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Biomass production and bioactive secondary metabolites of bioreactor grown callus suspension cultures of Swallow root (*Decalepis hamiltonii* Wight & Arn.)

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Abstract:

In the present study establishment of *in vitro* callus cultures of *D. hamiltonii* on MS medium followed by its biomass augmentation trials in Erlenmeyer conical flask and stirred tank bioreactor (STB) was investigated. The callus suspension culture grown in 150mL Erlenmeyer flask showed 9.1 g FW per 50 ml culture (182 gL^{-1} FW of biomass) and 0.097±0.21mg $100g^{-1}$ DW of 2H4MB in 4 weeks of growth. The methanol extract of the callus showed 13.1±0.2 mg $100g^{-1}$ DW and 0.46±0.03 mg $100g^{-1}$ DW of TPC and TFC respectively. Whereas in callus culture grown in 2 lit medium in STB, 33.5 g FW of biomass was recorded and 0.024± 0.007 mg 100g-^{1} DW 2H4MB upon 7 days growth. The methanol extract of fermenter-grown-callus showed 2.13± 0.39 mg 100g-^{1} DW and 0.12± 0.4 mg $100g^{-1}$ DW of TPC and TFC respectively. The study infers *D. hamiltonii* callus culture in a bioreactor can be an alternative method for producing vanillin isomer in large-scale production and further optimization of culture conditions in this regard is required.

Key words: **Callus suspension cultures, 2H4MB, Stirred tank bioreactor, Swallow root**

INTRODUCTION

Flavours come in high demand for food additives that are used in the food industry. The palatability of food could be increased through flavour and to date, vanillin tops second among all flavour molecules in both its usage and market [1]. Owing to high demand, long processing conditions of vanillin bean synthetic vanillin flavour stood as a great alternative product. However, in many countries, there was restricted usage of synthetic Flavours and has persistent demand for natural Flavours. Besides vanillin, there is a wide range of unique Flavours are reported from natural sources [2]. *Decalepis hamiltonii* Wight and Arn is one such plant which is having vanillin-flavour-rich tubers [3, 4]. *D. hamiltonii* (Swallow root) is an endemic monogenetic climbing shrub native to the Deccan peninsula that belongs to the

family Asclepediaceae and is used as a flavouring agent because of its strong aromatic tubers. It has an aromatic compound, namely 2-hydroxy-4-methoxy benzaldehyde (2H4MB), which is one of the structural isomers of vanillin [5] and its content varies with the habitat [6]. Large-scale production of flavour from this plant source can use as an alternative to synthetic flavour.

The plant due to its high-demanding tubers and its destructive harvesting currently enlisted as endangered [7]. Several techniques like micropropagation are used to revive the plant and its sustainable production. Micropropagation is, therefore, become a commercially lucrative enterprise and provides marked advantages over conventional horticultural propagation practices by facilitating the production of large numbers of homogenous plants year-round, generation [8]. In addition to 2-Hydroxy-4-methoxy benzaldehyde (2H4MB), other flavour metabolites, including vanillin, 4-Methoxy Cinnamic acid derivatives, aromatic alcohols, etc., have been identified [3]. *In vitro* cultures of plants viz., callus [2], roots[9], and embryos[10, 11] also produce secondary metabolites just like that of the *ex vitro* plant or may produce other metabolites. Such *in vitro* plant cultures are known to give a high potential for the production of secondary metabolites of economic value [12]

Bioreactors have been reported to be configured and effectively used for the large-scale production of metabolites [2]. The *in vitro* cultures produced through micropropagation can be scaled up to reactor level and high product yield; concentration can be achieved by optimizing bioreactor operating conditions [2]. During bioreactor culture, bioactive compound accumulation is affected by various factors such as the efficiency of oxygen transfer, shear forces, hydrodynamic forces, and control of the physicochemical environment. Studies have focused on controlling these parameters to enhance the yields of biomass and bioactive compounds during bioreactor culture [11]. The present research work aims to the establishment of *in vitro* callus suspension cultures of *D. hamiltonii* and to evaluate TPC, TFC, and 2H4MB production in conical flasks and stirred tank bioreactor.

