Eco. Env. & Cons. 30 (August Suppl. Issue) : 2024; pp. (S90-S96)
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ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2024.v30i05s.015

Effect of Ferulic acid and Chitosan on cell suspension cultures of *Decalepis hamiltonii* Wight & Arn.

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(Received 21 January, 2024; Accepted 30 March, 2024)

ABSTRACT

The tuberous roots of *Decalepis hamiltonii*, known for their rich flavor profile including Vanillin, are endangered due to over-exploitation. The present study aimed to establish cell suspension cultures from such root segments and their utility in enhancing the production of flavor metabolite, particularly through the incorporation of Ferulic acid (FA) and Chitosan (CH). Friable mass of callus stock after three cycles of subculture of *D. hamiltonii* on MS supplemented with 2,4-D (1 mgl-1) is the source for establishing cell suspension cultures. The addition of 0.1–1.5 mM FA and CH as precursors to cell suspension showed 1.34-fold and 1.14-fold increase respectively, with a significant (p\0.001) impact on the biomass and Vanillin accumulation as compared to control. These elicitation experiments increase in flavor metabolite levels in cell suspension cultures, indicating the potential for large-scale production of these metabolites. This research highlights the importance of developing sustainable methods for obtaining flavor-attributing metabolites from *D. hamiltonii* to meet the increasing demand while conserving the species.

Key words: Decalepis hamiltonii, *Roots, suspension culture, Ferulic acid (FA), Chitosan (CH), Vanillin, LCMS.*

Introduction

Decalepis hamiltonii Wight & Arn.belongs to the familyApocynaceae, an endangered plant indigenous to the southern and Western ghats of India, is rich in vanillin and its isomer 2-Hydroxy-4-methoxy benzaldehyde (2H4MB), also known as the principal flavor compound in its tubers. Extensive research has explored its antimicrobial Thangadurai *et al*. (2002). Antioxidant Srivastava *et al*. (2006). and antihepatotoxic properties Zarei and Shivanandappa (2013), as well as its traditional medicinal uses as an appetizer and blood purifier Pradeep *et al*. (2016 a). Concerns about the plant's survival due to destructive harvesting methods have prompted the investigation of alternative methods for producing flavor metabolites. Plant tissue and cell culture methods offer a controlled environment for extracting secondary metabolites, with *in vitro* production being an attractive alternative to chemical or microbial methods.

Significant studies have focused on producing various secondary metabolites through callus cell cultures, including terpenoids Giridhar *et al.* (2005), alkaloids Kumar *et al.* (2008), glycosides, volatiles Sarfaraj *et al.*, Berlin *et al.* and Ravishankar *et al.* (2012, 1993 and 2003), diterpenes Sridevi *et al.* (2010), and flavonoids Devi and Giridhar *et al.* (2014). Biotransformation methods, which utilize microbial or plant cell cultures to convert inexpensive precursors into valuable final products show promise in the food industry for producing natural

Flavors. Researchers have explored the biotechnological production of bio-Flavors using microbes and *in vitro* plant materials to meet the growing demand for natural products. George and Ravishankar *et al.* (1996). However, the use of bio elicitation in obtaining these metabolites has the potential for commercial application through suspension culture.

This research aims to optimize production methods while conserving natural resources and preserving the species viability. By harnessing biotechnological approaches, the potential exists to produce valuable secondary metabolites efficiently and sustainably, contributing to the growing market for natural Flavors and enhancing the economic value of *D. hamiltonii*Furuya *et al.* and Karuppusamy (1998, 2009).

Materials and Methods

Preparation of explant

Freshly harvested roots of *D. hamiltonii* Wight & Arn. have been gathered from the western ghats, Amboli Ghat (Maharashtra) India (Fig. 1A & 1B). These roots have been washed under the running tap water 3-4 times. Surface sterilization of these roots was done with different chemicals such as Tween 20 (HI media grade) for 20 mins, 2% Bavistin for 45 mins, 0.5% NaOCl, i.e. Sodium hypochlorite (HI media grade) for 20 mins, and mercuric chloride [0.1 %, (w/v)] solution for 1min with alternate sterile distilled water wash after every chemical treatment.

