) for 5 days showed optimum diosgenin content (0.635% DW) followed by cholesterol (100 μM) for 5 days (0.562% DW). In all the cases 10 days harvesting time showed least diosgenin production. Squalene at 6 mM produced highest withanolides in *Withania somnifera* cell suspension culture [28]. In shoot cultures of *Digitalis purpurea*, cardiotonic glycosides production was greatly affected by squalene and cholesterol but less effective than progesterone [12].

Conclusions

This work demonstrated that the elicitation practice using exogenous elicitors could significantly improve the pharmacologically active diosgenin content in shoot cultures of *D.deltoidea* as compared to control. The results of the current study also suggest that type, concentrations and exposure time of different elicitors and precursors used in this study, remarkably stimulate the diosgenin content in *D. Deltoidea* shoot cultures. Among the different elicitors used, SA significantly influenced the concentration of

diosgenin in *D. Deltoidea* shoot cultures. The results revealed that the use of β-sitosterol act as potent precursor and elicited highest amount of diosgenin as compared to squalene and cholesterol.

The study provides valuable insights into the potential manipulation of precursors on a large scale for the production of diosgenin. In addition, current findings also provide a reference for enabling the scale-up production of valuable compounds by the aid of bioreactor system. Moreover, further studies are crucial to design metabolic engineering approaches that would enhance the synthesis of valuable bioactive compounds in *in vitro* cultures.

Materials And Methods

Chemicals and reagents

Squalene, cholesterol, β-sitosterol, salicylic acid, methyl jasmonateand standard diosgenin (>99% purity) were procured from Sigma-Aldrich, USA. HPLC grade chemicals like ethanol, hydrochloric acid, chloroform, analytical grade methanol was procured from HiMedia Laboratories Pvt. Ltd. (India).

Plant growth regulators such as N^6-benzyladenine (BA) and Indole-3-butyric acid (IBA) were purchased from Sigma-Aldrich, India.

Plant material and culture conditions

Mother plants of *D. deltoidea* were collected in the month of July, 2017 from Baramulla, Kashmir located at an elevation of 1690 m. The authentication of plant species was done by the curator, submitted the specimen in KASH Herbarium, Centre for Biodiversity and Taxonomy, University of Kashmir (Voucher Specimen No. 2614-KASH).

Explant selection and sterilization

Healthy shoots were obtained from mother plants kept in greenhouse. The explants were rinsed in running tap water (10 min), followed by tween-20 (10 min) and finally washed with distilled water thrice. Surface sterilization of explants was performed inside the Laminar Air Flow Chamber with 0.1 % (w/v) of mercuric chloride ($HgCl_2$) for 3 min and then thoroughly rinsed with sterilized distilled water in order to remove the $HgCl_2$ traces. Sterilized nodal segments were carved into proper size (1.5-2.0 cm) before inoculation.

Culture conditions and shoot initiation

Nodal segments were inoculated onto the Murashige and Skoog (1962) medium supplemented with 3% sucrose, 0.8 % agar (w/v) and BA (2.0 mg/l) and IBA (1.5 mg/l) for direct shoot organogenesis. The pH of the medium was adjusted to 5.8 with 1 N NaOH or 1 N HCl. The medium was autoclaved at 121ºC for 15 minutes. The cultures were maintained at (25±2 °C) with photoperiod (16 h light/8 h dark) and photosynthetic photon flux (PPF) of 40-50 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. After 21

days plantlets were transferred into liquid MS medium with similar composition of PGRs for 5 week and then used for further process. Optimal harvest time was evaluated in terms of biomass accumulation at 7 weeks of culture in liquid media when plant biomass reached a maximum level of 1.95 g DW (6.6 g FW) and further increase or decrease in harvest time has led to reduction of biomass accumulation.

