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Original paper

In vitro elicitation supports the enrichment of 2H4MB production in callus suspension cultures of *D. hamiltonii* Wight & Arn

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Abstract

I nfluence of elicitation on *in vitro* production of 2-Hydroxy-4-Methoxy Benzaldehyde (2H4MB), a structural isomer of vanillin from callus suspension cultures of *Decalepis hamiltonii* was investigated. *In vitro* culture conditions were optimized to induce callus, suspension culture and biomass followed by metabolite production. Suspension cultures were established using leaf generated friable callus. Maximum content of 2H4MB production 0.079 ± 0.01 mg $100g^{-1}$ DW and biomass 197.5 ± 1.5 gL^{-1} were observed by 4th week of culturing. Elicitation was induced to suspension cultures by using *m*-topolin (*m*T), sodium nitroprusside (SNP), and pectin individually at different concentrations. *m*-topolin (15 µM), pectin (15 µM) and SNP (10 µM) supported 0.31 mg$100g^{-1}$ DW, 0.27 mg$100g^{-1}$ DW and 0.21 mg$100g^{-1}$ of 2H4MB production respectively by 4th week. This data infers that the elicitation improves 2H4MB content in callus suspension cultures of *D. hamiltonii*.

Keywords

Biomass, Elicitor, Callus cell suspension cultures, *Meta*-topolin (N6-(meta-hydroxybenzyl) adenine), Sodium nitroprusside (SNP), Pectin

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Introduction

Secondary metabolites produced by the plants have wide range of application as industrial raw materials, in medicines, food products as enzymes, and flavour etc**.** Flavors originate from the natural sources mostly from plants have high commercial value. Vanillin is one such metabolite obtained from plants which has wide application in flavoring industry. *Vanilla planifolia* and *V. tahitensis* are major natural sources for the production of vanillin flavor. Due to its high demand and inadequate availability of natural vanillin, synthetically manufactured vanillin is brought in to application to meet the demand. But this has not been readily accepted in some countries due to regulatory guidelines thereby natural vanillin is having persistent demand (Vaithanomsat P & al [1]**).** 2-Hydroxy-4-methoxybenzaldehyde (2H4MB) is an isomer of vanillin, has sweet fragrance can be readily used as alternative to synthetic vanillin. 2H4MB is reported to synthesized through phenyl propanoid pathway and present in few plant species such as *Decalepis hamiltonii, Hemidesmus indicus* (Mehmood Z & al [2]), *Mondia whytei* and root bark of *Periploca sepium* (Yamashita Shi J &al [3]). Biochemical characterization of key step involved in 2H4MB production in *D. hamiltonii* was reported (Kamireddy K & al [4]. In *Decalepis hamiltonii* tubers 2H4MB constitutes 96% of volatile oil, hence is the major aromatic compound and its flavor extract is recognized as GRAS by United States Food and Drug Authority (Nagarajan S & al [5]. This flavor metabolite 2H4MB along with other bioactives reported to have many bioactive properties like antioxidant antimicrobial, and hepatoprotective etc. (Pradeep M & al [6]. This endemic plant *D. hamiltonii* familiar as swallow root, has been constantly over exploited from ages for various applications for its rich aroma and bioactives and currently in the state of endanger.

Plant tissue culture is a promising technique in which *in vitro* production of secondary metabolites in plant cell suspension cultures has been reported from various food and medicinal plants (Dias MI & al [7]). In *D.hamiltonii,* tissue culture technique is reported to be extensively used for cultivation of plants using nodal explants (Obul Reddy B & al [8]), (Sharma S & al [9]) shoot tips (Giridhar P & al [10]), and *in vitro* rooting of micro shoots (Reddy BO & al [11]). Similar, efforts were made to enhance production of flavor metabolites through precursor feeding in callus cell suspension cultures (Pradeep M & al [12]). Nitric oxide as a bioactive molecule is biosynthesized during plant pathogen interactions and reported to exhibit pro-oxidant as well as antioxidant properties in plants (Delledonne M & al [13]). It induces plant defense genes and accumulation of cyclic acid that leads to expression of systemic acquired resistance (Crawford NM & al [14]). Prior art indicates the presence of NO in the plant kingdom and its connection in growth, development (Beligni MV &al [15]), senescence (Jie TU & al [16]), defense responses (Arun M & al [17]) and *in vitro* regeneration (Tan BC & al [18]). SNP at high concentrations stimulated various secondary metabolites such as catharanthine in *Catharantuse roseus* (Xu J & al [19]), hypericin in *Hypericum perforatum* (Xu MJ & al [20]), phenols and flavonoids in Ginkgo biloba *in vitro* cultures (El-Beltagi HS & al [21]). *Meta*-topolin (N^6-(*meta*-hydroxybenzyl) adenine) is a highly active aromatic, unconventional cytokinin isolated and characterized from poplar leaves (Strnad M & al [22]). *Meta*-t opolin (*m*T) has been shown to promote *in vitro* shoot proliferation and improve quality of shoots in several plant species (Aremu AO & al [23]). The potential of *m*T to induce organogenesis from somatic embryo derived cotyledon explants in Cassava (Chauhan RD & al [24]), *Opuntia stricta* (Souza M & al [25]) and *Corylus colurna* (Gentile A & al [26]) was assessed. It is reported to prevent oxidative stress in sugar cane micropropagation (Souza M & al [27]) and production of flavonoids in *Amburana cearensis* (Vasconcelos JNC & al [28]).

