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Research article

Establishment of *In Vitro* Cell Suspension Culture, Kinetics of Cell Growth, pH, Nutrient Uptake and Production of 2-hydroxy-4-Methoxybenzaldehyde from the Germinated Root of *Decalepis hamiltonii* Wight & Arn. -An Endangered Plant

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Abstract

Keywords

Decalepis hamiltonii;
secondary metabolite;
2-hydroxy-4-methoxybenzaldehyde;
steam condensate;
gas chromatography

Decalepis hamiltonii Wight & Arn. belongs to Asclepiadaceae family which is an ethno-pharmaceutically important monogenic plant species that is native to the Deccan peninsula forest areas of India. It is endangered due to habitat loss and over exploitation for volatile 2-hydroxy-4-methoxybenzaldehyde (HMB), which is an aromatic bioactive secondary metabolite. HMB is of great biological significance and is present in the plant's tuberous roots. Plant cell culture is a viable alternative method for *in vivo* plant cultivation for secondary metabolites production. Callus induction with high biomass from the germinated root as explants for HMB production was optimised on Murashige and Skoog's (MS) medium containing 3% (w/v) sucrose, supplemented with 1 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 1 µM α- naphthaleneacetic acid (NAA) and 10 µM 6-benzylaminopurine (BAP) in dark incubation. Cell suspension cultures were established in 250 ml shake flasks, and each flask contained 50 ml of the same induction medium. Moreover, the pH 5.8, 3% sugar concentration and 120 rpm agitation speed in dark condition were suitable for high biomass production and the specific growth rate (µ) was 0.086 /day. Extraction of HMB was done by steam condensate methods from the biomass of cell suspension culture. Qualitative and quantification analyses of HMB were performed with gas chromatography (GC) and observed that 0.92 ± 0.02 mg/ml (0.092%) HMB were synthesized in the cell suspension culture.

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1. Introduction

Decalepis hamiltonii Wight &Arn. is commonly known as swallow root. It is a perennial wood climber shrub and belongs to the family Asclepiadaceae. It is an endangered medicinal plant, found in the moist as well as the dry deciduous forests of peninsular India [1]. Traditionally, *D. hamiltonii* root has been utilised for the purification of blood, and for the treatment of fever, intrinsic haemorrhage, wound healing, Kushtha, erysipelas, bronchial asthma, and food poisoning. It has also been used in paediatric rejuvenating, and as a general vitaliser in Siddha, Ayurveda, and folk medicines systems [2]. The tribal societies of the peninsular Indian region chew the roots and partake of an herbal drink named Nannari, which is prepared from roots by Yanadi tribe, as an appetizer and digestive aid. Due to the medicinally voluble nature of the roots of *D. hamiltonii*, they are consumed as pickles and juices preparation [2].

Decalepis hamiltonii tuberous roots have high quantities of secondary metabolites, especially 2-hydroxy-4-methoxy benzaldehyde (HMB), which has numerous medicinal properties [3]. HMB is a volatile compound, and an isomer of vanillin which is principal flavour compound in the roots of *D. hamiltonii*. Moreover, *D. hamiltonii* tuberous roots have various volatile oils, including 0.018% salicylaldehyde, 0.17% benzaldehyde, 0.044% methyl salicylate, 0.081% 2-phenyl ethyl alcohol, 0.016% benzyl alcohol, 0.01% *p*-anisaldehyde, 0.038% ethyl salicylate, and 0.45% vanillin [2].

Decalepis hamiltonii is an economically valuable plant and its natural distribution is in a limited area; it is found only in the southern states of India. Therefore, this plant is an endemic plant. The tribal people collect the roots of *D. hamiltonii* regularly to sell in the local markets. These regional tribal families are economically dependent on this plant as they take various extracts from the tuberous roots and sell them at the local markets throughout the year. The natural wild populations of the plant have been declining continually decade by decade and the plant has become endangered due to overexploitation with indiscriminate harvesting for the tuberous root and habitat destruction [2]. In contrast to the way medicinally valuable plant roots have been harvested in traditional practices for drugs, D. *hamiltonii has been harvested on an 'industrial scale', with* more than 70% of the plants harvested in a destructive manner; and with very little cultivation of the plants occurring [4]. At this junction, several high-valued secondary metabolites produced from *in vitro* plant tissue cultures have been explored for industrial production. *In vitro* cell suspension cultures have shown high potential for the production of secondary metabolites using roots (either from natural or *in vitro* sources) as explants [3, 5]. Over the last few decades, several studies have reported on the production of medicinally valuable secondary metabolites including terpenoids, alkaloids, glycosides, diterpenes, volatile, and flavonoids. Their production depends on the selected explant-induced callus for cell suspension cultures [6, 7]. The plant cell suspension cultures provide a simple dynamic system which can be used to understand and assess cell growth and production kinetics and to apply standardized physical and chemical parameters to the production of highly demanded secondary metabolites in adequate quantities [8].