Experimental procedure

Effect of elicitors on biomass and diosgenin production

SA and MeJa were used as elicitors. Stock solutions of SA and MeJa were prepared individually by dissolving them in aqueous ethanol (50% ethanol: 50% water v/v) and filter sterilize through a syringe filter (0.22 µM). The multiple shoots (5 g FW) was allowed to grow at different concentrations of SA (100 µM and 200 µM) and MeJa (100 µM and 200 µM) at different time duration (4,8,16 h) on 5^{th} week of culture since the maximum biomass was reached on 5^{th} week of culture. After treatment of SA and MeJa treatment in different time period immediately the multiple shoots were transferred to the liquid MS medium supplemented with BA (2.0 mg/l) and IBA (1.5mg/l) aseptically. After elicitation, the shoots were harvested on the 7^{th} week for production of biomass and diosgenin. All sets were done in triplicates and for each trial control cultures were sustained and 50% ethanol (v/v) was used in control cultures.

Effect of precursors on biomass and diosgenin production

Squalene, β-sitosterol and Cholesterol, the precursors in diosgenin pathway were used in different concentrations (100 and 200 µM) respectively. Stock solution of squalene, β-sitosterol and cholesterol were prepared in 99% ethanol and filter sterilized with 0.22 µM of syringe filter. Filter-sterilized squalene, β-sitosterol and cholesterol were added to the liquid MS medium fortified with BA (2.0 mg/l) and IBA (1.5 mg/l) aseptically on the 5^{th} week of culture. Cultures were harvested after 5^{th} and 10^{th} day after the addition of precursor and were done in triplicates.

Biomass quantification

Fresh weight of control shoots and treated shoots were recorded after harvesting. *In vitro* harvested shoots were freeze-dried and lyophilized and dry weight measurement was recorded.

Sample preparation

The shoots were pulverized into a fine powder after drying and 1 g DW of fine biomass powder of each set was macerated with aqueous ethanol (50% v/v) for 24 h at room temperature. The extract was filtered through Whatman filter paper No 1 and dried with help of rotary evaporator at 40 ºC. 20 ml of HCL (10%) was mixed to the dried residue and hydrolysed at 98 ºC for 1 h. After cooling, chloroform (15 ml) was added two times for washing and collective mixture was extracted and isolated, lower layer i.e. chloroform layer was collected and other 20 ml chloroform was used to extract upper layer. Chloroform layers were combined and concentrated to dryness. An appropriate amount of methanol was added to

residue and final concentration was filtered through 0.2 µM syringe filter and preserved in refrigerator (4°C) for further analysis.

GC-MS analysis

An Agilent 7890A Gas chromatography coupled to a 5875C mass spectrometer detector (XL MSD) with triple axis and mass hunter work station software (USA) was used for the analysis of diosgenin. Chromatography was performed on DB-5: 30 m x 0.25 mm i.d. x 0.25 µM film thickness column. Helium works as a carrier gas at a flow rate of 0.5 mL/min. The Gas chromatography oven temperature was raised from 200 °C for 2 min to 280 °C for 20 min at a heating rate of 10 °C/min. The injection volume was 5 µl using split ratio (1:1). The Mass spectra were recorded in electron impact mode with ionization energy of 70 eV and scan rate of 0.5 s/scan with scan range of 50-600 amu. Inlet and transfer line temperature were set 250^0C.Component identification was achieved by Wiley, NIST libraries. Compounds were also recognized by peak enrichment on co-injection with available authentic standards. Peak area percentages were achieved electronically from the TIC response without the use of correction factors. GC-MS chromatogram of diosgenin is shown in Figure 2 (a, b).

Statistical analysis

The values were represented in triplicates as mean ± standard deviation. Statistical analyses were done by implementing analysis of variance (ANOVA) with Tukey's test using SPSS ($p < 0.05$).