However, there are no reports regarding the influence of elicitation on callus suspension cultures of *D. hamiltonii*. As there is a demand in the market for natural flavor (Giridhar P & al [29]), experiments to obtain flavor metabolites through tissue culture will facilitate effective potentiation. Elicitation is technique which was reported to be most significant approach in enhancement of metabolites (Saini RK& al [30]), (Sridevi V & al [31]). Biotic and abiotic elicitors are reported to increase accumulation of metabolites in plant *in vitro* and *in vivo* (Kim HJ & al [32]), (Perez-Balibrea S & al [33]). Accordingly, in the present study, investigations were carried out to determine potential benefit of *m*-topolin, sodium nitroprusside (SNP) and pectin for efficient augmentation of flavour metabolites, since no such studies on *D. hamiltonii* and its metabolites are available.

Materials and methods

Chemicals

Murashige and Skoog medium, 2, 4-Dichlorophenoxyacetic acid, (2, 4-D), Kinetin (Kn), *m*-topolin and Sodium nitroprusside (SNP) of plant tissue culture grade were purchased from Hi-media (Mumbai, India). 2H4MB standards was supplied by Fluka (Switzerland) and pectin from Sigma-Aldrich India. HPLC grade methanol and acetonitrile obtained from Merck (Mumbai, India)

Plant material and culture conditions

D. hamiltonii Wight & Arn., fruits were collected from 12-year-old plant grown at Departmental Garden in CSIR-Central Food Technological Research Institute, Mysore, India. The seeds were separated from the dried capsule and surface sterilization of seeds was done using 0.1% (w v^{-1}) mercuric chloride (Hi-media, Mumbai) for 5 min followed by washing (4-5 times) with autoclaved distilled water (Saini RK & al [30]). Sterilized seeds were inoculated in the MS media with 0.2 µM gibberellic acid (GA_3), (Murashige T,& Skoog F [34]) containing 3% (wv^{-1}) sucrose (Hi-media, Mumbai) and 0.8% (wv^{-1}) of agar (Hi-media, Mumbai) at 25 ± 2 °C under 45 µmol.m^{-2} s^{-1} light for 16 h photoperiod. The leaves of seedling were used as explant for callus induction.

Callus induction and development of suspension cultures

Leaves from 6 weeks old seedling plant were used as explants for callus induction. Leaves from seedling plant were chopped (leaf 1 sq.cm) and inoculated in 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 (not for suspension culture) (Pradeep M & al [12]). The pH of the medium was adjusted to 5.8 and autoclaved at 121 °C for 15 min. Callus cultures were maintained at 25 ± 2 °C in 45 µmol.m^{-2} s^{-1} light for 16 h photoperiod. These calluses were sub-cultured every 4 weeks. The friable callus mass that was obtained after two subcultures was used for preparation of suspension cultures. This callus was transferred to 150 ml conical flasks containing 40 ml liquid medium and grown for 8 weeks on a rotary shaker at 95 rpm at 24 ± 2 °C in 16 h photoperiod.

Elicitor treatment

Preparation of elicitor stock solution

Stock solution of 1M concentration *m*-topolin was prepared by dissolving in 0.5N KOH solution. Similarly, sodium nitroprusside, and pectin stock solutions were prepared in sterile distilled water. These stock solutions were sterilized by filtering through 0.22 µm PVDF membrane filters (Durapore) and were inoculated in known concentration to *in vitro* callus cell suspension cultures of *D. hamiltonii*.