Despite being an endangered plant having several medicinal values, to date there has been no dynamic investigation of the biosynthesis of HMB in *D. hamiltonii* cell suspension cultures from *in vitro* roots of germinated seeds. The present study is concerned with the establishment of cell suspension cultures from the best biomass yield cell lines of *D. hamiltonii* from *in vitro* germinated root cultures, and the establishment of the most favourable operating conditions for shake flask cell suspension cultures for HMB production. The kinetics of wet and dry cell concentrations, variations in pH, consumption of nutrients, fortified carbon sources, and agitation speeds were analysed for the cell suspension cultures.

2. Materials and Methods

2.1 Explant preparation

Fresh seeds of *Decalepis hamiltonii* Wight & Arn. were collected from wild-grown plants in Nellore (location coordinates: 14.45°N 79.99°E), Andhra Pradesh, India. Seeds were brought to the laboratory and processed for surface sterilization. First, seeds were thoroughly washed for 10 min under running tap water. The washed seeds were then sterilised with 0.10% (w/v) mercuric chloride solution for 7 min, and washed three times with sterile distilled water for 5 min each. Afterwards, surface sterilised *D. hamiltonii* seeds were inoculated for germination on Murashige and Skoog (MS) [9] basal medium fortified with gibberellic acid (GA_3) (0.2 µM), 3% (w/v) sucrose and solidified with 0.8% (w/v) bacteriological agar (Himedia, India) in complete dark incubation at room temperature (i.e. 25±2°C) [10]. After one week of incubation, the germinated seeds (Figure 1a) were taken out and their root tips were cut into approx 2mm length and blotted on sterile filter paper. Next, the cleaned and blotted explants (2mm cut root tips) were cultured on MS medium with or without combination of 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) (Table 1), and incubated in dark for induction of callus. MS medium without growth regulator was the control for induction and growth of callus experiments. The effects of individual and combinations of BAP, NAA and 2,4-D, were also examined. The pH of medium (5.8) was adjusted with 1N sodium hydroxide and 1N hydrochloric acid. The media were solidified with 0.8% (w/v) bacteriological agar, and then autoclaved at a pressure of 1.06 kg/cm^2 for 20 min at 121ºC.

2.2 Establishment of suspension cultures

Cell suspension cultures were initiated when the high biomass producing cell lines were obtained on semi-solid medium. Healthy, fresh, friable and loosely attached soft calli were utilized for initiation and establishment of cell suspension cultures. A medium having the same concentration of plant growth regulators (2,4-D + NAA + BAP) that had given high biomass yield and selected features of calli was used for establishing the cell suspension cultures (without bacteriological agar). One hundred milligrams of soft healthy friable callus (gained after three continuous subcultures of callus at 7-week interval) was inoculated to 250 ml conical flasks (Borosil, India), each flask containing 50 ml of liquid medium. Incubation of all the cell suspension cultures was done in a rotary shaker at 25±2ºC in complete dark conditions.

2.3 Optimisation of initial pH, sucrose concentration and agitation speed

The effect of different pH (5.2, 5.4, 5.6, 5.8, 6.0 and 6.2) and optimization of sucrose concentrations (10, 20, 30, 40 and 50 g/l) were investigated for the production of biomass. The effect of agitation speed (90,120 and 160 rpm) as a parameter was also investigated. All these parameters were investigated and optimised in terms of cell growth (in g/l), i.e. biomass in cell suspension culture. For all the mentioned parameters, 100 mg of fresh suspension cells were inoculated in 50 ml of the liquid media in 250 ml Erlenmeyer flasks (Borosil, India) for 35 days. The cells were incubated at 25±2ºC in completely dark conditions.