Abbreviations

ANOVA : analysis of variance

BA : N^6-benzylaminopurine

DW : dry weight

GC-MS : gas chromatography-mass spectrometry

IBA : indole-3-butyric acid

JA : jasmonic acid

MS : Murashige and Skoog (1962)

MeJa : methyl jasmonate

PTC : plant tissue culture

PGRs : plant growth regulators

SA : salicylic acid

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors'contributions: RN did the experiment, SG help in writing the paper, AD helps in collection of plant samples, AJ helped in GC-MS analysis, PD helped in statistical analysis, VK and TM did literature survey while DKP conceived the idea, and supervised the work. All authors read and approved the final Manuscript

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Tables

Table 1: Elicitors effect on biomass accumulation and diosgenin content in shoot cultures of *D.deltoidea*

Elicitor (µm)	Incubation Time (h)	Harvest after 10 days	
		Biomass (g DW)	Diosgenin (%)
MeJa			
0	4	$1.74 \pm 0.091^{a,b}$	$0.269 \pm 0.014^{i,j}$
100	4	0.727 ± 0.057^{d}	0.348 ± 0.026^{g}
200	4	0.487 ± 0.059^{g}	0.432 ± 0.013^{f}
0	8	$1.748 \pm 0.043^{a,b}$	$0.266 \pm 0.010^{i,j}$
100	8	**$0.674 \pm 0.045^{d,e}$**	**0.814 ± 0.013^{b}**
200	8	$0.632 \pm 0.031^{e,f}$	0.765 ± 0.020^{c}
0	16	$1.726 \pm 0.024^{a,b}$	0.255 ± 0.053^{j}
100	16	$0.663 \pm 0.026^{,d,e}$	0.533 ± 0.018^{e}
200	16	$0.523 \pm 0.024^{f,g}$	0.628 ± 0.019^{e}
SA			
0	4	1.826 ± 0.020^{a}	$0.265 \pm 0.006^{j,j}$
100	4	$1.742 \pm 0.037^{a,b}$	0.471 ± 0.016^{f}
200	4	**$1.664 \pm 0.038^{b,c}$**	**0.912 ± 0.011^{a}**
0	8	$1.810 \pm 0.136^{a,b}$	$0.263 \pm 0.005^{i,j}$
100	8	$1.552 \pm 0.330^{b,c}$	0.819 ± 0.012^{b}
200	8	$1.767 \pm 0.028^{a,b}$	0.664 ± 0.019^{d}
0	16	1.817 ± 0.025^{a}	$0.267 \pm 0.005^{i,j}$
100	16	1.656 ± 0.1946^{b}	0.625 ± 0.007^{d}
200	16	1.539 ± 0.0223^{c}	0.549 ± 0.026^{e}

Each value represents mean ± SD of three replicates. Within a column, means followed by the same letter are not significantly different ($P \leq 0.05$) according to Tukey Test.

Table 2: Precursors effect on biomass accumulation and diosgenin content in shoot cultures of *D.deltoidea*

Precursor (µm)	Harvest Time (Days)	Biomass (g DW)	Diosgenin %
Squalene			
0	5	1.780 ± 0.092 ^{a,b}	0.319 ± 0.008^{i,j}
100	5	1.121 ± 0.017^{d}	0.947 ± 0.021^{a}
200	5	1.034 ± 0.022^{d}	0.636 ± 0.048^{c}
0	10	1.737 ± 0.191 ^{a,b}	0.307 ± 0.019^{i}
100	10	0.736 ± 0.019^{e,f}	0.485 ± 0.012^{e,f}
200	10	0.638 ± 0.016^{g}	0.412 ± 0.010^{f,g,h}
β-Sitosterol			
0	5	1.721 ± 0.024^{a,b}	0.315 ± 0.014^{i,j}
100	5	1.559 ± 0.025^{c}	0.782 ± 0.011^{b}
200	5	0.745 ± 0.028^{e,f}	1.006 ± 0.001^{a}
0	10	1.823 ± 0.012^{a}	0.301 ± 0.010^{j}
100	10	1.121 ± 0.016^{d}	0.432 ± 0.009^{f,g}
200	10	0.517 ± 0.011^{h}	0.486 ± 0.011^{e,f}
Cholesterol			
0	5	1.730 ± 0.014 ^{a,b}	0.313 ± 0.013^{i,j}
100	5	1.032 ± 0.017^{d}	0.562 ± 0.011^{d}
200	5	0.725 ± 0.011^{f}	0.635 ± 0.012^{c}
0	10	1.777 ± 0.019^{a,b}	0.414 ± 0.007^{f,g,h}
100	10	0.819 ± 0.017^{e}	0.474 ± 0.009^{e,f,g}
200	10	0.559 ± 0.015^{g,h}	0.518 ± 0.009^{d,e}

Each value represents mean ± SD of three replicates. Within a column, means followed by the same letter are not significantly different ($P \leq 0.05$) according to Tukey Test.