Elicitation in callus suspension cultures

m-topolin, pectin, and SNP were added to callus suspension cultures in different concentrations (10, 15 and 20 µM) for evaluation of callus growth and 2H4MB content. Suspension cultures without elicitor was also maintained in similar conditions as a control. Callus biomass was harvested from the suspension culture medium after every week (1st to 4th week) to record callus biomass accumulation as fresh weight (FW) and content of 2H4MB. All the experiments were performed with three replicates for each concentration treatment and the experiment was conducted twice.

Extraction of metabolites

The 2H4MB content from the callus was extracted as described earlier (Pradeep M & al [12]). Callus from suspension cultures were separated using Whatman no.1 filter paper. 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 35^0 C for 12 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 were analyzed.

Screening of flavour metabolites by HPLC and MS analysis

The major flavour ascribing metabolites quantification and confirmation from the solvent extracts of *D. hamiltonii* callus was done by HPLC (SPD-20AD, Shimadzu, Kyoto, Japan) and MS(QTof Ultima Waters Corporation, Micro Mass UK) as reported earlier but with slight modification (Gururaj HB & al [35]). 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 (Sunfire column, Waters Corporation, MA, USA) for the sample separation and analysis. The flow rate was maintained at 1 ml min^{-1} 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 standard (Fluka, Switzerland) for which known amount was injected. 2H4MB in the samples were identified based on the retention time for the corresponding standard. The mass spectral data were accompanied by a Mass Lynux 4.0 SP4 data acquisition system. The ionization mode was negative(Pradeep M & al [11]).

Statistical analysis

The experiments were conducted in a completely randomized design with three replicates. Data were subjected to an analysis of variance (ANOVA). To determine the significant difference, Duncan's multiple range test (DMRT) was performed using SPSS software (Version 17).

Results and discussion

Culture establishment

Cell suspension cultures were developed on MS medium (without agar) containing 3% sucrose, with the combination of 2 mgL^{-1} 2,4-D and 0.5 mgL^{-1} Kinetin , wherein the biomass and 2H4MB content is observed to be good (Pradeep M & al [12]). The callus cultures biomass was observed at regular interval, which shows that the growth of the callus in suspension cultures had some physical changes that include color and texture. During the first week to second week, the light greenish colored callus cells start changing their color to green, later by 4th week it was friable and dark green with 197.52 ± 1.48 gL^{-1} of biomass and after 8 weeks they turn brown with 184.23 ± 1.84 gL^{-1} of biomass (Fig 2a). Maximum 2H4MB production was recorded by 4th week (0.079 ± 0.01 $mg100g^{-1}$ DW), but showed decline from 6th week (0.003 ± 0.014 $mg100\ g^{-1}$ DW) and 8th week (0.002 ± 0.031 mg 100 g^{-1} DW).

Elicitation with m-topolin (mT)

Callus cultured on MS medium supplemented with different concentration of *m*-topolin gave the highest level of 2H4MB among the treatment when compared to control. The results obtained for *D. hamiltonii* callus cultures suggest that their degree of differentiation had a significant influence on the biochemical synthesis of 2H4MB. However, significant accumulation of all the analyzed flavour metabolites 2H4MB was evident with maximum content at 4 weeks and then a depletion in 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 2H4MB was recorded as 0.31 ± 0.01 mg 100 g^{-1} DW in 4 weeks grown culture that supplemented with 15 µM *mT* as a cytokinin inducer (Fig.4). HPLC analysis of ethyl acetate extract of suspension cultures resulted in the detection of 2H4MB with a retention time of 8.5 min at wavelength of 280 nm in standard HPLC chromatogram. Quantitative HPLC determination (Fig. 3) of respective metabolites in samples showed that the contents of 2H4MB were notably higher in *m*T treated suspension cultures. *m*- topolin is reported to be used as an alternative to cytokinin in tissue culture as it has a great influence on physiological property and in production of quality explant (Aremu AO & al [23]), (Ahmad N & al [35]). It was reported to improve *in vitro* morphogenesis, rhizogenesis and increase biochemical content in differ plants (Chauhan RD & al [23])(Gentile A & al [26], (Ahmad A & al [37]). *Meta*-topolin induced *in vitro* regeneration and enhanced the secondary metabolites in the leaf tissues (Khanam MN & al [38]). (Aremu AO & al [39]) reported higher phenolic production in 'Williams' banana treated with *mT* when compared to the BA-treated and control (PGR-free) plants. It is reported to prevent oxidative stress in sugar cane micro propagation (Souza M & al [24]) and production of flavonoids in *Amburana cearensis* (Vasconcelos JNC& al [28]).

