



In vitro Diosgenin Augmentation in Microtubers of *Dioscorea floribunda* (M. Martens & Galeotti)

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ABSTRACT

Medicinal yam *Dioscorea floribunda* is known to possess pharmacologically active diosgenin, a steroidal saponin, usually found in greater quantities in the tubers. It is used for the commercial synthesis of cortisone, pregnenolone, progesterone, and other steroid products. Diosgenin is also implicated in reduction of serum cholesterol, estrogenic activity and anticancer effects.

In the present study, multiple shoots were initiated from nodal explants and microtuberization, from budding nodal segments of *D. floribunda* using Murashige and Skoog medium supplemented with varying quantities and combinations of plant hormones; benzyl amino purine, indole-3-butyric acid, naphthalene acetic acid and kinetin. There was a differential response in the medium consisting of hormones and their concentrations on diosgenin accumulation in tubers. The best performance was observed in media supplemented with 2 mg L⁻¹ naphthalene acetic acid and 0.5 mg L⁻¹ kinetin producing viable tubers only in 3 months of culture period and accumulating the higher amount of diosgenin content. Also, results reveal that the faster multiple shoot formation and microtuberization is achieved more efficiently in liquid media. Thus, we have achieved diosgenin production and augmentation in microtubers of *D. floribunda*.

Introduction

Dioscorea, monocotyledonous tuber plant of the family Dioscoreaceae with about 600 species recorded so far, is commonly referred to as yam. Diosgenin (Figure 1) is a steroidal saponin possessing estrogenic and antitumor properties. The pharmacological property of a steroidal saponin has been described including their hypocholesterolemic, antidiabetic and antioxidant activities¹⁻⁴. Diosgenin (Figure 1) is biosynthesized in plant *Dioscorea*. Different species originate from different parts of the world; Africa, Asia, the Caribbean's South America and the South Pacific islands etc. The dominant zone for yam production in the world is in West Africa⁵. Different species of *Dioscorea* found in India are *Dioscorea deltoidea*, *Dioscorea prazeri*, *Dioscorea floribunda* and *Dioscorea composita*⁶. The amount of Diosgenin naturally present in field grown yam species e.g. *Dioscorea floribunda* is very little, about 3-3.5% on dry weight basis⁶, too little to meet current demands. *Dioscorea* are plants of very high value in the tropics and semi tropical areas of the world⁷ though listed as underutilised species by the WHO. Millions of people in Africa, Asia and South America depend on the tubers for food and medicines. To date, diosgenin and related steroidal saponins were commercially obtained from the tubers of various *Dioscorea* species⁸. Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological activities, higher safety margins and lesser costs. For past few decades, compounds from natural sources have been gaining importance because of the vast chemical diversity that they offer. *Dioscorea* species leaves were also reported for the presence of multicellular glandular trichome⁹ and elemol rich essential oil from the glandular trichome of *Dioscorea* species.

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There are several reports available in literature suggesting diosgenin quantification in *in vitro* grown callus of *Dioscorea* species. The callus and suspension cultures derived from *Dioscorea deltoidea* can accumulate the steroid diosgenin up to a level exceeding 1% of the dry weight and the yield can be increased substantially by feeding the cultures with the precursor cholesterol¹⁰. Diosgenin accumulation in callus or undifferentiated tissue cultures of *Dioscorea* seed have been reported by Staba *et al.*¹¹. Tissue cultures of callus obtained from seeds of various *Dioscorea* species have been established, including the preferred *D. deltoidea* as well as *D. composita*, *D. spiculiflora* and *D. floribunda*. *D. deltoidea* tissue cultures have been found to yield diosgenin in amounts far in excess of the amounts obtained from tissue cultures of the other *Dioscorea* species¹¹. It has been reported that diosgenin production was improved by as much as 72% compared to control cultures by addition of autoclaved mycelia of non-host specific fungi to cell suspension cultures of *Dioscorea deltoidea*¹². While phytoalexin elicitor's laminarin, arachidonic acid and chitin added to *D. deltoidea* cultures had no stimulating effect on the diosgenin level. In the present report, diosgenin quantification in tissue cultured rhizoids of *D. floribunda* is reported for the first time. Plantlet regeneration *in vitro* for vegetative propagation of some economically important *Dioscorea* species has been achieved using nodal cuttings¹³⁻¹⁴. Tissue culture is recognised as a powerful tool for plant regeneration as production is independent of season, climate or weather and one could develop true type plants, clones of the mother plant or with hormonal manipulations, modify by increasing or decreasing phytomolecule levels. Tissue culture techniques are well established in yam¹⁵ in the quest to break yam dormancy and increase size of tubers. These studies provide a logical method for investigating the role of specific plant growth regulators (PGRs) in yam development with regards to morphogenesis and rhizogenesis. Naphthalene acetic acid (NAA) is a synthetic plant hormone in the auxin family and is a rooting agent. 6-Benzylaminopurine, benzyl adenine (BAP) is cytokinin that elicits plant growth and development responses by stimulating cell division. In plant tissue culture Indole-3-butyric acid (IBA) and other auxins are used to initiate root formation *in vitro* in micropropagation protocol. Kinetin is a type of cytokinin, that promotes cell division. The objective of the present