2.4 Estimation of cell growth and nutrient uptake kinetics

For quantification of cell dry weight, the cell suspensions were harvested every 5th day. The harvested cells were washed with sterile distilled water and filtered under vacuum. Afterwards, the harvested cells were kept at temperature in the oven for drying until the constant weight was achieved. A drying temperature of 60±2°C was maintained to prevent decomposition of thermolabile compounds [11]. The change of medium pH, sucrose uptake, nitrate uptake and phosphate uptake by cell suspension cultures were monitored every 5thdays. The utilization of

sucrose was estimated using anthrone reagent. Anthrone reagent was prepared by dissolving 0.2 g anthrone in 100 ml 17.8 M sulphuric acid (95% w/w). After 30 to 40 min, 1 ml of standard solution including samples (filtrate of medium for estimation) and 5 ml of anthrone reagent were added. This mixture was incubated for 5 min in a boiling water bath, and then absorbance at 629 nm was recorded. For making standard curve, glucose was utilized [8]. Phosphate uptake was calculated using a standard calibration curve made from NaH_2PO_4 [11, 12]. For estimation of phosphate, 0.5 ml solution of standard or sample (filtrate of medium for estimation) and 4 ml of reagent (2.5 M H_2SO_4, 10 mM ammonium molybdate.$4H_2O$ and acetone mixed with the ratio of 1:1:2) were mixed and vortexed. Then 0.4 ml of citric acid (1 M) was added and mixed by a vortex. After proper mixing, absorbances of all samples were measured at 355 nm by UV visible spectrophotometer (Varian Cary 300). In the same way, nitrate uptake was estimated; the calibration curve was prepared from a 0.01 N solution of KNO_3 that had been preserved in chloroform [11, 13]. After acidification of all samples with 95% (w/w) sulphuric acid (i.e. 17.8 M), absorbance was taken at 275 nm by UV visible spectrophotometer.

2.5 Extraction of plant metabolites

Cell separation (at 30thday of growth) was performed by filtration cell suspension culture of MS (3% sucrose) + 2,4-D (1 µM) + NAA (1 µM) + BAP (10 µM) with pH 5.8 and at 120 rpm with Whatman No.1 filter paper. Then, the filtered cells were washed 4 times with SDW (sterile distilled water) and weighed. With slight modification, extraction of 2-hydroxy-4-methoxy benzaldehyde from the cell suspension method was done according to Giridhar *et al*. [3, 14]. Separated cells were first dried in the oven at 60ºC. The cells (1 g) were then lightly ground in a mortar and pestle. Extraction of the volatile compounds was done by a steam condensate method with dichloromethane (50 ml × 4). Extract mixed with water was passed through a separating funnel that contained anhydrous sodium sulphate to remove the water content. Then, extracts were concentrated in a vacuum evaporator. After concentration and weighing for estimation of yield, the concentrated extracts were dissolved in 1 ml ethanol and stored in glass vials.

2.6 Preparation of standard dilution for GC analysis

Gas chromatography (GC) grade standard quality chemicals were used for GC analysis. Standard compound (2-hydroxy-4-methoxy benzaldehyde (HMB); 98 % purity) was procured from Sigma-Aldrich Chemical Co. Ltd (New Delhi, India). The stock solution was made with a concentration of 1mg/ml (by dissolving 5 mg HMB in 5 ml methanol) of standard or marker compound. Working HMB solutions of 20, 30, 40, 50, 100 and 200 µg/ml were made through the serial dilution method from standard stock solution with methanol. All the prepared solutions were stored for further use at 4ºC.

2.7 GC conditions and preparation of calibration graph

From each prepared HMB standard working solution (20 to 200 µg/ml), 1 µl was injected into the GC instrument (Shimadzu GC 2014) equipped with a capillary column (30m X 0.32 mm, 0.25 mm thickness of film). The oven temperatures were set as 60°C (2 min) rising 2°C/min up to 250°C (held for 5 min). The injection port temperature of the GC was 225°C, and the detector temperature was 250°C. The carrier gas was nitrogen and the flow rate was 30 ml/min. A calibration curve was prepared by plotting concentration against peak area on Microsoft Office Excel 2007. The correlation coefficients (r^2) were also generated with the same software by fitting the linear trend lines to the standard curve obtained for HMB. The retention times of the obtained HMB standard and extracted samples peaks were analysed and quantification was carried out. For the quantification, a standard equation was generated from the standard curve for extracted samples.

2.8 Statistical analysis

All observed results were represented as the mean ± SD of three separate analyses. All the data were pooled in triplicate to check reproducibility. The data of multiplication of callus and optimization (pH, sucrose convention and rpm) of cell suspension cultures were analysed using one-way analysis of variance (ANOVA) by SPSS (version 16) software. The significance of differences among means was analysed by Duncan's test at 5% level ($P< 0.05$) of significance. The specific growth rate (μ) was evaluated by:

$$\mu = \ln (MT2 – MT1) / T2 – T1$$

In the above growth rate (μ) formula, MT2 and MT1 are biomasses at the different time points (T1 and T2; and T2>T1), respectively. For HMB production and analysis by GC, two repeated experiments with three replicate flasks in each experiment were considered.