Elicitation with Pectin

In the present study, pectin treatments to cultures resulted in only moderate change in their biomass with progressive increase from 2nd to 4th week (Fig. 5a) in comparison to control. The maximum accumulation of 2H4MB 0.29 ± 0.01 mg $100g^{-1}$ DW was found in 4 weeks of cultured cells supplemented with 15 µM of pectin (Fig. 5b). The addition of pectin has shown a significant influence on cell growth in *Gymnema sylvestre* R. Br (VeerashreeV & al [40]). Pectin involved in activating secondary metabolism in *M. citrifolia* cell suspension cultures (Dornenburg H & al [41]), *Calendula officinalis* (Wiktorowska & al [42]*)*, and *Hypericum adenotrichum* (Yamaner O & al [43]) was known. Pectin's structure is a practically and fundamentally diverse class of galacturonic acid rich polysaccharides which can go through plentiful alteration with an accompanying change in physicochemical properties (Wolf S & al [44]).

Elicitation with Sodium nitroprusside (SNP)

The results obtained for *D. hamiltonii* callus cultures with SNP suggest that their degree of differentiation had a significant influence on the biochemical synthesis of 2H4MB (Fig. 6). The maximum accumulation of 2H4MB 0.21 ± 0.01 $mg100\ g^{-1}$ DW was recorded in 4 weeks of cultured cells supplemented with 10 µM concentration of SNP and it was found to be significant (Fig. 6). In plants, various environmental and hormonal stimuli are transmitted either directly or indirectly by nitric oxide (NO) signalling cascades (Delledonne M & al [13]), (Crawford NM & al [14]). A good number of reports reveal the significance of NO, and the usage of sodium nitroprusside (SNP) that form a transition metal nitroxyl anion (NO-) complexes (Beligni MV & al [15]), (Jie TU &al [16]). SNP in combination with auxin 2,4-D is reported to improve cell division that leads to embryonic cell formation in *Medicago sativa* (Ahmad N & al [36]) and shoot multiplication in vanilla (Tan BC & al [18]).

Conclusion

On the basis of our study augmented production of 2H4MB from callus suspension cultures has been demonstrated. The feeding of m*T* , SNP and pectin to *D. hamil-*

tonii callus suspension culture medium supports the augmented accumulation of 2H4MB, with substantial increase in 4.5 folds under *m*-topolin elicitation, followed by 3.6, 2.4 folds in presence of pectin and sodium nitroprusside, which act as an inducer for the cells to increase the production of 2H4MB. The optimized culture conditions conceivably can be applied for scale-up studies for 2H4MB production.

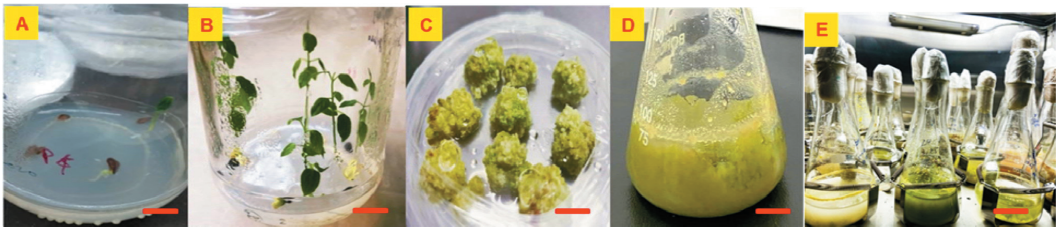

Figure 1. A *In vitro* plants germinated from seeds (bar=10 cm); **B** *In vitro* seedlings of *D. hamiltonii* (bar=10 cm); **C** Callus induction on medium bearing 2,4-D (9.06 µM) & kinetin (2.32 µM) (bar= 5 cm) **D** Callus cell suspension on medium with 9.06 µM 2,4-D and 2.32 µM kinetin after 4 weeks (bar=5 cm); **E** Rotary shaker with suspension cultures (bar=15cm)

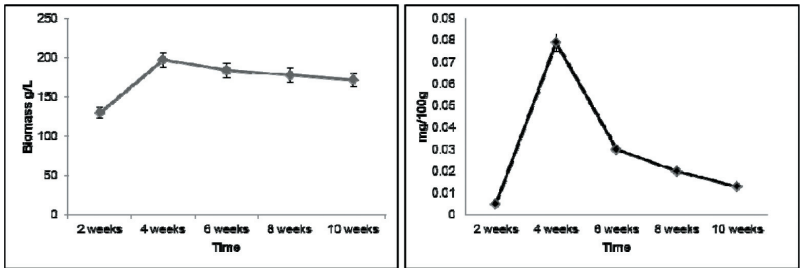

Figure 2. Callus growth and its 2H4MB quantification in suspension cultures. **a)** Callus biomass at different time intervals; **b)** 2H4MB content in callus during its growth.