Figures

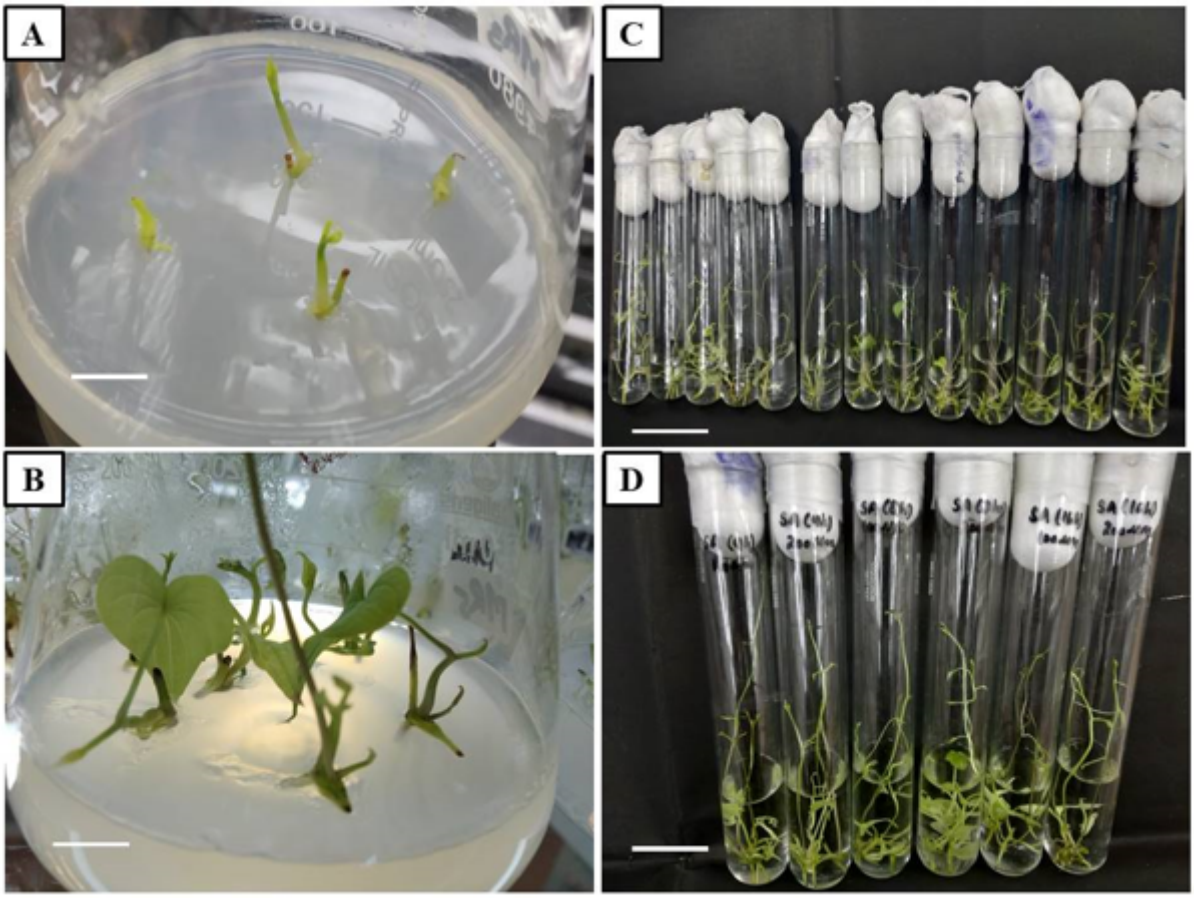

Figure 1

Tissue cultured stages in D. deltoidea (A) shoot initiation in 1 week of culture from MS medium supplemented with BAP and IBA (B) shoot proliferation and multiplication after 2 weeks (C) regenerants sub-cultured in liquid MS medium after 21 days of inoculation (D) treated regenerants.

