Enhanced production of vanillin flavour metabolites by precursor feeding in cell suspension cultures of *Decalepis hamiltonii* Wight & Arn., in shake flask culture

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Abstract The flavour rich tuberous roots of *Decalepis hamiltonii* are known for its edible and medicinal use and have become endangered due to commercial over-exploitation. Besides 2-Hydroxy-4-methoxy benzaldehyde (2H4MB), other flavour metabolites in tuberous roots include vanillin, 4-Methoxy Cinnamic acid derivatives, aromatic alcohols etc. So far, there are no reports on the pathway of 2H4MB biosynthesis nor there is an organized work on biotransformation using normal and cell suspension cultures for obtaining these metabolites using precursors. The main aim of the study is to develop a method for enhanced production of flavour attributing metabolites through ferulic acid (FA) feeding to the *D. hamiltonii* callus culture medium. Biomass of *D. hamiltonii* cell suspension cultures was maximum (200.38 ± 1.56 g/l) by 4th week. Maximum production of 2H4MB was recorded on 4th week (0.08 ± 0.01 mg/100 g dry weight) as quantified by HPLC. Addition of 0.1–1.5 mM ferulic acid as precursor in the culture medium showed significant ($p < 0.001$) effect on suspension cultures biomass and respective phenylpropanoid metabolites content and 2H4MB accumulation. The maximum accumulation of vanillin, 2H4MB, vanillic acid, ferulic acid were of 0.1 ± 0.02 mg/100 g, 0.44 ± 0.01 mg/100 g, 0.52 ± 0.04 mg/100 g, 0.18 ± 0.02 mg/100 g DW respectively in 4 weeks of cultured cells supplemented with 1 mM ferulic acid as a precursor. The results indicate that, substantial increase in the levels of flavour metabolites in *D. hamiltonii* callus suspension culture was achieved. This would be

having implications in biosynthesis of respective vanilla flavour attributing metabolites at very high levels for their large scale production.

Keywords Biomass · Callus cell suspension cultures · Ferulic acid · 2-Hydroxy-4-methoxy benzaldehyde (2H4MB) · Swallow root

Introduction

A vanillin isomer 2-Hydroxy-4-methoxy benzaldehyde is the principal flavour compound in tubers of *Decalepis hamiltonii* belongs to Asclepiadaceae. This endangered plant popularly familiar as swallow root (English) or makaliberu in vernacular language (Kannada) is endemic to southern peninsular region of India. During the last one and half decade this plant has been explored for its antimicrobial (Thangadurai et al. 2002; Mohana et al. 2008), antioxidant (Harish et al. 2005; Srivastava et al. 2006) and anticancer potential (Zarei and Shivandappa 2013). Also studies to some extent substantiated this plant tuber's usage in traditional medicine as an appetizer and blood purifier and also as an edible in making recipes (Pradeep et al. 2016a). Similarly, through biotechnological intervention, effective in vitro propagation methods and alternate mode of flavour attributing 2H4MB production by using in vitro root cultures was reported (Giridhar et al. 2005; Pradeep and Giridhar 2016, 2017). In view of the increasing demand for the tubers of *D. hamiltonii*, there is a concern on its survivability and existence as the local ethnic communities yank the tubers through destructive harvest method for economic gains. Under this sense, it is necessary to have alternate viable methods to produce the

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flavour metabolites for commercial applications and also to explore the potential molecules of interest.

