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Production of a root-specific flavour compound, 2-hydroxy-4-methoxy benzaldehyde by normal root cultures of *Decalepis hamiltonii* Wight and Arn (Asclepiadaceae)

P Giridhar, T Rajasekaran and GA Ravishankar*
Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore-570013, India

Abstract: The flavour compound 2-hydroxy-4-methoxy benzaldehyde from normal roots of swallow root (*Decalepis hamiltonii*) raised *in vitro* was extracted with dichloromethane, evaporated to dryness and dissolved in ethanol for qualitative (TLC) and quantitative (GC-MS) analysis. Maximum root biomass and the maximum content of flavour compound ($40 \pm 2.1\ \mu g\ g^{-1}$ dry weight) were recorded after 45 days of growth on Murashige and Skoog medium containing $1.0\ mg\ l^{-1}$ α-naphthaleneacetic acid.

Keywords: *Decalepis hamiltonii*; 2-hydroxy-4-methoxy benzaldehyde; flavouring agent; root cultures; GC-MS

INTRODUCTION

Decalepis hamiltonii Wight and Arn (Swallow root), a monogeneric endangered climbing shrub native to the Deccan peninsula and forest areas of the Western Ghats of India, finds use as a culinary spice due to its expensive aromatic roots,[1] and as a food preservative, appetizer and blood purifier.[2–4] The active principle in these roots is a volatile compound, 2-hydroxy-4-methoxy benzaldehyde (2H4MB), which has been shown to have insecticidal[5] and antimicrobial activity.[6] Over-exploitation during decades of indiscriminate harvesting for the aromatic tuberous roots has almost endangered this plant in its natural habitat. As there is no organized cultivation of this plant, immediate measures to standardize protocols for micropropagation have been documented.[7–9]

In approximately 60% of the medicinally important plants used in traditional systems of medicine, roots are the principal material for drug preparation.[10] Nearly 90% of the plant species used by the industry are collected from the wild[11] and more than 70% involve destructive harvesting; very few are in cultivation. In this context, the production of several high-value metabolites from plant tissue cultures is being investigated for industrial production.[12] *In vitro* root cultures have much significance for the production of various secondary metabolites, using either hairy roots[13] or normal roots.[14] Although tuberous roots are known to contain the flavour compound 2H4MB[15] as the major compound, no reports are available on the accumulation of this compound in normal roots raised *in vitro*, which offers unique opportunities for producing root metabolites in the laboratory for a wide range of applications. The main goal of this work was to evaluate the capability of *D hamiltonii* to accumulate the root-specific flavour compound 2H4MB.

MATERIALS AND METHODS

Source of plant material

Aseptic shoot cultures of *D hamiltonii* were raised through shoot formation from nodal explants derived from 1-year-old field-grown plants (originally collected from the Gumballi forest range located in the Biligiri Rangan Hills, 80 km from Mysore as in the Western Ghats, India) on Murashige and Skoog (MS)[16] medium with $3\ g\ l^{-1}$ sucrose, and supplemented with naphthalene acetic acid ($0.05–2.5\ mg\ l^{-1}$) and 6-benzyladenine ($0.05–2.5\ mg\ l^{-1}$), along with $7\ g\ l^{-1}$ agar, pH 5.7, as reported earlier,[17] and incubated at 25±2 °C with a light intensity of $40\ \mu mol\ m^{-2}\ s^{-1}$ and a $16\ h\ day^{-1}$ photoperiod using fluorescent lights (Philips India Ltd, Mumbai, India), and 60–70% relative humidity. Six-week-old *in vitro* shoots were used for rooting experiments.

* Correspondence to: GA Ravishankar, Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore-570013, India
E-mail: pcbt@cscftri.ren.nic.in
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In vitro rooting

In vitro shoots longer than 2.5 cm were placed on MS medium supplemented with various levels (0.5–2.0 mg l^{-1}) of α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA), either in combination or individually for root induction. Portions of 50 ml of MS liquid medium supplemented at the same hormonal concentrations as described for induction of rooting, along with 30 g l^{-1} sucrose were transferred to glass conical flasks (150 ml) inoculated with approximately 10 mg fresh weight of normal roots of *D hamiltonii* under aseptic conditions and maintained at 26 °C and 60–70% RH. The cultures were kept under continuous agitation at 90 rpm on a Model No G-52 gyrotary shaker (New Brunswick Scientific Co Inc, Edison, NJ, USA) and incubated under light (16 h day^{-1} photoperiod) at a photon flux density of 40 μmol m^{-2} s^{-1}. The fresh weight of roots taken at random was determined after 30, 45 and 60 days of growth. The same root material was then dried at 60 °C and dry weight was determined. Five flasks were harvested at a time and the experiment was repeated twice to determine the parameters studied and the content of the flavour compound 2H4MB.