MATERIALS AND METHODS

Materials

Plant growth hormones, Murashige and Skoog media, Sucrose, Inositol (Hi-Media, Mumbai), High-performance liquid chromatography (HPLC) grade solvents, viz., ethanol, methanol, acetonitrile and glacial acetic acid were purchased from Merck Millipore (India). Analytical-grade dichloromethane, sodium carbonate (Na_2CO_3), Folin–Ciocalteu's reagent, aluminium chloride ($AlCl_3$), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Sisco Research Laboratory (Mumbai, India).

Methods

Plants material and Medium preparation

Decalepis hamiltonii Wight and Arn., freshly harvested dry fruits from 10 years old plant of CSIR-CFTRI Mysore, India departmental garden were collected. The seeds are separated and inoculated after surface sterilization with 0.1% ($w.v^{-1}$) mercuric chloride for 5 min in culture media containing Murashige and Skoog medium [13] supplemented with 0.2 µM gibberellic acid (GA_3), 3% (w/v) sucrose and 0.75% (w/v) Agar [14]. The pH of the media was adjusted to 5.8 ± 0.2 with 0.8% (w.v-1) of agar. These cultures were maintained at 24 ± 2 ° C under 45 $\mu mol.m^{-2}\ s^{-1}$ light for 16 h photoperiod using fluorescent lights (Philips India Ltd, Mumbai).

Callus Suspension culture

Callus culture was developed from the leaves of a 6-week old seedling-plant, which were cut into appropriate sizes (~ 1sq.cm), and used as explants. The explants were inoculated on MS medium containing 9.06 µM 2,4-D (2,4-D, Hi-media, Mumbai), in combination with 2.32 µM Kinetin (Kin, Hi-media, Mumbai), 3% (w/v) sucrose, and 0.8% (w/v) agar for solid media [14]. The pH of the medium was adjusted to 5.7 and autoclaved at 121 °C for 15 min. Callus cultures were maintained at 25 ± 2 °C in 45 $\mu mol.m^{-2}\ s^{-1}$ light for 16 h photoperiod. The callus culture obtained was separated from the explants and sub-cultured onto the same medium and cultured for 4 weeks, and the same was repeated for two more cycles. The friable callus mass that was obtained was used for the preparation of suspension cultures. This callus was transferred to 150 ml conical flasks containing 50 ml liquid medium and grown for 15 days on a rotary shaker at 95 rpm at 24 ± 2 °C.in 16 h photoperiod, to get fine suspension cultures without clumps. The suspension culture was further subcultured to the same medium to get week old fine suspension that was used as an inoculum.

Fermenter

Stirred-tank bioreactor (BioFlo 3000, M/s New Brunswick Scientific, USA) having 3L capacity, was inoculated with the callus inoculum (0.1%) MS medium supplemented with 9.06 µM 2,4-D, and 2.32 µM Kinetin, The pH of the medium was adjusted to 5.6-5.8 and autoclaved in a 3L fermenter (working volume of 2L). The inoculum was prepared from callus culture which was a subculture from 10 days grown suspension cultures. The biomass was chopped finely and with inoculated into the fermenter under sterile conditions (1% i.e. 10g callus mass for 1L medium). The agitation of the impellers was kept constant throughout the experiments to allow proper mixing throughout the culture (60 rpm). The

temperature of the reactor was maintained at 24±2 ˚C with the help of a chiller and aeration in the entire proceeding time was regulated by using a flow meter to pass sterile air through an air filter (Whatman) from an air pump. The total biomass accumulated was harvested, transferred to a filter membrane which is attached to a vacuum pump to separate callus from media. The biomass obtained was weighed (both fresh and dry weight). The metabolic extract of the callus was extracted (dry callus) and TPC, TFC, and 2H4MB content was analyzed.