Plant tissue and cell culture methods can be established under controlled conditions from various explants such as leaves, stems, roots, and meristems for extraction of various secondary metabolites (Karuppusamy 2009). In vitro production of secondary metabolites in plant cell suspension cultures has been reported from various edible food and medicinal plants (Hussain et al. 2012). This method is considered as an alternative to chemical or microbial methods which are having certain limitations. During the last few decades significant studies have been made in this area for producing wide range of secondary metabolites belong to terpenoids (Rajasekaran et al. 2007), alkaloids (Kumar et al. 2008), glycosides, volatiles (Hussain et al. 2012; Berlin et al. 1993; Ravishankar et al. 2003), diterpenes (Sridevi et al. 2010) and flavonoids (Devi and Giridhar 2014), which depends on the capacity of respective callus cell cultures of plants in performing methylation, oxidation, reduction, hydroxylation, glucosylation-acylation and amino-acylation, to transform a wide range of substrates. The attention in the production of high value secondary metabolites even in relatively low quantity is having relevance in food industry. To realize this, considerable interest has been shown by employing biotransformation method wherein the ability of microbial cells or plant cell culture to catalyze the conversion of readily available or low-cost precursor into a more valuable final product occurs (Hussain et al. 2012). The projected potentials of such methods use in industry lie in conversion of less expensive, bulk products to value added, more bioactive and low volume products (George and Ravishankar 1996). Essential oils of higher plants, fruit juices, vegetable extracts and a very few products of animal origin (amber, musk, zivet) were for a long time the sole sources of natural flavours. Biotechnological production of bioflavours by using microbes has been investigated (Bicas et al. 2010). The surge in consumer's preference for natural flavours over synthetic compounds and increase in demand at global market for natural products provoked the researchers to develop in vitro plant materials as potential factories for secondary phytochemicals production (Furuya et al. 1998; Karuppusamy 2009) and aromatic alcohols (Suga and Hirata 1990; Gholamreza et al. 2004). An et al. (2013) developed a process for the production of γ -Dodecalactone a fragrant compound from 10-Hydroxystearate by *Waltomyces lipofer* cells. Recently, Kiran et al. (2017) demonstrated the 2H4MB biosynthesis in *D. hamiltonii* with known phenylpropanoid pathway inhibitors and its precursor feeding experiments to know the immediate substrate molecule involved in its biosynthesis. Accordingly, the main aim of the study is to develop a method for enhanced production of flavour attributing metabolites

through ferulic acid feeding to the *D. hamiltonii* callus culture medium.

Materials and methods

Preparation of explants

Freshly harvested seeds of *Decalepis hamiltonii* Wight & Arn., were collected from the 7 year old plant grown at Departmental garden in CSIR-Central Food Technological Research Institute, Mysore, India. These seeds were washed under running tap water for 10 min. Surface sterilization of these seeds was done with mercuric chloride [0.1%, (w/v)] solution for 5 min, and washed thoroughly (three times, 5 min each) with sterile distilled water. Subsequently, seeds were inoculated on MS basal medium (Murashige and Skoog 1962) supplemented with 0.2 μ M gibberellic acid (GA_3), 3% (w/v) sucrose and 0.75% (w/v) Agar (Saini and Giridhar 2012). The leaves of 6 weeks old seedlings (Fig. 1a) were cut into appropriate sizes (leaf 1 sq.cm), and used as explants. After blotting with sterile filter paper, the explants were cultured on MS medium with or without 6-benzylaminopurine (BA), kinetin (Kn), α -naphthaleneacetic acid (NAA), or 2,4-dichlorophenoxyacetic acid (2,4-D), each at different concentrations (Table 1). The effect of combinations of 2,4-D and BA or Kn was also studied. The medium pH of 5.7 ± 0.1 was maintained, and solidified with 0.8% (w/v) agar for solid medium (but not for liquid medium), and autoclaved at a pressure of 1.06 kg/cm^2 for 20 min at 121°C . For suspension culture, 100 mg of friable callus mass (obtained after three subcultures of callus at 4 week interval) was transferred to 150 ml conical flasks each containing 40 ml of liquid medium. The suspension cultures were grown for 10 weeks on a rotary shaker at 95 rpm in 16 h photoperiod at $24 \pm 2^\circ\text{C}$.

Incorporation of precursor

Four week-old cell suspension cultures of *D. hamiltonii* were used for the experiment. Initially ferulic acid (FA) was dissolved in ethanol [50% (v/v)] followed by filter sterilization (Millipore 0.22 μ m). Then different concentrations ferulic acid (Johnson et al. 1996) viz., 0.1, 0.5, 1 and 1.5 mM were incorporated into medium and then cell suspension cultures were inoculated into these flasks. The levels of phenylpropanoids including 2-hydroxy-4-methoxy-benzaldehyde were analyzed at every 2-week intervals for 6-weeks. All the experiments were performed with three replicates for each concentration of FA treatment and the experiment was repeated twice.