Isolation of the flavour compound

The dried roots (5 g) were powdered and subjected to steam distillation for 5 h. The steam condensate was extracted with dichloromethane (50 ml × 4). The combined extracts were passed through a funnel containing anhydrous sodium sulphate to remove the water content, concentrated in a flash evaporator, dissolved in 1 ml ethanol and stored in a closed vial. Quantification of the flavour compound was determined by gas chromatographic analysis (GC) (Fisons GC-8000 series, Thermo Quest Italia SPA, Rodano, Milan, Italy) using flame ionization detection (FID) (Perkin-Elmer Autosystem XL, Perkin-Elmer, Norwalk, CT, USA) and GC-MS (GC 17A QP 5000, Shimadzu, Kyoto, Japan) analysis.

Estimation of flavour compound

Determination of 2H4MB, which is an isomer of vanillin with a molecular weight of 152, was done by spotting the root extracts on TLC plates along with a standard (Fluka Chemicals, Buchs, Switzerland) and run in a solvent system comprising hexane:benzene (1:1). The spot coinciding with that of the standard (2H4MB; with R_f 0.47) was extracted in the same solvent and the UV spectrum was determined on a Perkin-Elmer Autosystem XL UV–vis recording spectrophotometer UV-160 (Perkin-Elmer, Norwalk, CT, USA). Maximum absorption was obtained at 278 nm. Quantitative detection was done by GC-MS and FID. The constituent was identified by matching its mass spectrum with GC-MS library user generated mass spectral libraries, and also confirmed by comparison with the GC retention time of the standard sample. First the standard solution of 2H4MB and root extracts were separated by a gas chromatograph GC-8000 series (Fison Instruments, Thermo Quest Italia SPA, Rodano, Milan, Italy) using flame ionization with a capillary column; column temperature was raised from 60 to 250 °C at an incremental rate of 2 °C min^{-1}. The carrier gas was nitrogen with a flow rate of 30 ml min^{-1}. Detection temperature was 250 °C and injection temperature 100 °C. Both standard and root extract (alcohol) samples (1 μl) were injected separately.

Gas chromatographic analysis

The retention times of the individual peaks in the standard sample and root extract samples were compared and quantitative percentage calculations carried out.

GC-MS method

In order to identify the constituents of 2H4MB and the concentrations of desired compounds, mass spectral analysis was carried out using GC-MS (Shimadzu, Kyoto, Japan), GC-14B coupled with a QP 5000 MS system under the following conditions: SPB-1 column (Supelco, Bellefonte, PA, USA, 30 m × 0.32 mm, 0.25 μm film thickness); oven temperature programme, 60 °C for 2 min, rising at 2 °C min^{-1} to 250 °C, held for 5 min; injection port temperature 225 °C; detector temperature, 250 °C; carrier gas helium flow rate 1 ml min^{-1}. The amount of sample (dissolved in alcohol) injected was 1 μl. The GC-MS separation profile of the alcoholic extract of the root extract indicated the presence of 2H4MB being predominant. The standard and root-extract-derived samples showed identical compounds during FID and GC-MS analysis. Identification and quantification were based on FID and GC-MS analysis, respectively.

Statistical analysis

The experiment was repeated twice with five replicates each for different concentrations of auxin treatments to grow roots and subsequently for flavour compound analysis. The mean (± SE) values of the results were determined. Significant differences between the control and treatments were determined using Turkey Multiple Comparison (Software Prism 3.0, Prism Software, Rosewell, GA, USA).

RESULTS AND DISCUSSION

As illustrated in Table 1, during the first 30 days the root growth was slow, but between 30 and 45 days growth was more rapid. Although auxins such as IAA and IBA initiated root formation and further growth, it was the 1.0 mg l^{-1} NAA which initiated very early rooting (by the 10th day) and also maximum root growth. The roots initiated on medium containing NAA (0.5–2.0 mg l^{-1}) were thick and short compared with the long, thin roots produced on medium containing IAA and IBA. The medium supplemented with 1.0 mg l^{-1} NAA was best

Table 1. Influence of auxins on growth *in vitro* of normal roots of *Decalepis hamiltonii* and production of 2H4MB