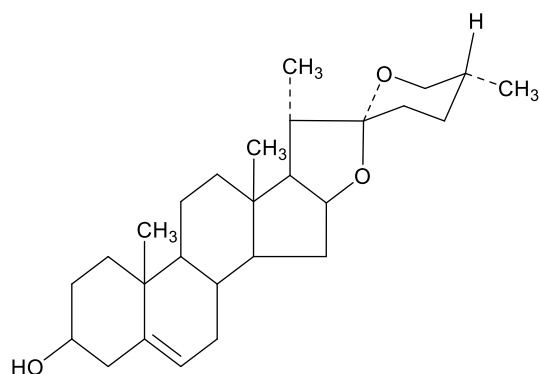


Figure 1: Chemical structure of Diosgenin

study was to determine the effects of naphthalene acetic acid (NAA), benzyl amino purine (BAP), indole-3-butyric acid (IBA) and kinetin on *in vitro* yam morphogenesis, micro-tuber initiation and diosgenin accumulation in rhizoid of *Dioscorea floribunda*.

Materials and Methods

Plant Materials and Surface Disinfection

The resource explants for tissue culture were obtained from the experimental lines maintained at the experimental farm of the CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow. Nodal segments (explants) from the field plants were used for the generation of sterile plant material for *in vitro* studies. The explants were thoroughly washed under running tap water with 5% (v/v) commercial bleach for 1 hour. The explants were surface sterilized by soaking in 70-percent ethanol with 4 percent (w/v) hypochlorite for 10 mins. Later explants were quickly rinsed with 70-percent ethanol, and again rinsed properly with sterile distilled water. The surface sterilized explants were aseptically transferred to MS medium.

Shoot Induction

The surface sterilized explants were used for generation of multiple shoot. Multiple shoot cultures were established aseptically, *in vitro*, nodal segments and maintained in Murashige and Skoog (MS) medium supplemented with the cytokinins BAP (0, 1, 2, 3 and 4 mgL⁻¹) and kinetin (0, 1, 2, 3 and 4 mgL⁻¹) alone and in combination (Table 1). Each treatment of hormonal combinations consisted of five replicates making 24 treatments. The cultures were evaluated in terms of number of buds after 10 days of inoculation. Cultures were maintained at 25 ± 2°C under a 14 h photoperiod (30 μmol m⁻²s⁻²) with cool white fluorescent tubes.

Shoot Elongation and Root Induction

Obtained shoots of 1 to 2 cm long was excised and transferred to MS rooting medium supplemented with five different combinations (A1 to A5) of kinetin, IBA, BAP and NAA. A1 treatment was composed of Kinetin 0.5 mgL⁻¹, BAP 1 mgL⁻¹ and IBA 1 mgL⁻¹; composition for treatment A2 was NAA 1 mgL⁻¹ only; composition for treatment A3 was IBA 1 mgL⁻¹ only; composition for treatment A4 was Kinetin 0.5 mgL⁻¹ and NAA 2 mgL⁻¹; and composition for treatment A5 was 1/2 strength MS medium, Kinetin 0.5 mgL⁻¹ and NAA 2 mgL⁻¹. For treatments A1 to A4 1 strength of MS medium was used while for treatment A5 ½ strength of MS medium was used. The detail of treatment A1 to A5 was given in figure 2. The days to rooting and microtuberization was examined for different treatment. All the cultures were maintained at 25 ± 2°C under a 14 h photoperiod (30 μmol m⁻²s⁻²) with cool white fluorescent tubes.

Isolation of Phytomolecule

Diosgenin was extracted from plant tissue (tubers) after three months of inoculation was extracted by using methanol as extraction solvent. Extraction of diosgenin was conducted by refluxing 5 g of oven-dried (40-45°C) rhizome (powdered by liquid nitrogen) in 50 mL of methanol for 12 h at 25°C. Then the methanol phase was filtered and evaporated to dryness in a fume hood.

The procedure was repeated three to four times. The dried extract was reconstituted in 1 mL of HPLC grade methanol and centrifuged at 12,000 x g to remove the impurities. The supernatant was filtered through the 0.2 μ filter for HPLC analysis.

Calibration Solution

The linear detection range for diosgenin was established by preparing standard stock solutions in HPLC grade methanol. Aliquots of these solutions were diluted and analyzed to determine method linearity. Limit of quantification (LOQ) values were estimated from serial dilution and analyzed. Calibration ranges for diosgenin 0.01 to 0.1 mg/mL were prepared. Triplicate 20 μL injections were made for each concentration of standard solution to see the reproducibility of the detector response at each concentration level. The peak area of diosgenin was plotted against the concentration to obtain the calibration graph. The ten concentrations of diosgenin were subjected to regression analysis to calculate calibration equation and correlation coefficients.

Procedure for HPLC Analysis

The analysis was performed with an LC system consisting of Waters HPLC system which was equipped with a 515 programmable pump, in-line degasser AV, 717plus auto sampler, and 2996 photodiode array detector. The system was controlled and data analysis was performed with Empower software. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of peak area. A reversed phase column (Nova Pack, C18, 4 μm, 150 mm X 3.9 mm) was used for separation by using mobile phase of methanol-water (80:20, v/v) at a flow rate of 0.80 mL/min at 25°C. Changes in absorbance at 205 nm were recorded, and spectra from 190 to 400 nm were recorded on-line for peak identification. The peak area was calibrated to diosgenin content with a standard.