Fig. 1 **a** In vitro raised seedlings of *D. hamiltonii*, Bar 10 cm; **b** Callus induction on medium bearing 2,4-D (9.06 μ M) + kinetin (2.32 μ M), Bar 5 cm; **c** Callus cell suspension on medium with 2,4-D (9.06 μ M) + kinetin (2.32 μ M), Bar 7 cm

Table 1 Callus induction from leaf explants grown on MS medium with growth regulators after 90 days of culture

Growth regulators (μ M)				Callus fresh weight (mg)
2,4-D	NAA	BA	Kinetin	
2.26	0	0.55	0	–
4.53	0	1.11	0	730 $\pm$ 1.25 ^f
9.06	0	2.22	0	1334 $\pm$ 1.5 ^d
11.32	0	3.33	0	1117 $\pm$ 1.14 ^e
0	2.68	0.55	0	–
0	5.37	1.11	0	610 $\pm$ 0.98 ^f
0	10.74	2.22	0	1786 $\pm$ 0.85 ^b
0	13.42	3.33	0	1478 $\pm$ 1.23 ^c
2.26	0	0	0.58	254 $\pm$ 1.74 ^g
4.53	0	0	1.16	984 $\pm$ 0.95 ^e
9.06	0	0	2.32	1897 $\pm$ 1.65 ^a
11.32	0	0	3.48	1484 $\pm$ 1.12 ^c
0	2.68	0	0.58	–
0	5.37	0	1.16	–
0	10.74	0	2.32	291 $\pm$ 0.82 ^g
0	13.42	0	3.48	874 $\pm$ 0.56 ^f

Values are represented as mean $\pm$ SE of three replicates. Significance was tested by Duncan Multiple Range Test at $p < 0.01$, and values with same superscript were found not significantly different from each other

Extraction of metabolites

Cells were collected from suspension cultures after filtration using Whatman no.1 filter paper. The culture medium was extracted with ethyl acetate (1:2) and pooled. The solvent extract thus obtained was evaporated to dryness under vacuum. The cell mass in the filter paper was thoroughly washed with distilled water and dried in an oven at 35 °C for 48 h, weighed and powdered with mortar and pestle. The powder thus obtained was extracted with 2 volumes of ethyl acetate and centrifuged at 10,000 rpm for 15 min. The supernatant was concentrated and dried under vacuum. The dried residue was dissolved with a known volume of methanol and the flavour attributing compounds such as 2H4MB, vanillin and vanillic acid along with ferulic acid were analyzed by HPLC.

Screening of flavour metabolites by HPLC and MS analysis

Quantification and confirmation of major flavour attributing metabolites that are found to be characteristic for *D. hamiltonii* was done by HPLC and MS as reported earlier but with slight modification (Gururaj et al. 2012). A sensitive, accurate and specific method coupling high performance liquid chromatography (HPLC) with SPD-20AD, Shimadzu, Kyoto, Japan was used, for this analysis. The isocratic mobile phase contained Methanol: Acetonitrile: Water: Acetic acid (47:10:42:1). C18 column (250 × 4.6 mm and 5-μm diameter) was used (Sunfire column, Waters Corporation, MA, USA) for the sample separation and analysis. The flow rate was maintained at 1 ml/min throughout analysis and the detection wavelength was 280 nm. The samples were respectively injected and mean area for three replicate analyses were calculated. Quantitative analysis was done based on the area of standards (Fluka, Switzerland) for which known amount was injected. Ferulic acid, Vanillin, Vanillic acid and 2H4MB in the samples were identified based on the retention time for the corresponding standards. Changes in the pH value of the mobile phase had a significant effect on the resolution of these compounds, especially the phenolic acids ensures better sample separation but shortens the HPLC column lifetime and affects for that eluent compound mass was determined by Electrospray Ionization Mass Spectrometry (ESI-MS) unit model QToF Ultima Waters Corporation, Micro Mass UK, Manchester. The mass spectral data were accompanied by a Mass Lynx 4.0 SP₄ data acquisition system. The ionization mode was -ve. The interface and the MSD parameters were as follows. The source capillary was set to 3.5 kV, with a temperature of 80 °C. The cone temperature was operated at 100 °C, and the dissolvation temperature at 30 °C. The cone was

operated at 100 °C, Dry gas (N₂)-dissolvation gas 500 l/h, cone voltage 35 V, dissolvation temperature at 150 °C, collision temperature 10 kV, TOF-9.10, connected on scan range *m/z* 100–1000 and data type accurate mass developed new method for the separation and identification of metabolites, by direct injection of collected fractions into the MS system.