3. Results and Discussion

3.1 Induction of callus from root tip

Surface sterilised seeds of *D. hamiltonii* were germinated and radicals appeared on the 3rd day; roots elongated by approximately 1.5 cm in 6 days after incubation (Figure 1a). Based on the literature, 2,4-D, NAA and BAP were selected for induction of callus [2, 7, 15]. The effects of various concentrations of 2,4-D, NAA and BAP alone, and in combination, on induction of callus from root tip are shown in Table 1. MS basal medium without growth regulator (control) and MS medium with 2,4-D, NAA or BAP alone did not induce callus from the root tips. The use of various concentrations of 2,4-D, NAA and BAP (i.e. two auxins and one cytokinin) in combination did efficiently cause the induction of callus from germinated root explants (Table 1). Initially, all calli were white in colour and hard to semi-hard in nature. The subculturing of calli was done over a 7-week interval. After the second subculturing (i.e. 14 weeks), the calli became fragile and cells were loosely attached on MS fortified with 2,4-D (1μM), NAA (1μM) and BAP (10μM) (Figure 1b). After 49 days, the significantly maximum callus growth, 1210±27mg, was observed in MS (3% sucrose) + 2,4-D (1μM) + NAA (1μM) + BAP (10μM) (Table 1).

3.2 Cell line establishment

Initiation of cell suspension culture was reported to be possible when cell lines were established on semi-solid medium [7]. For the establishment of suspension culture, MS medium with the same concentration and plant growth regulators as present in the induction and growth of callus were used. So, the establishment of cell suspension culture were done in liquid (without agar) MS (3% sucrose) medium + 2,4-D (1 μM) + NAA (1 μM) + BAP (10 μM) (Figure 1c) and cells were healthy and loosely attached (Figure 1d). Carbohydrates as carbon sources are the major constituents of plant cell culture [8, 11]. In the present study, sucrose was used as a carbon source. The optimum sucrose concentration for higher biomass yield was checked in suspension culture, and it was observed that 30 g/l (i.e. 3%) sucrose gave the highest biomass yield (105.6±4.5 g/l) followed by 40 g/l (Figure 2). Similarly, in other species of cell suspension culture, sucrose was shown to significantly increase biomass [8, 11]. Variation of the initial pH of the medium was also optimised, and pH 5.8 (Figure 3) was observed to be suitable as it yielded the highest biomass (105.6±4.5 g/l). The growth period of cell suspension culture was 30 days (for detail see in Section 3.4), but callus growth on semi-solid medium was 49 days. The advantage of reduced growth period in the suspension culture was probably due to superior availability of nutrients or nutrient transport in the case of suspension cultures compared to semi-solid medium cultures [11, 16].

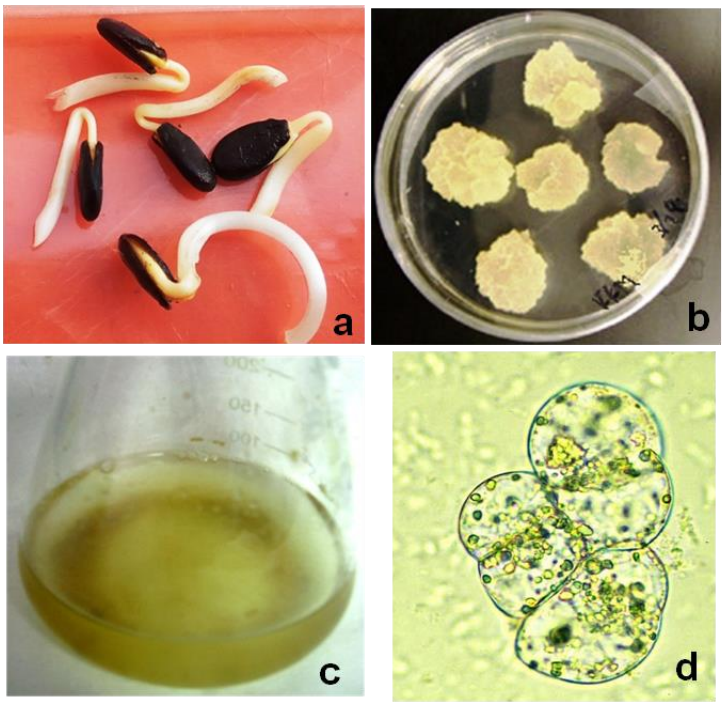

Figure 1. Establishment of cell suspension culture
a. Germinated seed in MS (3% sucrose) + GA_3 (0.2 µM) at 6th day
b. Grown callus biomass at 49th day of culture on MS (3% sucrose) + 2,4-D (1µM) + NAA (1µM) + BAP (10µM) being white, soft and fragile in nature
c. Suspension culture in MS (3% sucrose) + 2,4-D (1µM) + NAA (1µM) + BAP (10µM) in shake flask
d. Loosely attached healthy growing cells in cell suspension culture at 120 rpm