Detection and Quantification Limits

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration curves as kσ/S, where k = 3.3 for LOD, and k = 10 for LOQ, σ is the standard deviation of the Y-intercept of regression line and S is the slope of the calibration curve. Both of LOD and LOQ were expressed by the detection quantity (μg) of diosgenin in the mentioned methods. LOD and LOQ were experimentally verified by six injections of diosgenin at the LOD and LOQ concentrations.

Linearity

Ten different concentrations of diosgenin were analyzed and their calibration curve was constructed in the specified concentration range (0.01-0.10 mg/mL). The calibration plots were generated by replicate analysis (n = 3) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel @program.

Results and Discussion

In order to examine the effect and efficiency of BAP and kinetin alone and in combination on shoot induction of *D. floribunda* explants, different concentration and combination of BAP and kinetin (Table 1) supplemented in MS medium was used for shoot induction Shoots developed in each combination of BAP and kinetin as a result (Plate 1) using the following treatment plan, MS medium supplemented with cytokinin; BAP (0.5, 1, 2 and 4 mgL⁻¹) and kinetin (0.5, 1, 2 and 4 mgL⁻¹). Budding in treatment 1 to 4 was observed after 5 days post-inoculation (Plate 2A, B, C and D) while by day 10 budding showed in all the remaining treatment. 100% budding was observed in treatment 4 (BAP 4 mgL⁻¹ only). The percentage of budding for trt 1 (BAP 1 mgL⁻¹), trt 2 (BAP 2 mgL⁻¹), trt3 (BAP 3 mgL⁻¹) was 50.00%, 62.50% and 75% respectively. In combination of hormone (BAP and Kinetin), the highest percentage of budding was 62.50% and was observed in trt 7 (BAP 3 mgL⁻¹ and KIN 1 mgL⁻¹), trt 8 (BAP 4 mgL⁻¹ and KIN 1 mgL⁻¹), trt11 (BAP 3 mgL⁻¹ and KIN 2 mgL⁻¹), and trt 12 (BAP 4mg L⁻¹ and KIN 2 mgL⁻¹).

Table 1: Growth media hormonal concentration permutations

Serial no (Treatments)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
BAP concentration mg/L	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	0	0	0	0
KIN concentration mg/L	0	0	0	0	1	1	1	1	2	2	2	2	3	3	3	3	4	4	4	4	1	2	3	4

KIN treatment alone (trt 21 to trt 24) was found to be the least efficient in budding. The percentage of budding for Trt 21 (KIN 1 mgL⁻¹) was 0%, for trt 22 (KIN 2 mgL⁻¹) and trt23 was (KIN 3 mg L⁻¹) was 25.00% and for trt 24 (KIN 4 mgL⁻¹) was 37.50% (Table 1). Shoots were also induced on solid medium (Plate 2E) and necrosis appeared on the shoot (Plate 2F) after some time. Subsequently, all future experiments were carried out in liquid media (without agar).

Micro-tubers were generated in solid and liquid medium supplemented with NAA 2 mgL⁻¹ and Kinetin 0.5 mgL⁻¹ (Plate 3, Plate 4A and B). Microtuberization was achieved more efficiently in liquid media in comparison with the solid. Induced shoot from trt4 (BAP 4 mg L⁻¹) was excised and used for shoot elongation and root induction by supplementing MS medium with hormone BAP, kinetin, IBA, and NAA alone or in different combination. The effect of applied hormone on plant morphology and rooting system was visualised clearly in plate 5 (A1-A5). Treatment A2 (NAA 1 mgL⁻¹) and A3 (IBA 1 mgL⁻¹) appeared most efficient in shoot and root induction while treatment A1 (IBA 1 mgL⁻¹; KIN 0.5 mgL⁻¹; BAP 1mgL⁻¹) appeared least efficient. Treatment A4 (NAA 2 mgL⁻¹; KIN 0.5 mgL⁻¹) and A5 (1/2 strength MS medium; NAA 2 mgL⁻¹; KIN 0.5 mgL⁻¹) showed intermediate effect on shoot and root induction (Plate 5). Elongated shoots of 1 to 2 cm could form roots only after transfer to rooting media with different concentrations of IBA and IAA. Maximum frequency (100%) of root induction occurred in MS medium supplemented with 2 mgL⁻¹ IBA.