Extraction and Quantification of flavour metabolite

The flavour metabolite present in callus was extracted according to [14] using dichloromethane (DCM) in order to quantify 2H4MB which is the principal flavour molecule. Callus suspension cultures were separated by filtration from the media and the medium around the cell mass was removed by washing with distilled water and dried in an oven at 35^0C for 12 h. The dried mass was weighed and powdered with mortar and pestle and the powder thus obtained was extracted with 1:2 volumes of DCM and centrifuged at 10,000 rpm for 5 min. The supernatant was concentrated and dried under a vacuum. The dried residue was dissolved with a known volume of methanol and the flavour attributing compounds such as 2H4MB were analyzed.

The flavour metabolite 2H4MB was quantified from the solvent extracts of *D. hamiltonii* callus by HPLC (SPD-20AD, Shimadzu, Kyoto, Japan) as reported earlier for PPP molecules, but with slight modification[15]. The isocratic mobile phase contained Methanol: Acetonitrile: Water: Acetic acid (47:10:42:1). C18 column (250 x 4.6 mm and 5-µm diameter) was used (YMC column, Waters Corporation, MA, USA) for the sample separation and analysis. The flow rate was maintained at 1 ml min^{-1} throughout the analysis and the detection wavelength was 280 nm. The samples were respectively injected and the mean area for three replicate analyses was calculated. Quantitative analysis was done based on the area of 2H4MB standard (Fluka, Switzerland) for which a known amount was injected. 2H4MB in the samples was identified based on the retention time for the corresponding standard.

Biochemical analysis

Determination of total phenolic content

The TPC in methanol extract was determined ‘‘Folin-Ciocalteu’s’’ reagent assay [16]. The 80% methanol extract from the tuber was mixed with Folin Ciocalteau’s reagent followed by 20% Na_2CO_3 solution and kept for incubation. The samples after incubation were measured for phenolic content at 650 nm and the amount of total phenolic present in the samples was obtained by plotting against the standard graph.

Determination of total Flavonoid content

The TFC in methanol extract was estimated using the spectrophotometric method as reported by Pradeep et al., (2019) with little modification. The flavonoid content in 80% methanol extract of callus was determined and the amount of TFC present in the samples was obtained by plotting against the standard graph.

Statistical analysis

The callus suspension cultures were grown in conical flasks (20 no.) and the callus biomass of five flasks were recorded (± SD). The TPC, TFC and 2H4MB content of the callus was shown as three replicate analyses (± SD).

3. RESULTS

Callus and suspension cultures

D. hamiltonii seeds were observed to germinate after 20 d and showed 80% seed germination [17]. The seedlings grown after 45 days were used in preparing the leaf explants to induce callusing (Fig 1A). The callus initiation from the *in vitro* seedling plant leaf explants was noticed by 10-14d after inoculation into the callus induction medium comprising MS medium supplemented with 9.06 µM 2,4-D and 2.32 µM kinetin. After 4 weeks, the green color callus developed from the leaf explant cut edges were separated and sub-cultured onto the same medium and allowed to grow for 4 weeks for further proliferation. Two more cycles of subculturing at 4weeks intervals were carried out, to obtain friable and green callus, which was used for further experiments. (Fig 1B). To develop callus suspension culture, known quantity (500mg) callus mass was transferred to 150 ml conical flasks containing 50 ml culture medium i.e. MS liquid medium containing 3 % sucrose, 9.06 µM 2,4-D and 2.32 µM kinetin [14]. Callus suspension culture was obtained in MS liquid medium containing 3 % sucrose, 9.06 µM 2,4-D, and 2.32 µM kinetin [14]. Initially, the callus growth was not completely uniform and some small clumps were noticed. Two cycles of subculturing at 15d intervals were done, to get green color callus suspension that was further used as inoculum. In our study, after one week of growth in a conical flask, the callus biomass was very low (0.75 g FW). Similarly, TPC, TFC, and 2H4MB contents are substantially low (Table 1). After 4 weeks of culturing, 182 ±1 gL^{-1} FW of biomass was recorded. The methanolic extract of the callus cultures showed TPC 13.1±0.2 mg $100g^{-1}$ DW and 0.46±0.03 ±0.1mg $100g^{-1}$DW respectively upon spectrophotometric analysis. The dichloromethane extract of suspension cultures was used for quantification of flavour metabolite i.e., 2H4MB. The compound showed its peak at a retention time (RT) of 8.2 min, at the wavelength of 280 nm with the standard compound (Fig.2A). In the four-week-old callus suspension culture, 0.097±0.21mg $100g^{-1}$ DW of 2H4 MB production was recorded.