Table 1. Fresh weight of callus from root tip grown on MS medium with different concentrations of plant growth regulators after 49 days of culture

Growth regulators concentration in µM			Callus fresh weight (mg)
2,4-D	**NAA**	**BAP**	
0	0	0	-
1	0	0	-
0	1	0	-
0	0	1	-
1	1	1	360±20^{c}
1	1	5	864±23^{b}
1	1	10	1210±27^{a}
1	2	1	423±30^{c}
1	2	5	346±21^{c}
1	2	10	773±31^{b}
2	1	1	125±18^{d}
2	1	5	259±24^{cd}
2	1	10	460±34^{c}

Means ± SD followed by the same letter are not significantly different at the 5% (P< 0.05) level according to Duncan's multiple range tests.
- means callus induction and growth did not occurred in this medium.

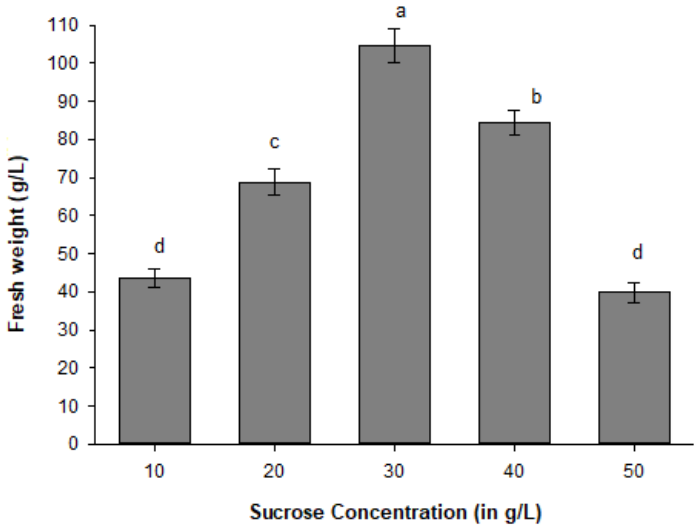

Figure 2. Effects of sucrose concentration on cell biomass of *D. hamiltonii* cell suspension culture. Same letters do not differ significantly ($p < 0.05$) according to Duncan's multiple range test.

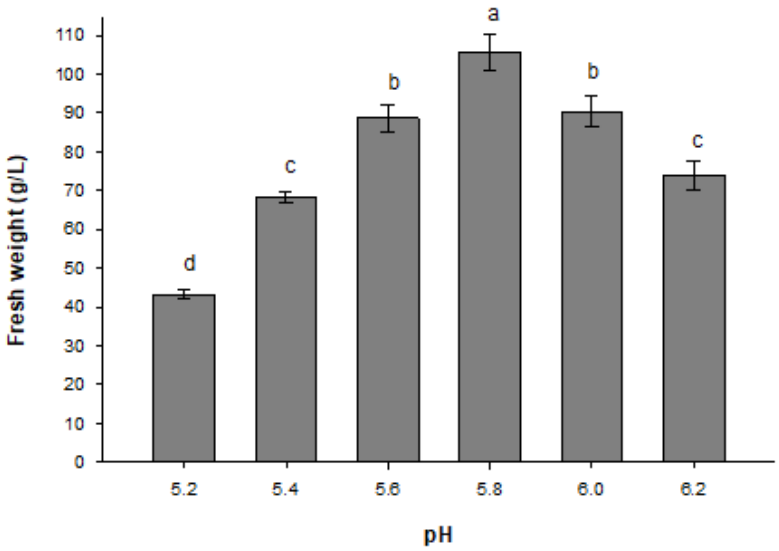

Figure 3. Effect of pH on cell biomass of *D. hamiltonii* cell suspension culture. Same letters do not differ significantly ($p < 0.05$) according to Duncan's multiple range test.