Fig. 1a. Mother plant

Fig. 1b. Wild Roots of *Decalepis hamiltonii*

Such roots were treated with 70% alcohol for 2 mins before slicing into and then they were inoculated on MS basal medium Murashige and Skoog (1962), supplemented with 0.25 mgl^{-1}, 0.50 mgl^{-1}, 1.0 mgl-1 2,4-dichlorophenoxyaceticacid (2,4-D) and Kinetin and Picloram, and Benzyl amino purine (BAP) individually and in a few combinations. 3% (w/v) sucrose has been used (Fig. 2A). The medium pH of 5.7±0.1 has been maintained, and solidified by using 0.8 % (w/v) agar to prepare semi-solid medium for induction of callus and proliferation of friable stock mass. For establishing cell suspension cultures, liquid media are used without adding agar and autoclaved at a pressure of 1.06kg/cm^2 for a time of 20 min at a temp of 121^0 C. Cultures were incubated under 16hr photoperiod and 8 h Dark (Fig. 2B). For initiating cell suspension cultures, 100±10mg of friable callus mass which was attained after 3 cycles of subculturing with an interval of 4 weeks has been transferred to conical flasks of150ml consisting 40ml of liquid medium. The suspension cultures have been maintained on a rotary shaker at the speed of 95rpm for the time of 16hr photoperiod at the temp of 24 ± 2^0 C for 10 weeks (Fig. 2C).

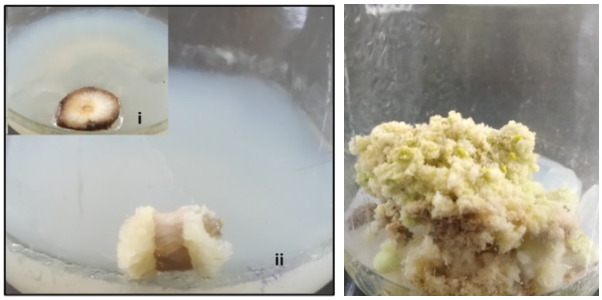


Fig. 2a. i. Root segment culture 2b. Proliferated mass of root callus ii. Callus induction

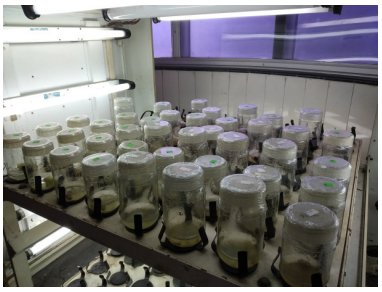


Fig. 2c. Maintenance of Cell suspension

Fig. 2d. Plant Cell suspension

Incorporation of precursor

For experimentation, Ferulic acid (FA) [50% (v/v)] is dissolved in ethanol and filter sterilized using a Millipore filter (0.22 µm). The variable concentrations of FA were made such as 0.1, 0.5, 1,& 1.5 mM Johnson *et al.* (1996) and Chitosan has been dissolved in 5 % (v/v) 1N HCl with gentle heating as well as stirring, followed by pH adjustment to 5 using 1N NaOH. The final concentration was set to 1mg/ml using an MS liquid medium. Homogenates

have been autoclaved for 15 minutes at the temp of 121°C before utilization as an elicitor at varying concentrations. This process is likely aimed to prepare chitosan-based elicitors for biological applications. Hasanloo *et al.* (2014) and incorporated in liquid medium and then 150 ml of cell suspension was inoculated into experimental flasks (Fig. 2D). During the experiment, the level of Vanillin was analysed on 2nd, 4th, 6th, and 8th weeks. Experiments were performed in triplicates.

Extraction of metabolites

The filtration was used for the cells separation from suspension culture using Whatman filter paper no. 1. After the filtration process separated cells were collected. Ethyl acetate (1:2) was used to extract the culture medium, and after pooling the results, the solvent extract was vacuum-dried until it was completely dry (Kiran *et al.*, 2017). The filter's cell mass was thoroughly cleaned with distilled water before being dried for 48 hours at 35°C in an oven. It was weighed and then ground into a powder in a mortar and pestle. After extracting the powder using twice as much ethyl acetate, it was centrifuged for 20 minutes at 10,000 rpm. The vacuum was used for the drying of concentrated supernatant. The dried form of residue has been dissolved into a known volume of methanol and vanillin concentration with respect to FA and CH was analysed by LCMS.

Analysis and Quantification of Vanillin by LCMS

Quantification and confirmation of major flavour-attributing metabolites in *D. hamiltonii* were carried out using HPLC and MS, with slight modifications to previously reported methods Gururaj *et al.* (2012). The experiment utilized Tret Yakov, K.V. as the source for retention data, sourced from the NIST Mass Spectrometry Data Centre in 2008. The mobile phase comprised of 0.1 % FA in water for phase A, while phase B contained acetonitrile. An Agilent Por shell 120 C18 column, with dimensions of 2.7 um x 4.6 mm x 100 mm, was employed for sample separation and analysis using a Shimadzu 8045 UPLC-MS/MS instrument.