Statistical analysis

All values presented are mean ± SD of three analytical replicates. Data were subjected to one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) using SPSS 17 (SPSS Inc., Chicago, IL, USA) for determining significant differences. A difference was considered significant when $p < 0.01$.

Results

The use of various concentrations of NAA, 2,4-D, BA, and Kn alone, and combinations of 2,4-D and BA or Kn was efficient in inducing callus from leaf explants (Table 1), but the texture, amount, and colour varied depending on the type and concentration of the growth regulators. Callus developed on MS medium containing 9.06 μM 2,4-D in combination with 2.32 μM Kn or 2.22 μM BA (Fig. 1b) was friable and green coloured, while the callus grown on other media with 10.74 μM NAA in combination with 2.22 μM BA was hard to semi-hard and pale green to dark green. Similarly, cell suspension cultures were developed on MS liquid medium containing 3% sucrose, with same combination of 9.06 μM 2,4-D and 2.32 μM Kn, in which they grew faster and also yielded good biomass (Fig. 2c). Monitoring of biomass of the callus cultures at regular interval revealed that the growth of the suspension cultures showed some physical changes that include colour and texture. During the first week to second week the light greenish coloured callus cells start changing their colour to green, later by 4th week it was friable and dark green with 200.38 ± 1.56 g/l of biomass and after 8 weeks they become brown with 191.41 ± 1.28 g/l of biomass (Fig. 2a). Maximum 2H4MB production was recorded by 4th week (0.08 ± 0.01 mg/100 g DW), then stable up to 5th week (0.08 ± 0.02 mg/100 g DW), but showed slight decline from 6th week (0.02 ± 0.009 mg/100 g DW) to 8th week (0.003 ± 0.001 mg/100 g DW) (Fig. 2b). Addition of ferulic acid as a precursor in the culture medium showed significant ($p < 0.01$) effect on suspension cultures growth and 2H4MB accumulation, along with other flavour metabolites content. As compared to control, all the FA treatments resulted in only moderate change in their biomass with progressive increase from 2nd to 6th week

Fig. 2 Estimation of biomass and phenylpropanoid pathway metabolites in *D. hamiltonii* callus suspension cultures. **a** Standardization of Biomass for cell suspension at different week intervals; **b** major phenylpropanoid pathway metabolites at different week intervals.

(Fig. 3). However, significant accumulation of all the analyzed flavour metabolites including 2H4MB was evident with maximum content by 4 weeks and then a depletion in all the metabolites content was observed. There was a gradual increase in levels of flavour metabolites from 1st day to the 4th week. The maximum accumulation of vanillin, 2H4MB, vanillic acid, ferulic acid were of 0.1 ± 0.02 mg/100 g, 0.44 ± 0.01 mg/100 g, 0.52 ± 0.04 mg/100 g, 0.18 ± 0.02 mg/100 g DW respectively in 4 weeks of cultured cells supplemented with 1 mM ferulic acid as a precursor (Fig. 3).

HPLC analysis of ethyl acetate extract of suspension cultures resulted in the detection of respective flavour metabolites with a retention time of 9.05 min and ferulic acid with a retention time of 3.895 min at wavelength of 280 nm in standard HPLC chromatogram (Fig. 4a, b). Quantitative HPLC determination (Fig. 4a, b) of respective metabolites in samples showed that the contents of 2H4MB were notably higher than vanillin, except in lower concentration of ferulic acid treated suspension cultures. In this study, totally four compounds were characterized. 2H4MB was confirmed by comparing retention times (Rt), UV and MS data with those of the reference standards (Fig. 5). The most important variations in phenolic acids structure are in the level of oxygenation (hydroxyl or methoxyl groups), when tandem mass spectrometric experiments are performed on instruments with ion trap analyzers, it is possible to perform tandem experiments many times (MS^n) on sequential product ions. MS–ESI was used for determination of molecular mass of wash peak. We presumed four flavour metabolites because of the ionization mode was negative, most of the mass data compounds identified are given in (Table 2). Based on the mass fragmentation (Fig. 5) interpretation, the 2H4MB, vanillin, vanillic acid, ferulic acid were identified in in vitro callus cell suspension cultures of *D. hamiltonii*.