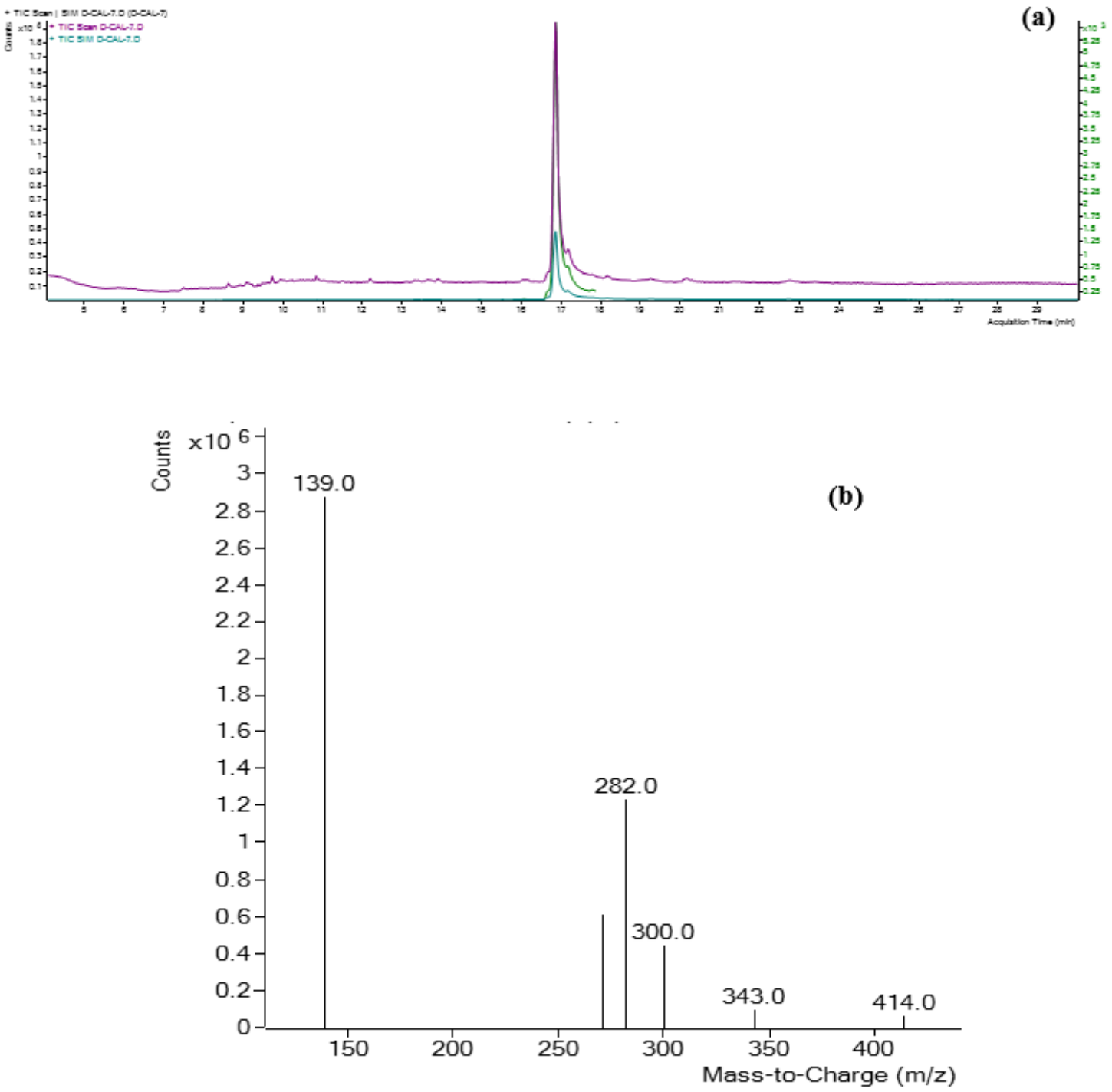

Figure 2

TIC (a) and SIM (b) chromatograms of diosgenin