Finally, the steroid, diosgenin was extracted from tubers of regenerated plants of treatments A1 to A5 separately and quantified by HPLC with PDA detector. A simple, sensitive, accurate HPLC-PDA method was developed for the determination of diosgenin in the herbal extract. To optimize the proposed HPLC method, all of the experimental conditions were investigated. For the choice of stationary phase, reversed-phase separation was preferred due to the drawbacks of the normal phase, e. g., hydration of silica with water that can cause peak tailing. To optimize the mobile phase, different composition were tried for chromatographic separation of the compounds. The best resolution was achieved using a mobile phase consisting of methanol: water in the ratio of 80:20 v/v, which gave satisfactory result with shape well defined and resolved peak with minimum tailing as compared to other mobile phase. Here, methanol was used as mobile phase for diosgenin detection and quantification. Changes in absorbance at 205 nm were recorded, and spectra from 190 to 400 nm were recorded on-line for peak identification. Diosgenin peak were confirmed by their retention time and PDA spectrum in comparison with pure standards. The peak area was calibrated to diosgenin content with a standard. The calibration curves (n=3) constructed for the standard were linear over the concentration range of 0.01-0.1 mg/ml diosgenin. Linear regression was used to establish the calibration curve. Good linearity for diosgenin (r² = 1) was found between 0.01 mg/mL to 0.1 mg/mL. Peak areas of the standard were plotted versus the concentration and linear regression analysis performed on the resultant curve. The equation of the regression line formula and correlation coefficient obtained were Y = 246422X-0.0033 and r² = 1 for diosgenin. The LOD and LOQ were found to be 1.32 and 4.01 µg/ml respectively for diosgenin. The retention time obtained for diosgenin peak was 4.7 minute the developed method was applied for the determination of diosgenin content in regenerated samples. Figure 2 shows the representative chromatogram of diosgenin standard as well as chromatogram of diosgenin in sample. The retention time for diosgenin peak was recorded 4.70 min. Several attempts were made earlier to increase the productivity of compounds of interest in plants the help of tissue culture^{16-17,19-20,25}. The diosgenin content (µg g⁻¹ DW) obtained from tubers of A1(1 strength MS medium; IBA 1 mgL⁻¹; KIN 0.5 mgL⁻¹; BAP 1mgL⁻¹) treated plant was 217.82 ± 10.23 µg g⁻¹ DW, from tubers of A2 (1 strength MS medium; NAA 1 mgL⁻¹) treated plant was 271.96 ± 12.35 µg g⁻¹ DW, from tubers of A3 (1 strength MS medium; IBA 1 mgL⁻¹) treated plant was 202.41 ± 15.34 µg g⁻¹ DW, from tubers of A4 (1 strength MS medium; NAA 2 mgL⁻¹; KIN 0.5 mgL⁻¹) treated plant was 1526.99 ± 26.32 µg g⁻¹ DW and from tubers of A5 (1/2 strength MS medium; NAA 2mgL⁻¹; KIN 0.5 mgL⁻¹) treated plant was 821.03 ± 20.34 µg g⁻¹ DW (Figure 2). Thus, the data showed clearly that the maximum diosgenin content was obtained for tubers under treatment A4 and A5 and both of which was supplemented with 2 mgL⁻¹ NAA and 0.5 mgL⁻¹ KIN. Treatment A2 have third highest diosgenin content in their tubers was also supplemented with NAA (1 mgL⁻¹).

Dioscorea Tissue Culture

The shoot induction experiments confirmed the efficacy of the use of plant growth regulator BAP and Kinetin as already carried out in different accessions of medicinally important *Withania somnifera* plant¹⁶⁻²⁰. Prabakaran *et al.*²¹ reported the use of benzyl adenine (BA) along with NAA for callus and shoot induction in *Ceropegia pusilla*. Kondamudi *et al.*²² have used BAP, auxin, IAA, IBA and NAP at different concentrations either alone or in combination for callus and shoot induction in endangered medicinal plant *Ceropegia pusilla*. Faisal *et al.*²³ reported the use of different hormones such as 6-benzyladenine (BA), kinetin, thiadiazuron (TDZ), IAA, IBA and NAA either alone or in combination on *in vitro* morphogenesis on endangered medicinally important plant *Typhora indica*. Gour *et al.*²⁴ used 2, 4-dichlorophenoxyacetic acid for callus induction in *Balanites aegyptiaca*. The data showed clearly that BAP alone was more efficient in shoot induction than in combination with KIN while the efficiency of budding for KIN alone was least. Sabir *et al.*^{16-17,25} report is in agreement with present report. Sabir *et al.*¹⁶ had reported that MS medium with cytokinins indicated that BAP in combination with kinetin had the maximum shoot proliferation. Increasing the concentration of kinetin in combination with BAP decreased the shoot numbers and shoot length. Low and high levels of BAP in combination with kinetin induced fewer shoots than the BAP in combination with kinetin. BAP was the most effective cytokinin for promoting shoot multiplication and proliferation from nodal segments^{16,17, 25}. Prabakaran *et al.*²¹ reported effective growth from nodal explants of *Ceropegia pusilla* on MS medium supplemented with BAP which is in agreement with the present report. Kondamudi *et al.*²² reported the induction of callus from the Thin Cell Layer (TCL) explants of *Ceropegia pusilla* were initiated on the medium supplemented with various concentrations of auxins and cytokinins. Callus was observed from TCL explants on MS medium containing combinations of BAP, 2, 4-D, IAA, IBA and NAA. The callusing response of most of these PGRs has been studied on multiplication of *Gymnema sylvestre*²⁶; and *Hemidesmus indicus*²⁷. The effect of BA on multiple shoot bud differentiation has been demonstrated in a number of cases using a variety of explants^{23,28}. The results from rhizogenesis experiment shows clearly that NAA alone and IBA alone was more efficient in shoot and root induction. Sabir *et al.*^{16,17} had reported that shoots induced in the presence of BAP and other cytokinins generally lacked roots. Faisal *et al.*²⁹ had optimized rooting response of plantlets raised *in vitro*, using different auxins (IAA, IBA, and NAA) which were tested at various concentrations and observed IBA to induce a strong rooting response in *T. indica*. The success of IBA in promoting efficient root induction has been reported for *Swaivona formosa*³⁰, *Hemidesmus indicus*³¹, *Cunila galoides*³², and *Mucuna pruriens*.²³ Whereas Kondamudi *et al.*²² reported that the medium containing NAA along with BAP produced good amount of callus with rhizoid like structures and showed the direct proportionality in NAA concentration and rooting ability which is in agreement with the present data.