The wavelength at which detection took place was 280 nm, and the flow rate has been kept constant at 0.8ml/min. Following the injection of samples, the mean area over three replicate analyses was computed. On the basis of area of standards (Fluka, Switzerland) with known amounts injected, quantitative analysis was carried out. The retention period for the matching standards was used to determine the presence of vanillin in the samples. Using a "Mass Lynux 4.0 SP4 data acquisition system, the mass spectral data was analysed. The interface and parameters have been set as follows: the source capillary has been set to 4.0 kV, with an interface temp of 300 °C, DL temp of 250 °C, and column temperature at 35 °C. Dry gas (N_2) dissolvation gas was set at 500 l/H, associated on a scan range of m/z 100–1000, and data type accurate mass was recorded.

Statistical Analysis

The provided results are from a Tukey HSD (Honestly Significant Difference) test, which is used for multiple comparisons of means.

Results and Discussion

The variable concentrations of PGRs like 2, 4-D, Kinetin, Picloram, and BAP were used individually and in a few combinations. Efficient callus induction was observed from root segments as explants (Table 1). However, the amount, colour, and texture changed depending on the concentrations and type of the regulator.

Represented values are as mean ±SE of triplicates

Callus created on MS medium consists of 2,4-D (1mgl-1) alone depicted maximum biomass (1695 ± 1.11 g/l) by 4th week from whitish to Dark greenish in color along with the friable mass and minimum at (208±0.54 g/l) by 4th week on MS+BAP (0.50 mgl-1) starting from whitish to light yellow in color with the semi-hard in the texture.

However, the four-week growth period of cell suspension cultures revealed the presence of vanillin conc. as 527mg/100 g DW on control media, whereas at 0.5mM Ferulic acid treatment, the highest vanillin conc. i.e., 707mg/100 g DW was observed (Table 3). With 1.0mM Chitosan treatment, vanillin conc. 604mg/100 g DW was the maximum with respect to CH treatments only (Table 2).

The biomass of suspension cultures and the accumulation of vanillin were significantly ($p<0.001$) affected by the addition of 0.1–1.5 mM ferulic acid as a precursor in the culture medium. Through this elicitation experiment, there is a 1.14-fold increase when the control is compared with the CH (Fig. 6). a 1.34-fold increase when the control is compared with the FA (Fig.4), and when results of both elici-

Table 1. Effect of PGRs on fresh weight of root induced callus at 6 weeks growth period

MS + Picloram (mgl^{-1})	MS+BAP (mgl^{-1})	Callus fresh weight (mg)
0.25	0	354±0.22
0.50	0	723±1.30
1.0	0	830±0.40
0	0.25	-
0	0.50	208±0.54
0	1.0	241±0.25
0.25	0.25	332±1.1
0.50	0.25	345±0.26
1.0	0.25	350±0.81
0.25	0.50	312±0.34
0.50	0.50	496±0.22
1.0	0.50	290±1.25
0.25	1.0	472±0.51
0.50	1.0	282±0.32
1.0	1.0	265±1.4
MS+2,4-D (mgl^{-1})	MS+Kinetin (mgl^{-1})	Callus fresh weight (mg)
0.25	0	1270 ± 1.23
0.50	0	1582 ± 0.52
1.0	0	1695 ± 1.11
0	0.25	-
0	0.50	-
0	1.0	592±0.42
0.25	0.25	822±0.55
0.50	0.25	870±0.21
1.0	0.25	1262±0.38
0.25	0.50	790±1.32
0.50	0.50	1142±1.5
1.0	0.50	732±1.0
0.25	1.0	995± 0.82
0.50	1.0	648±0.23
1.0	1.0	683±1.41

Table 2. Cell suspension treated with CH (0.1 mM -1.5 mM)

	Chitosan				
Weeks	MS+2,4-D ($1mgl^{-1}$)	0.1 mM	0.5 mM	1.0 mM	1.5 mM
2	520	561	563	570	564
4	527	570	574	**604**	592
6	525	565	567	595	580
8	523	563	565	584	576

Represented values are as mean ±SE of triplicates.

tors are compared with each other we came out with a 1.17-fold increase in FA over the CH.

So, according to the data of LCMS from the two to fourth-week growth period, a rise in the quantity of vanillin was observed, from the fourth to sixth week quantity of vanillin almost remained constant, but from the sixth to eighth-week quantity of vanillin started declining.