Values are represented as Mean $\pm$ SE of three replicates. Significance was tested by Duncan Multiple Range Test at $p < 0.01$, and values with same superscript were found not significantly different from each other

Discussion

One of the important observations in the present study was the production of very high concentration of vanillic acid in cell suspension cultures fed with 1 mM ferulic acid for 4 weeks. The formation of vanillic acid at high levels is reported (Zenk 1965) in cell cultures of *Vanilla planifolia* on medium fed with ferulic acid. Besides 2H4MB, other flavour metabolites reported include vanillin, several 4-methoxy cinnamic acid derivatives, aromatic alcohols etc. So far, there are no reports on the pathway of 2H4MB biosynthesis nor there is an organized work on biotransformation using cell suspension cultures for obtaining these metabolites using precursors. Giridhar et al. (2004) have made use of elicitors for improving the 2H4MB quantities through normal root cultures. Priefert et al. (2001) has compiled a detail report on the mechanism for the enzymatic degradation of ferulic acid. The Ferulic acid degradation leading to vanillic acid and vanillin formation could be due to decarboxylation of ferulic acid to 4-vinyl guaicol catalysed by ferulic acid decarboxylase (Karmakar et al. 2000), which leads to the formation of vanillin. Barthelmebs et al. (2000) reported reduction of FA to dihydroferulic acid through ferulic acid reductase leading to the formation of homovanillic acid (or) vanillic acid. Another possibility of vanillin production is through coenzyme-A-independent deacetylation of FA wherein the elimination of an acetate moiety from the unsaturated side-chain of FA takes place that leads to vanillin production (Huang et al. 1994) in addition to these is a coenzyme-A-dependent deacetylation of ferulic acid was also investigated. However, recently Kiran et al. (2017) reported that ferulic acid is channelled through the 4-CL (4-coumarate CoA ligase) enzyme, where it would be converted to feruloyl-CoA and could be further converted to 2H4MB in *D. hamiltonii*.

Fig. 3 Estimation of phenylpropanoid pathway metabolites in *D. hamiltonii* callus suspension cultures upon ferulic acid feeding. **a** Biomass production of callus cell suspension; **b** 2H4MB content; **c** vanillic acid content; **d** vanillin content; **e** ferulic acid content.

Values are represented of three replicates. Significance was tested by Duncan Multiple Range Test at $p < 0.01$, and values with same superscript were found not significantly different from each other

Fig. 4 **a** HPLC chromatogram depicting separation of phenylpropanoid intermediates in standard mix 01—vanillin, 02—ferulic acid, 03—vanillic acid, 04—2H4MB; **b** HPLC chromatogram

depicting separation of phenylpropanoid intermediates in cell suspension cultures on 4th week on 1 mM ferulic acid 01—vanillin, 02—ferulic acid, 03—vanillic acid, 04—2H4MB

Fig. 5 Major MS chromatogram of flavour metabolites in an aliquot fraction of cell suspension culture of *D. hamiltonii*. **a** 2H4MB (151.2986); **b** Vanillin (151.2937); **c** Vanillic acid (167.2491); **d** Ferulic acid (195.2515)

Table 2 Peak assignment of metabolites in ethyl acetate extract of *D. hamiltonii* using MS in negative ionization mode

No.	Retention time (RT)	Molecular weight	[M-H] ⁻ m/z	Compound name
01	3.1	152.15	151.2937	Vanillin
02	3.7	194.18	195.2515	Ferulic acid
03	4.8	168.14	167.2491	Vanillic acid
04	9.75	152.15	151.2986	2-Hydroxy-4-methoxybenzaldehyde