3.3 Effect of agitation speed on biomass yield

Agitation speed directly affects the growth of cells and biomass yield (g/l) in cell suspension cultures due to agitation and aeration. The yield of biomass at different agitation speeds (rpm) was optimised. The maximum fresh weight (105.6±4.5 g/l) was observed at 120 rpm (Figure 4). At this rpm, the cells were healthy and loosely attached to each other (Figure 1d). At higher

agitation speeds (150 and 180 rpm), biomass was highly unsatisfactory. At speeds lower than 120 rpm, e.g., agitation speed at 60-90 rpm, the biomass yield was lower and clumps/aggregates of cells occurred. In the same ways, several other plant species reports explained that cell suspension is highly sensitive to agitation speed [8, 11]. Agitation ensures the proper mixing of plant cells in the medium and thus facilitates the homogeneous distribution of nutrients for all cells equally, and also providing a sufficient O_2 and CO_2 supply. Agitation shear force can cause various changes in morphology and cell physiology such as shape and size of cell aggregates, the integrity of cells, cell viability and cell biomass. All these morphological and physiological changes affect the production of secondary metabolites [8].

3.4 Cell growth and nutrient uptake kinetics

In the present study, cell growth kinetics are expressed as fresh and dry cell weights of *D. hamiltonii* suspension culture (Figure 5). Our results suggest that *D. hameltonii* cell suspension culture was in a lag phase at initial 5 days. After that, cells growth entered an exponential growth phase and cell biomass increased till the 30th day as of a stationary phase. A death phase or decline of biomass observed after the 30th day onwards. Similarly, after 4 weeks, a decrease in biomass production was also observed in *D. hamiltonii* suspension culture in shake flask from *in vitro* leaf explant calli [16]. This decrease of cell suspension biomass is due to the unavailable or very much redueced availability of plant nutrients and oxygen deficiency in the medium [8]. For the cell growth curve in our study, cell biomass decreased in the death phase, and the same type of pattern was also reported for *Spilantes acmella* [8]. The maximum specific growth rate (μ) of *D. hamiltonii* cell suspension culture is calculated using the equation that was earlier mentioned in the statistical analysis (Section 2.8). The maximum specific growth rate (μ) of *D. hamiltonii* cell suspension culture was 0.086/day. Initially, up to the 5thday, pH of the medium decreased. Then, it decreased very quickly during different stages of cell culture. In the death phase, slight increases of the medium pH were observed (Figure 6). The consumption profiles of nitrate, phosphate and sucrose for the complete growth of cell suspension culture are shown in Figure 6. The uptake rate of phosphate is relatively slower than that of nitrate. Nitrate and phosphate were present in the medium until the end of cultivation (Figure 6). Consumption of sucrose proceeded at a relatively uneven rate and decreased continuously till the 35th day. In comparison to sucrose, phosphate and nitrate concentration in the medium was invariably observed and was almost finished by the 30th day. From the 30th day onwards, a slight increase in phosphate and nitrate concentration in the medium had been observed until the culture was examined. The slight increase of nitrate and phosphate concentration in the medium may have been due to cell stress, cell death and cell lysis, which resulted in leakage of phosphate and nitrate into the medium from the cells. A similar profile of phosphate and nitrate concentration in cell cultures was also reported in different plant species [8].

3.5 Analysis of 2-hydroxy-4-methoxy benzaldehyde production

The extraction of produced HMB for CG analysis from the dried cell of *D. hamiltonii* by steam condensate methods was mention in Section 2. Volatile metabolites were obtained at a yield of 0.16%. The calibration curve was prepared by plotting the concentration (20-200 μg/μl HMB) versus obtained GC peak area. The retention times for HMB in GC analyses were 25.5 min (Figure 7a). The final calibration curve was found to be linear. From the regression analysis, the r^2 value was found to be 0.985.

Gas chromatography was used for analysing volatile oil which was extracted from *D. hamiltonii* cell suspension. The identification and quantification for the production of HMB were also done (Figure 7b). At the 30th day (i.e. stationary state), HMB production was identified and estimated to be 0.92 ± 0.02 mg/ml.

After calculation, HMB availability in cell suspension culture was 0.092%. The highest productions of HMB were previously reported as 0.14 and 0.12% in tissue culture raised roots

of *D. hamiltonii* and acclimatized *D. hamiltonii*, respectively [17]. The extraction of volatile oil