Fermenter

We evaluated the biomass growth and 2H4MB production in *D. hamiltonii* callus suspension cell cultures (Fig 1d). These results show the leaf origin suspension cell culture can be considered a well-growing culture in bioreactor (Table 2). In the bioreactor, 1% of the callus suspension culture after two cycles of subculturing at a 15d interval was used as inoculum. In the culture after 7d growth, the biomass was observed to be 33.5± 5 (gL^{-1} FW). The methanol extract of the harvested callus was evaluated for its TPC and TFC content and found to be 2.13± 0.39 mg $100g^{-1}$ DW and 0.12± 0.4 mg $100g^{-1}$ DW respectively. The flavour compound 2H4MB content was recorded as 0.024± 0.007 mg $100g^{-1}$ DW (Table 2).

Discussion

In the present study, compared to the conical flask culture of *D. hamiltonii* in bioreactor TPC, TFC, and 2H4MB content showed a varied response. Our earlier studies confirmed the ability of the suspension culture to proliferate in culture vessels for up to 4 weeks [14]. In the case of the bioreactor, the callus suspension cultures growth for more than 1 week did not support further growth and turned to brown. Accordingly, in the present study we have confined to 4 weeks and one week for the callus growth in conical flask and bioreactor respectively. Since the concentration of the inoculum is important for final biomass production in the culture vessels, we have used the uniform concentration of the inoculum i.e., 1% for both conical flask and bioreactor. The culture vessel volume, aeration pH also influences the callus cells' growth [11]. The callus cells biomass production in a bioreactor under specified culture conditions in one week was found to be ~ 50% of that of conical flask culture on by rotary shaker in 4 weeks. But from the incubation time perspective, the response for biomass production in the bioreactor in 1 week is notable. The TPC, TFC, and 2H4MB content of bioreactor grown culture for 1 week showed less to that of 4 weeks grown conical flask culture, but at the same time, it is more to that of 1 week grown conical flask culture. Based on these observations it is understood that it could be under optimized culture conditions. Similar observations for *in vitro* cultivation of plant cell suspensions in a liquid medium for scale-up in the bioreactor were stated [2, 9].

Conclusion

In this study *in vitro* callus suspension cultures from seedling leaf explants of *D. hamiltonii* were achieved. The total phenolics and flavonoids content along with flavour metabolite 2H4MB production were observed in the bioreactor grown cultures and also in conical flask culture. These preliminary leads in improving the biomass and bioactives production by *D. hamiltonii* callus cultures in bioreactor needs to be further investigated to scale up by optimizing culture conditions and also by advocating elicitors and growth-promoting agents.

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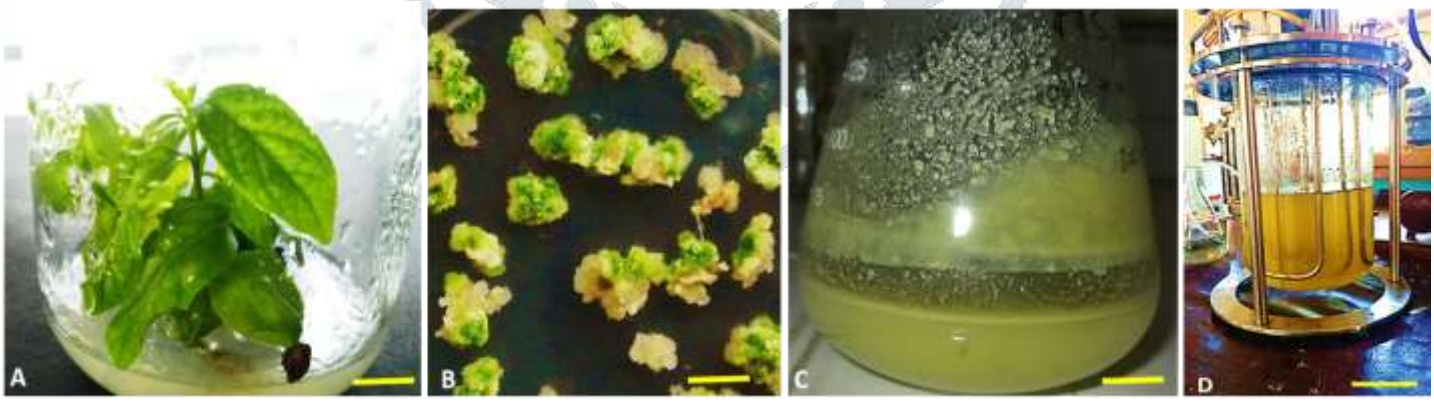