Hormones (mg l^{-1})			Dry weight of roots (mg per flask) after 30 days	2H4MB (μg g^{-1} dry weight) after 30 days	Dry weight of roots (mg per flask) after 45 days	2H4MB (μg g^{-1} dry weight) after 45 days	Dry weight of roots after (mg flask^{-1}) after 60 days	2H4MB (μg g^{-1} dry weight) after 60 days
IAA	IBA	NAA						
0.5	0	0	66 ± 2.3	2.0 ± 0.40	100 ± 5.0	16 ± 0.85	120 ± 10	18 ± 0.45
1.0	0	0	90 ± 3.4[a]	3.4 ± 0.25[a]	180 ± 6.7[a]	14 ± 0.94[a]	220 ± 9.4[a]	15 ± 0.28[a]
2.0	0	0	120 ± 3.5[a]	9.5 ± 0.18[c]	170 ± 4.8[c]	20 ± 1.1[c]	210 ± 11.3[b]	20 ± 0.94[c]
0	0.5	0	130 ± 4.2[b]	11 ± 1.2[b]	190 ± 12.3[c]	23 ± 1.5[c]	230 ± 10.4[a]	22.1 ± 0.43[a]
0	1.0	0	160 ± 7.5[b]	11 ± 0.8[a]	240 ± 10.4[a]	25 ± 1.7[b]	250 ± 12.5[b]	23.5 ± 0.98[c]
0	2.0	0	178 ± 5.3[a]	10 ± 0.75[a]	235 ± 13.8[b]	20 ± 1.2[a]	246 ± 15.6[c]	20.2 ± 0.74[a]
0	0	0.5	190 ± 8.2[c]	22 ± 1.2[a]	240 ± 18.5[c]	26.8 ± 1.7[b]	265 ± 12.8[a]	26.0 ± 1.9[b]
0	0	1.0	243 ± 11.4	28 ± 1.8[b]	360 ± 12.5[b]	40 ± 2.1[a]	378 ± 17.2[c]	38.2 ± 2.1[b]
0	0	2.0	200 ± 10.6[b]	21 ± 0.98[a]	285 ± 18.2[c]	27 ± 1.5[b]	299 ± 12.5[a]	24.3 ± 1.4[a]
0	0.5	1.0	65 ± 2.3[a]	10 ± 1.4[c]	105 ± 9.4[a]	18 ± 1.3[a]	120 ± 9.5[a]	16.9 ± 1.4[a]
0	1.0	1.0	72 ± 4.5[aa]	12 ± 0.95[a]	140 ± 7.8[c]	22 ± 1.9[c]	162 ± 8.7	18.4 ± 1.6[a]
0	2.0	1.0	70 ± 3.8[c]	10 ± 0.74[c]	110 ± 9.2[a]	23 ± 1.2[a]	124 ± 9.2[a]	21.2 ± 1.2[c]
0	0	0	—	—	—	—	—	—

Each flask contained 50 ml culture medium.
[a] $p < 0.01$; [b] $p < 0.05$; [c] $p < 0.001$ compared with culture harvested after first day.

to produce maximum biomass (Table 1). Even the number of roots (4–3) was greater in the presence of 1.0 mg l^{-1} NAA compared with IAA or IBA (1–2) along with more laterals (10–15) and an elongation of 1.8–3.2 cm. The initiation of laterals was started from the 18th day. The maximum growth of the roots was noticed at 45 days of culture, and beyond that growth slowly declined (Table 1). By 7–8 weeks of growth, the roots became brown, sagging and frail. The decline of root growth upon prolonged culture on the same batch of medium might be due to the accumulation of endogenous hormones or to nutrient exhaustion. The loss of root differentiation ability is probably one of the drawbacks of maintaining normal root culture for a long time, as observed in *Duboisia* species.[18] It can be concluded (Table 1) that, under the conditions developed for this experiment, the dry weight of the roots and the accumulation of 2H4MB were variable; the yield was highest after the first 45 days and slightly lower or stable towards the later part of the experiment. Again the roots that were initiated on medium containing 1.0 mg l^{-1} NAA accumulated the maximum amount of 2H4MB after 45 days growth. The GC-MS analysis showed that *D hamiltonii* root cultures produced 2H4MB. The retention time and retention indices for 2H4MB were 26.23 and 825.93, respectively. From Table 1 it is clear that the accumulation of this compound was closely related to the biomass production. Our studies are supported by earlier work on a similar plant, *Hemidesmus indicus*.[19] Although hairy root cultures are more beneficial than normal roots, in view of their genetic and biochemical stability over long periods, normal root culture is an alternative method for those species which are resistant to *Agrobacterium rhizogenes* infection. Apart from this, before the establishment of the first hairy root culture, normal root culture had been established for a number of species and demonstrated their ability to accumulate secondary products similar to those found in the roots of the parent plant.[20] Field-grown one-year-old plants, with tuberous roots of size 1.0–1.5 cm diameter, contained 120–140 μg g^{-1} dry weight of 2H4MB.[15] However, in the present study in a 45 day period, *in vitro* raised normal roots produced 40 μg g^{-1} dry weight of this compound. At this rate, in a one-year period, 320 μg g^{-1} dry weight of the compound could be obtained using *in vitro* normal root cultures, which is 2.7 times more than normal field-grown plant roots. In conclusion, the system of normal root culture described here would be valuable for the sustainable utilization of this endangered endemic plant for its bioactive ingredients.

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