Augmentation of Diosgenin

The isolation and quantification of the steroid, diosgenin was successfully carried out. The possibility of confirming the augmentation of diosgenin using tissue culture is observed clearly from the HPLC results. This is the answer to one of the questions posed for the present work. Nino *et al.* reported mobile phase acetonitrile: water in ratio 90:10 (v/v) for diosgenin detection and quantification.⁴⁰ Similarly, Warke *et al.*³³ and Li *et al.*³⁴ also reported mobile phase acetonitrile: water in ratio 90:10 (v/v) for diosgenin detection and quantification. In the present study, methanol and water were used in ratio 80:20 (v/v) for diosgenin detection and quantification. Sabir *et al.*¹⁷ had reported a marked increase in withanolide A and decrease in withaferin A production in the *in vitro* shoot cultures compared to the shoots of field-grown *Withania somnifera* plants. Multiple shoots cultures exhibited an increase in withanolide A accumulation compared to shoots of the mother plant. *In vitro* generated root cultures as well as callus and suspension cultures also produced withanolides though at lower levels¹⁷. Reinert *et al.*¹⁰ reported that callus and suspension cultures derived from *Dioscorea deltoidea* which can accumulate the steroid diosgenin up to a level exceeding 1% of the dry weight. Further, the yield can be increased substantially by feeding the cultures with the precursor cholesterol. Staba *et al.*¹¹ reported diosgenin accumulation in callus or undifferentiated tissue cultures of *Dioscorea* seed.