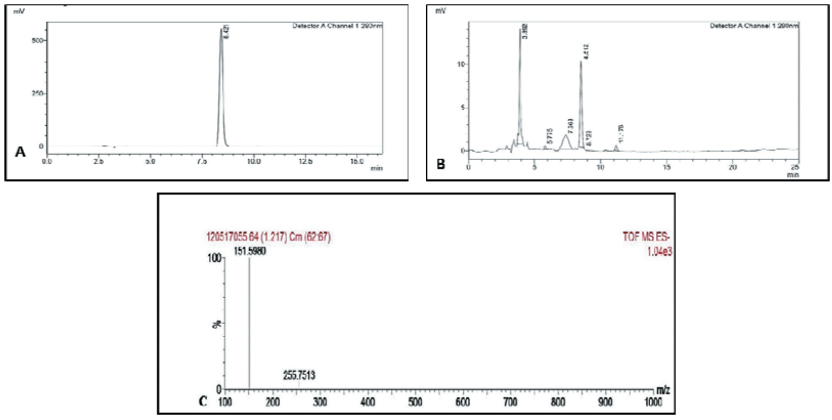

Figure 3. Quantification of 2H4MB using HPLC and confirmation by MS-ESI. **A)** 2H4MB standard; **B)** Chromatogram of Callus elicited with m-topolin ; **C)** MS fragment of 2H4MB Callus elicited with m-topolin

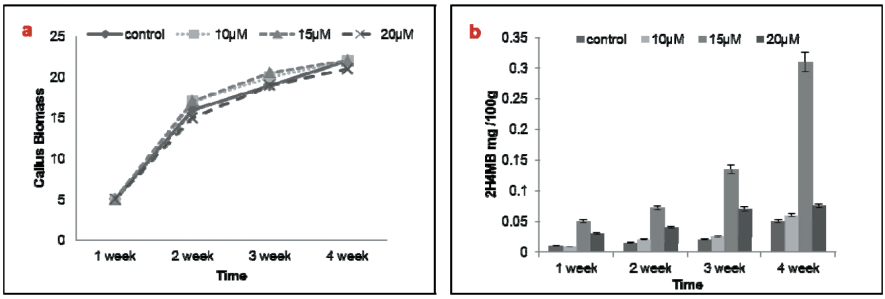

Figure 4. Influence of m-topolin on callus suspension cultures. a) Callus biomass (FW); b) 2H4MB content.

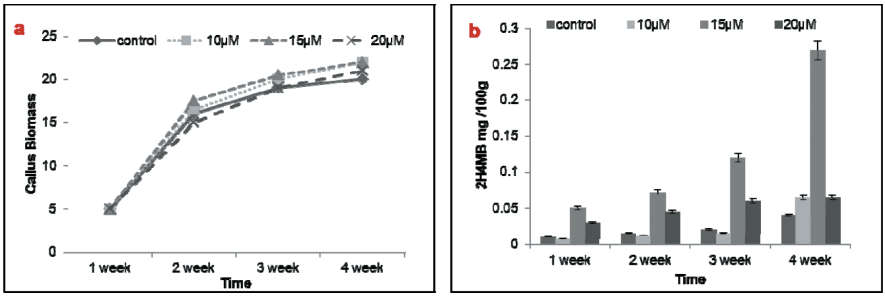

Figure 5. Influence of Pectin on callus suspension cultures. a) Callus biomass (FW); b) 2H4MB content

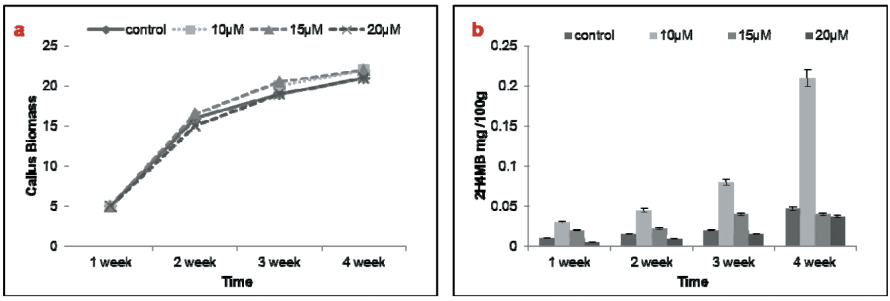

Figure 6. Influence of Sodium nitroprusside on callus suspension cultures. a) Callus biomass (FW); b) 2H4MB content

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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