Callus developed on MS medium containing Picloram (1 mgl-1) alone showed 830±0.40 mg fresh weight of callus, BAP (1 mgl-1) alone showed 241±0.25 mg fresh weight of callus whereas the combination of Picloram (0.50mgl-1) and BAP (0.50 mgl-1) showed 496±0.22 mg fresh weight of callus. it was semi-hard and light yellow in color. In a similar manner, MS liquid medium containing 3% sucrose was used to develop cell suspension cultures, and MS medium consisting 2,4-D (1 mgl-1) alone was used to develop Callus, which grew more quickly and produced good biomass (Fig. 2B).

The results obtained from the experiment align well with the biochemical pathways involved in vanillin production in *D. hamiltonii*. The transient accumulation of vanillic acid as well as trace amounts of protocatechuic acid detected in the culture medium at the time of growth on FA suggests the involvement of FA as a precursor in vanillin biosynthesis. This is supported by the subsequent observation of maximum vanillin production by the fourth week, with stability observed until the sixth week, followed by a slight decline thereafter (Fig. 3 & 5).

The incorporation of ferulic acid as a precursor in the culture medium significantly affected the growth and vanillin accumulation in suspension cultures, indicating its importance in vanillin biosynthesis. The mechanism of vanillin biosynthesis involves the cleavage of the side chain of FA, leading to the formation of vanillin. This reaction is catalyzed by the enzyme vanillin synthase (VpVAN), which was identified in the study. VpVAN catalyzes the direct conversion of FA into vanillin, providing a molecular basis for the observed increase in vanil-

Table 3. Cell suspension treated with FA (0.1 mM -1.5 mM)

	Ferulic acid				
Weeks	MS+2,4-D ($1mgl^{-1}$)	0.1 mM	0.5 mM	1.0 mM	1.5 mM
2	520	638	652	642	642
4	527	649	**707**	687	665
6	525	643	692	668	651
8	523	641	675	662	645

Represented values are as mean ±SE of triplicates.

lin content in the culture medium. The presence of chitosan as a precursor also showed a significant effect on suspension culture growth and vanillin accumulation, albeit to a lesser extent than FA. The stability of vanillin production up to the sixth week suggests that the metabolic pathways involved in vanillin biosynthesis are actively regulated during this period. The decline in vanillin content observed after the sixth week may be attributed to metabolic processes such as degradation or utilization of vanillin by the cells.

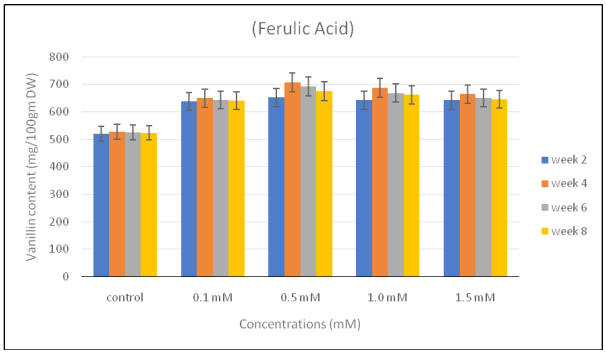


Fig. 3. Vanillin content with FA conc. From 2nd- 8th weeks old cell suspension culture

The callus cultures of *D. hamiltonii* underwent color and texture changes over time. Initially light greenish, they turned green by the second week and became friable and dark green by the fourth week. Maximum vanillin production has been observed by 4th week 707mg/100g DW (Fig. 4), then stable up to 6th week, but displayed a slight decline from 8th week.

When compared to the control, the addition of FA and chitosan as a precursor to the culture medium had a significant ($p<0.01$) impact on the growth of suspension cultures and the accumulation of vanillin. Only a moderate change in biomass was observed with the FA treatments, with a progressive increase from the second to the fourth week. However, a significant accumulation of vanillin was evident, reaching its maximum content by the fourth week of growth, followed by a depletion of metabolite content more so than chitosan.

Furthermore, the observation that cell suspension cultures created on MS medium consisting 2,4-D alone exhibited faster growth and higher biomass production is consistent with previous studies indicating the role of auxins in promoting cell proliferation and growth. This suggests that the optimization of culture conditions, including the choice of growth regulators, can have a significant impact on biomass production and metabolite accumulation in cell suspension cultures.