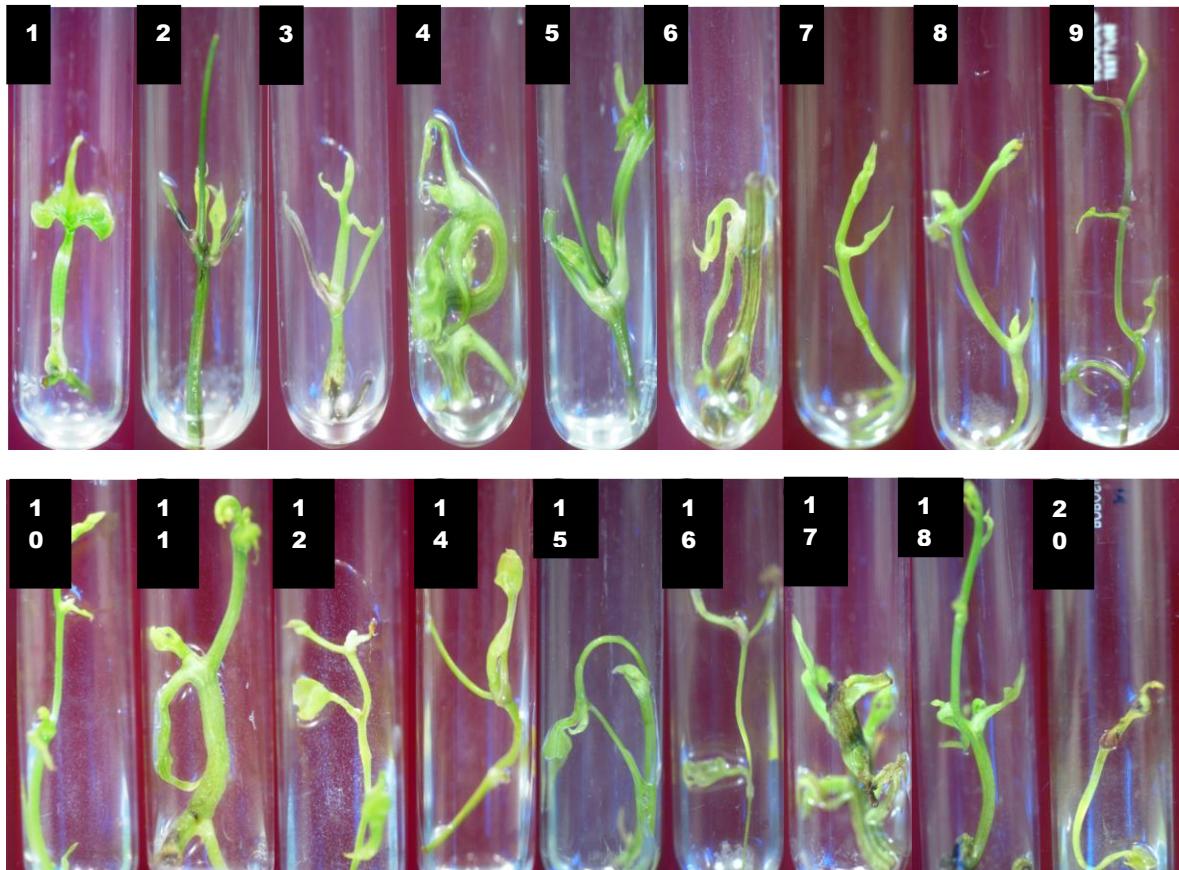


Plate 1: Shoot induction and multiplication in *D. floribunda* at different combination and concentration of BAP and kinetin. (1) BAP 1 mgL⁻¹; Kinetin 0, (2) BAP 2 mgL⁻¹; Kinetin 0, (3) BAP 3 mgL⁻¹; Kinetin 0, (4) BAP 4 mgL⁻¹; Kinetin 0, (5) BAP 1 mgL⁻¹; Kinetin 1 mgL⁻¹ (6) BAP 2 mgL⁻¹; Kinetin 1 mgL⁻¹, (7): BAP 3 mgL⁻¹; Kinetin 2 mgL⁻¹ (8): BAP 4 mgL⁻¹; Kinetin 3 mgL⁻¹, (9) BAP 1 mgL⁻¹, Kinetin 2 mgL⁻¹ (10): BAP 2 mgL⁻¹, Kinetin 3 mgL⁻¹ (11) BAP 3 mgL⁻¹, Kinetin 2 mgL⁻¹, (12) BAP 4 mgL⁻¹, Kinetin 2 mgL⁻¹, (14) BAP 2 mgL⁻¹, Kinetin 3 mgL⁻¹, (15) BAP 3 mgL⁻¹, Kinetin 3 mgL⁻¹, (16) BAP 4 mgL⁻¹, Kinetin 3 mgL⁻¹ (17) BAP 1 mgL⁻¹, Kinetin 4 mgL⁻¹ (18) BAP 2 mgL⁻¹, Kinetin 4 mgL⁻¹, (20) BAP 4 mgL⁻¹, Kinetin 4 mgL⁻¹.

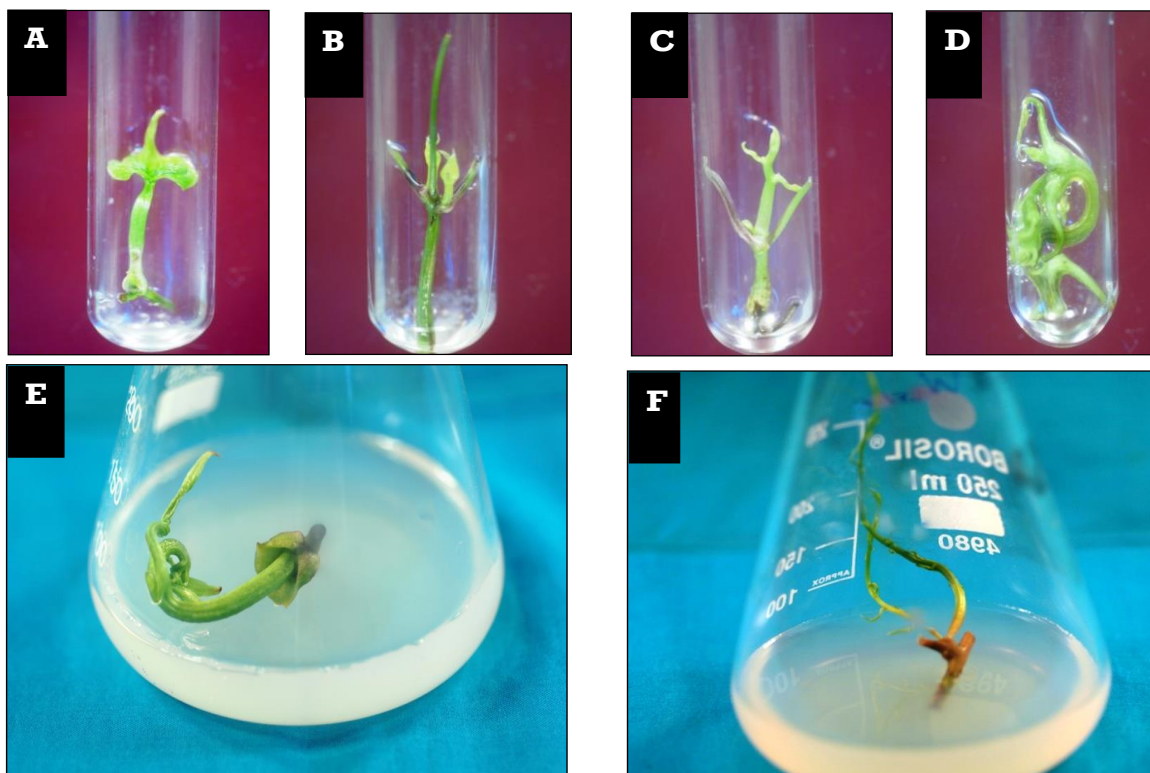


Plate 2: Shoot induction and multiplication in *D. floribunda* at different concentration of BAP (A: 1 mgL⁻¹ BAP; B: 2 mgL⁻¹ BAP; C: 3 mgL⁻¹ BAP and D: 4 mgL⁻¹ BAP; E: shoot induction of *D. floribunda* on solid medium; F: shoot necrosis of induced shoot on solid medium).