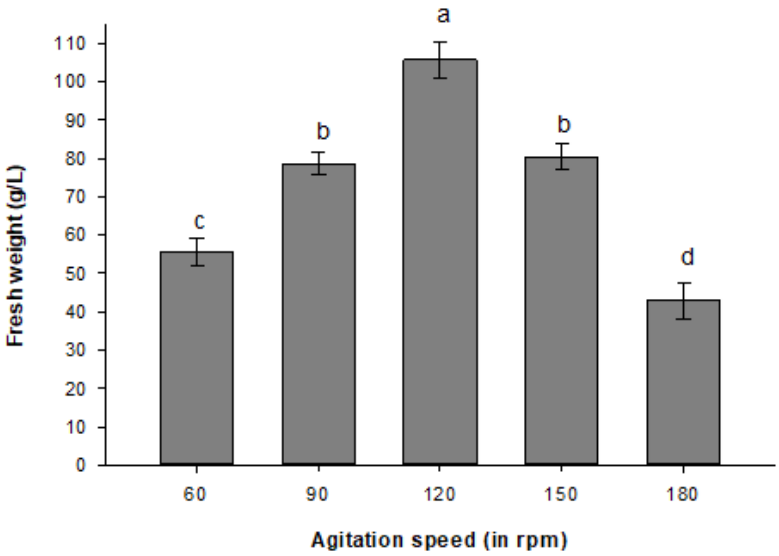

Figure 4. Effect of agitation speed on cell biomass of *D. hamiltonii* cell suspension culture. Same letters do not differ significantly ($p < 0.05$) according to Duncan's multiple range test.

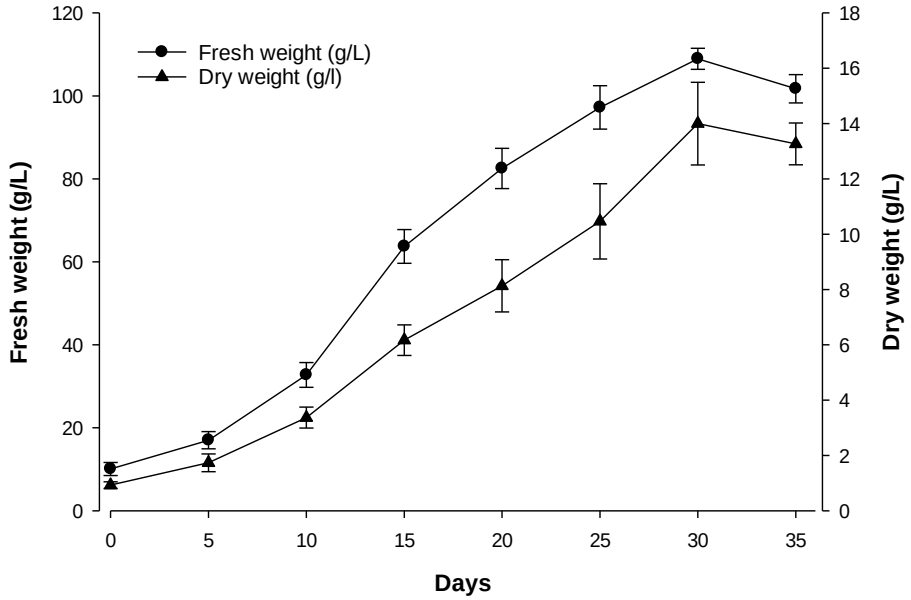

Figure 5. Growth profile of cell growth in *D. hamiltonii* cell suspension cultures in MS (3% sucrose) medium + 2,4-D (1 µM) + NAA (1 µM) + BAP (10 µM)

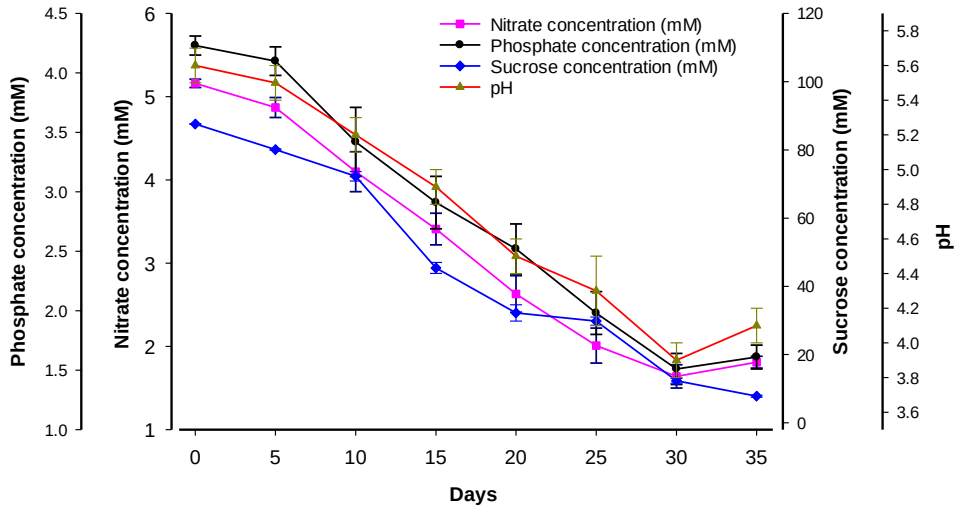

Figure 6. Profile of nutrient uptake and medium pH in *D. hamiltonii* cell suspension cultures