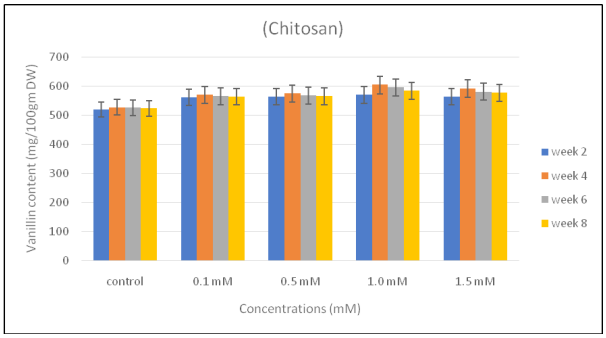
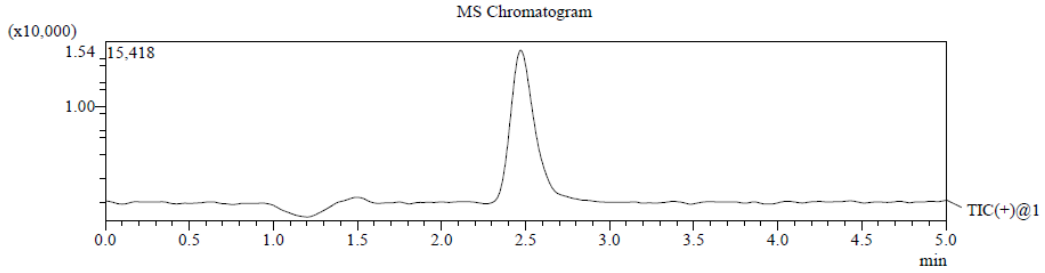


Fig. 5. Vanillin content with CH conc. From 2nd- 8th weeks old cell suspension culture

Conclusion

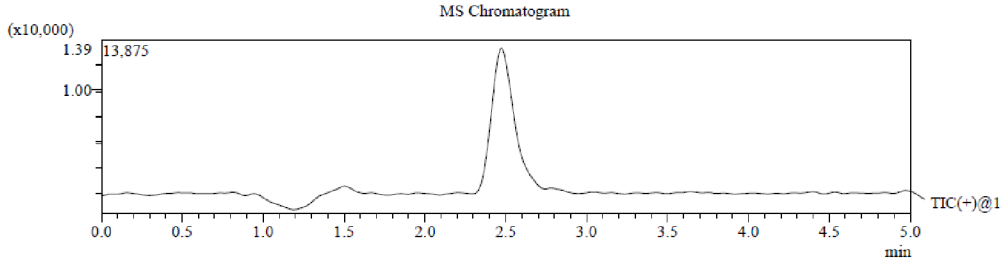
In conclusion, the study demonstrates the significance of FA and CH as precursors for vanillin bio-



MS Quantitative Table

ID#	Name	Ret. Time	Area	Dilution Factor	Conc.	Unit
1	Vanillin	2.471	115062	1000	707	mg/100g
Total			115062			

Fig. 4. LC MS Chromatogram of vanillin quantification Treated with FA 0.5 mM on 4th week old suspension culture



MS Quantitative Table

ID#	Name	Ret. Time	Area	Dilution Factor	Conc.	Unit
1	Vanillin	2.475	100110	1000	604	mg/100g
Total			100110			

Fig. 6. LC MS Chromatogram of vanillin quantification Treated with CH 1.0 mM on 4th week old suspension culture

synthesis in *D. hamiltonii* suspension cultures. The observed transient accumulation of vanillic acid as well as protocatechuic acid, followed by maximum vanillin production by the fourth week and stability until the sixth week, underscores the involvement of FA-derived pathways in vanillin biosynthesis. The outcomes shed light on the metabolic mechanisms underlying vanillin production and give insights for optimizing culture conditions to enhance vanillin accumulation in cell suspension cultures.

Acknowledgements

The grant from DBT (DBT BUILDER) to the institute is gratefully acknowledged. The research study was supported by Department of Plant Biotechnology, Rajiv Gandhi Institute of Information technology and biotechnology, Pune. The author would like to express gratitude towards authorities of Bharati Vidyapeeth (deemed to be) University for providing the facility at RGITBT and PCP, Pune, Maharashtra, India.

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 Biotechnology Department, RGITBT (BVDU), Pune, India.

Authors Contribution

Author contribution Dr. N. T. Meti conceived the idea of research and designed the experiments; D.R. Chikane, Dr. A. G. Namdeo executed the laboratory experiments; Dr. N. T. Meti, D.R. Chikane interpreted the results and wrote the manuscript. All the authors approved the final version of the manuscript prior to submission.

Funding

There is no any funding for this research work.

Ethics Statements

There is no any human and animal involve in this experiment.

Informed consent

Informed consent is not applicable due tothere is no any human and animal involve in this experiment.

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