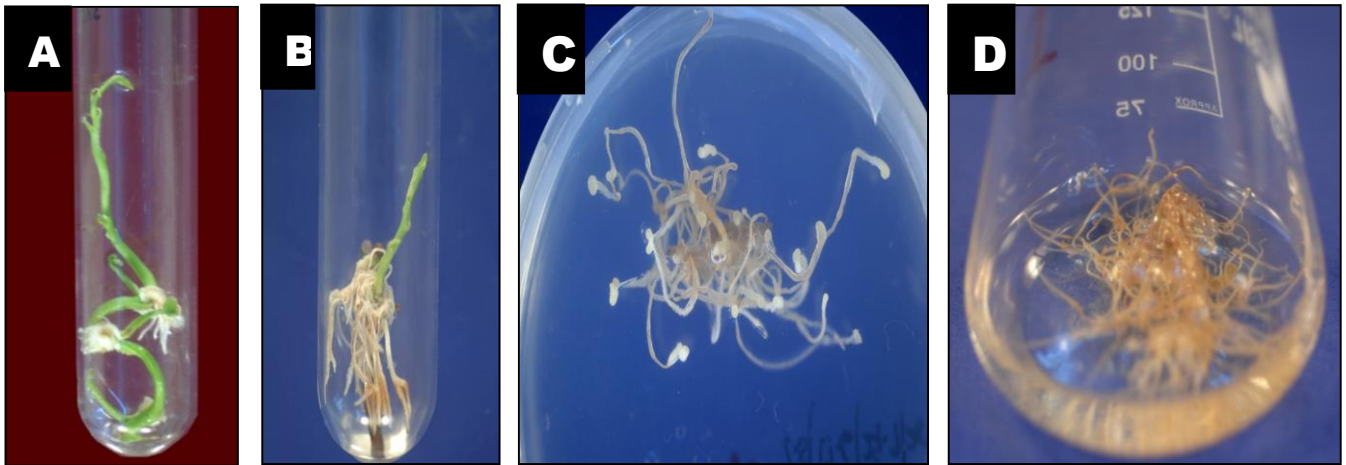


Plate 3: Rhizogenesis in *D. floribunda* plantlets. A: Root induction in morphotypes A4 (0.4 mgL⁻¹ NAA and 0.1 mgL⁻¹ Kinetin); B: Root induction in morphotypes A5 (½ MS, 0.4 mgL⁻¹ NAA and 0.1 mgL⁻¹ Kinetin); C: Root culture in solid medium supplemented with NAA 2 mgL⁻¹, Kinetin 0.5 mgL⁻¹; D: Root culture in liquid medium supplemented with NAA 2 mgL⁻¹, Kinetin 0.5 mgL⁻¹.