In the present study, 2, 4-D and kinetin were used as plant growth regulators (PGRs) for callus multiplication. Though 2, 4-D is a key auxin that facilitate callus multiplication in presence of cytokinins such as BA, kinetin and 2iP, among all the cytokinins kinetin gave good response with 2,4-D in *D. hamiltonii*. However, in combination of 2,4-D and kinetin substantially very low quantity of 2H4MB was noticed in HPLC analysis. The possibility of kinetin, as an elicitor or as an inducer of vanillic acid was reported (Funk and Brodelius 1992) in vanilla cell cultures, wherein it was shown that, kinetin induces catechol-4-*O*-methyl transferase activity, catalyzing the formation of 4-*O*-methyl cinnamic acids, which were the intermediates of hydroxybenzoic acid biosynthesis in Vanilla. It was proved that cinnamic acid and not ferulic acid is the precursor of vanillic acid biosynthesis. Moreover, enzyme activity may be influenced by variations in phytohormone concentrations and combinations, changes in culture conditions, and additions of metabolic precursors, elicitors (Dorenborg and Knorr 1996; Rao and Ravishankar 1999) reported an alternative route for the production of vanilla metabolites by biotransformation of isoeugenol by using freely suspended cells and immobilized cell cultures of *Capsicum frutescens*. The reactions are usually intracellular; the permeable substrates are taken across the cell wall, through enzymatic compartments, and the reaction products will be subsequently released extra-cellularly. But in the present investigation, FA incorporation to medium

enhanced the levels of vanilla flavour attributing metabolites such as 2H4MB along with vanillic acid. The actual sequential chemical reactions takes place in this regard are yet to be pursued at enzymatic and molecular aspects.

Present study focused on in vitro callus suspension cultures of *D. hamiltonii* and consequent flavour compounds production. Callus suspension culture is an alternative approach to the production of valuable secondary metabolites. Such suspension cultures are viewed as an efficient means of biomass production due to their fast growth rates and stable metabolic productivity. These cultures with regular subculturing on optimized medium and at appropriate growth conditions can serve as a continuous source of obtaining secondary metabolites. Therefore high productivity can be achieved by optimizing the in vitro culture conditions for scale up. In the present study, role of FA precursor (a-KGA) was analyzed for 2H4MB accumulation in suspension cultures. Earlier also elicitors and precursors were used to increase secondary metabolite production thus enabling their commercial exploitation. It is an established fact that the administered precursor molecule if it belongs to respective targeted molecule biosynthetic pathway, under goes a series of chemical conversions and leads to higher yield of targeted molecule. In the present study too, addition of ferulic acid might have helped to achieve higher content of 2H4MB and also other minor flavour metabolites. In all the microorganisms studied so far, vanillin produced in cultures is either

oxidized to vanillic acid, which is either *O*-demethylated to protocatechuic acid or decarboxylated to guaiacol. Since Vanillin is very reactive, it exhibits a toxic effect to most microorganisms (Matamoros-Leon et al. 1999). Thus, a rapid conversion of vanillin is commonly observed to less toxic intermediates (Overhage et al. 1999) may be 2H4MB, vanillic acid and therefore there is a less possibility of accumulation of vanillin in cultures. A proper time course experiment for all the enzymes involved in the synthesis of 2H4MB has to be performed for proper understand of the pathway for synthesis of this particular compound.

Conclusion

On the basis of our study and present literature, an alternative route for the production of 2H4MB from callus suspension cultures has been demonstrated. The precursor ferulic acid feeding to *D. hamiltonii* callus suspension culture medium for the accumulation of vanilla flavour metabolites i.e., 2H4MB, Vanillic acid, Vanillin gave a good response with substantial increase in the levels of these metabolites. This would be having implications in biosynthesis of respective vanilla flavour attributing metabolites at very high levels for their large scale production. Further studies on the purification and characterization of the enzyme responsible for the oxidative deamination is in progress.

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Author contribution Giridhar Parvatam—conceived the idea of research and designed the experiments; Pradeep Matam—executed the laboratory experiments; Giridhar Parvatam, Pradeep Matam, and Nandini P Shetty—interpreted the results and wrote the manuscript. All the authors approved the final version of the manuscript prior to submission.

Compliance with ethical standards

Conflict of interest The authors of this article declare that there is no conflict of interest. This publication is approved by the author and all coauthors. The work was carried out at Plant Cell Biotechnology Department, CSIR-CFTRI, Mysore, India.

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