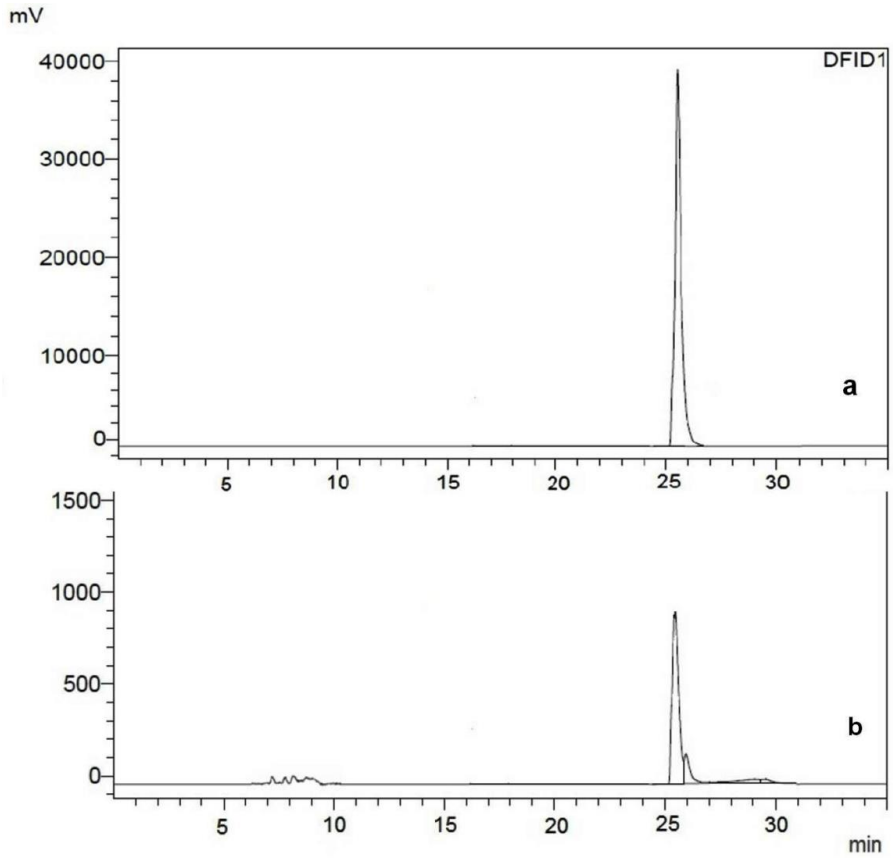

Figure7. GC chromatogram of 2-hydroxy-4-methoxybenzaldehyde analysis
a. Chromatogram of HMB standard showing retention time 25.5 min
b. Chromatogram of extract from suspension culture showing production of HMB (at same retention time 25.5 min)

was undertaken from wild or naturally grown *D. hamiltonii* fresh tuberous root by previous researchers [2, 18]. In those studies, the extraction yield of volatile oil was 0.68% and HMB production was 0.54%. The present study is the first report for suspension culture from root tip culture of *D. hamiltonii* that leads to valuable HMB production. In comparison with naturally grown and acclimatized micro-propagated plant, HMB production is lower in root tip callus derived suspension culture. Further adaptation of the media used and protocols followed, and improved techniques of elicitation in plant bioreactors, should lead to enhance the production of HMB.

4. Conclusions

This is the first report on the establishment of cell suspension culture from the root tip of germinated seed radicle that are explants of the endangered and economically valuable aromatic *D. hamiltonii* plant. In MS (3% sucrose) fortified with 2,4-D (1µM), NAA (1µM) and BAP (10µM) medium, the maximum callus amount was observed. The pH of this liquid medium at 5.8 and agitated at 120 rpm provided the best medium and conditions for the increasing of the cell biomass. A concentration of 0.092% of HMB, which was determined by GC, was obtained from cell suspension culture. Furthermore, the adoption of suitable elicitation and controlled conditions such as found in a plant bioreactor, may enhance the production of 2-hydroxy-4-methoxy benzaldehyde by biotechnological methods. Cell suspension cultures, as we have demonstrated in our work, may well be utilized in some food and pharmaceutical industries, and this should help to conserve the rare and valuable *D. hamiltonii* in its natural habitats.

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