Fig: 1: A *In vitro* seedlings of *D. hamiltonii* on MS basal medium with GA_3 (bar=10 cm); **B** Callus induction from leaf explants of the seedlings on MS medium supplemented with 2,4-D (9.06 µM) & kinetin (2.32 µM) (bar= 10 cm) **C** Callus cell suspension culture grown in medium with 9.06 µM 2,4-D and 2.32 µM kinetin after 4 weeks (bar=15 cm); **D** Bioreactor with callus culture (bar=30cm)

Table. 1. *D. hamiltonii* callus biomass and secondary metabolites production in conical flask grown cultures

Parameter	1 Week culture	4 weeks culture
Callus fresh weight (g FW/ 50 ml)	0.75±0.25	9.1±0.6
Biomass yield (gL^{-1} FW)	15	182
2H4MB (mg $100g^{-1}$ DW**)**	0.001±0.0005	0.097±0.21
Total Phenolic content (mg $100g^{-1}$ DW)	1.8±0.12	13.1±0.2
Total flavonoids content (mg $100g^{-1}$ DW)	0.09±0.01	0.46±0.03

Initial weight of the callus inoculum 500mg per 50 ml medium

*Values are mean ± SE of three replicate analyses FW- Fresh weight; DW- Dry weight

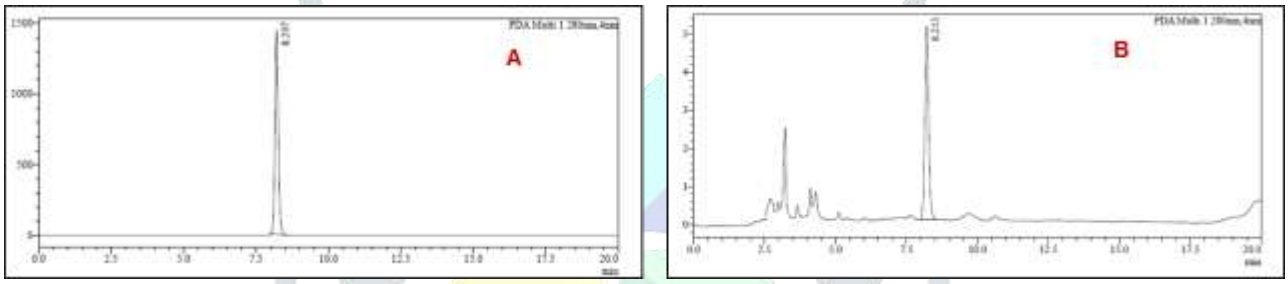

Fig: 2: HPLC analysis of flavour compound 2H4MB A**)** 2H4MB standard; **B**) 2H4MB in callus grown in bioreactor

Table. 2. *D. hamiltonii* callus biomass and 2H4MB production in Bioreactor grown cultures for one week

Biomass (FW)			**Flavour metabolite (DW)**		
Inoculum weight (g)	**Final weight (g)**	**Fold change**	**2H4MB** mg $100g^{-1}$	**Phenolic** mg $100g^{-1}$	**Flavonoids** mg $100g^{-1}$
20	33.5± 5	1.69± 0.2	0.024± 0.07	2.13± 0.39	0.12± 0.04

* Values are mean ± SE of three bioreactor run. The working volume of bioreactor is 2L; FW- Fresh weight; DW- Dry weight

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