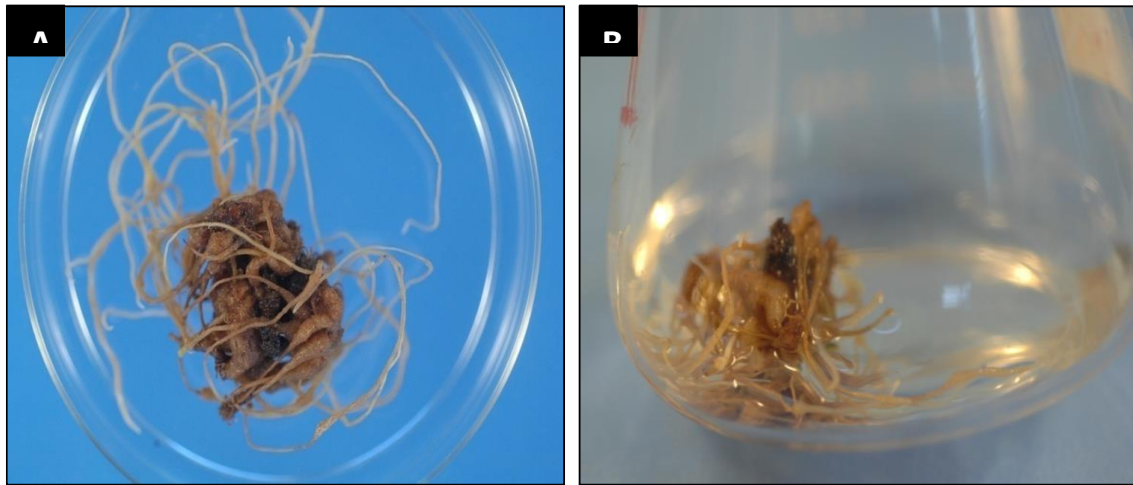


Plate 4: Microtubers generated after 90 days in liquid culture media

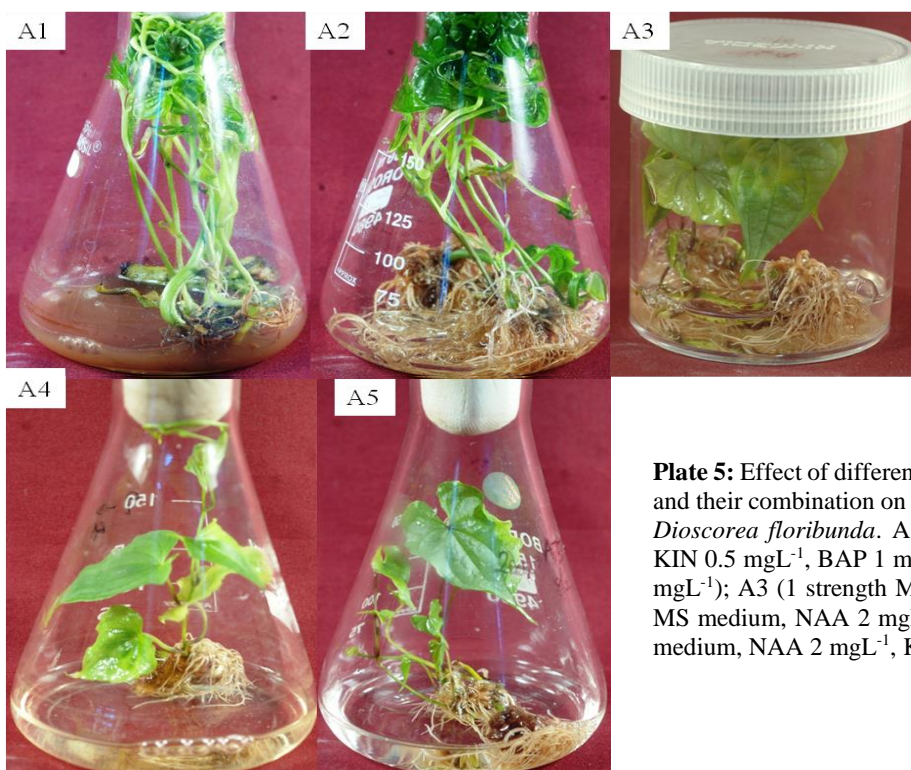


Plate 5: Effect of different hormones (NAA, BAP, IBA and Kinetin) and their combination on shoot and root induction on the explants of *Dioscorea floribunda*. A1 (1 strength MS medium, IBA 1 mgL⁻¹, KIN 0.5 mgL⁻¹, BAP 1 mgL⁻¹); A2 (1 strength MS medium, NAA 1 mgL⁻¹); A3 (1 strength MS medium, IBA 1 mgL⁻¹); A4 (1 strength MS medium, NAA 2 mgL⁻¹, KIN 0.5 mgL⁻¹); A5 (½ strength MS medium, NAA 2 mgL⁻¹, KIN

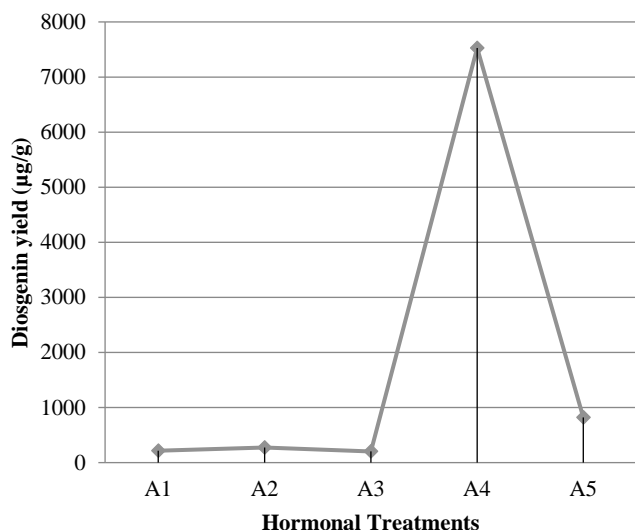


Figure 2: Graph of diosgenin content in microtubers ($\mu\text{g g}^{-1}$) dry weight according to different treatments. A1 (1 strength MS medium, IBA 1 mgL^{-1} , KIN 0.5 mgL^{-1} , BAP 1 mgL^{-1}); A2 (1 strength MS medium, NAA 1 mgL^{-1}); A3 (1 strength MS medium, IBA 1 mgL^{-1}); A4 (1 strength MS medium, NAA 2 mgL^{-1} , KIN 0.5 mgL^{-1}); A5 (1/2 strength MS medium, NAA 2 mgL^{-1} , KIN 0.5 mgL^{-1}).

Tissue cultures of callus obtained from seeds of various *Dioscorea* species have been established, including the preferred *D. deltoidea* as well as *D. composita*, *D. spiculiflora* and *D. foribunda*. *D. deltoidea* tissue cultures have been found to yield diosgenin in amounts far in excess of the amounts obtained from tissue cultures of the other *Dioscorea* species¹¹. The addition of autoclaved mycelia of non-host specific fungi to cell suspension cultures of *Dioscorea deltoidea* improved diosgenin production by as much as 72% compared to control cultures while phytoalexin elicitors' laminarin, arachidonic acid and chitin added to *D. deltoidea* cultures had no stimulating effect on the diosgenin level¹².

Several reports are available in literature with the report of diosgenin quantification in different species of *Dioscorea* as 0.2% in *D. polygonoides*³⁵, 0.2-2.3% in *D. althaeoides*³⁶, 1.92% in *D. prazeri*³⁷, 1.3% in *D. villosa*³⁸, 0.18-0.55% *D. zingiberensis*³⁹ and 0.02-2.64% in *D. polygonoid*⁴⁰. Here for the first time we are reporting the diosgenin quantification in tissue cultured microtubers of *D. floribunda*.

Conclusion

Multiple shoots were generated from nodal explants of *Dioscorea floribunda* and microtuberization was achieved from budding nodal segments using Murashige and Skoog (MS) medium supplemented with varying quantities of plant hormones as benzyl amino purine, indole-3-butylric acid, naphthalene acetic acid, and kinetin. The medium consisting of different combination of hormones and their concentrations showed differential responses on shoot and root induction and diosgenin accumulation in tubers. The media supplemented with 2 mgL^{-1} naphthalene acetic acid, and 0.5 mgL^{-1} kinetin produced viable tubers after only 3 months in culture media and accumulated a high amount of diosgenin.

The faster multiple shoot formation and microtuberization is achieved more efficiently in liquid media in comparison of solid medium. Greater amount of diosgenin was obtained from the tender tissues of the test plant as compared to the plants in the field.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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