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Research Article

Rhizogenesis in Cell Suspension Culture from Mango Ginger: A Source of Isosorbide and n-Hexadecanoic Acid

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Curcuma amada Roxb. belongs to the monocotyledonous family Zingiberaceae. It is commonly known as mango ginger and used as a spice and valuable medicine. In this study, adventitious roots of *C. amada* have been successfully established from cell suspension culture. The highest percentage of adventitious root production was obtained from friable callus derived cell suspension culture. The culture conditions of adventitious root were optimized and the maximum adventitious root production was obtained in half strength MS liquid medium containing 0.3 mg L^{-1} IBA along with 3% of sucrose after 5 weeks of culture. Among the different initial inoculum density, the best culture condition for root growth occurred at 10 g FW of initial inoculum density. GC-MS analysis revealed that the *in vitro* raised adventitious roots containing two valuable bioactive compounds, isosorbide and n-hexadecanoic acid. The outcome of the present work will be helpful for the large scale cultivation of adventitious roots for the production of valuable bioactive compounds.

1. Introduction

Curcuma amada Roxb. (mango ginger) belongs to the family of Zingiberaceae which is a unique perennial rhizomatous herb and morphologically resembles ginger and has a flavour of raw mango (*Mangifera indica*). There are 68 volatile aromas, more than 130 chemical constituents present in the mango ginger rhizome. The aromatic smell raised from *C. amada* is mainly attributed to the presence of car-3-ene and cis-ocimene compounds, which are used in still food, beverages, cosmetics, and medicines [1–8]. The rhizome is composed (fresh weight basis) of 86% moisture, 0.8% ash, 0.8% total sugars, traces of reducing sugars, 1.4% fibre, 0.1% essential oil, and 6.9% starch and on a dry weight basis, 5.7% ash, 5.8% total sugar, traces of reducing sugars, 10.6% crude fiber, 0.9% essential oil, and 45.6% starch [9]. The *C. amada* has been reported with high amylase activity that converts starch into simple metabolisable sugars and, in turn, into several valuable aromatic compounds [10]. Due to this metabolic advantage, curcumin-free portion is effective in lowering liver cholesterol in animals [11]. Recently, three terpenoid bioactive compounds (difurocumenonol, amadannulen, and amadaldehyde) were isolated from their rhizomes. They also exhibit potential actions such as antimicrobial, antioxidant, platelet aggregation inhibitor activities, and anticancer property [12]. It also contains antitubercular agent like labdane diterpenoid [13].

In plants, secondary metabolites accumulate in specific or specialized cells, tissues, or organs [14]. *In vitro*, tissues need dedifferentiation (callus formation) and redifferentiation (rhizogenesis and embryogenesis) process for biosynthesis and accumulation of secondary metabolites [15, 16]. Adventitious root culture is one of the valuable tools, especially cell suspension culture, and adventitious root induction is the best automation process biomass production. The present work reports a simple and reliable procedure for *in vitro* adventitious root induction from homogenous cell suspension culture of *C. amada* and examines bioactive compounds using GC-MS analysis.

2. Material and Methods

2.1. Callus Induction. Microrhizome segments were excised from 3-month-old *in vitro* grown plants [17]. For callus induction, these segments were placed on MS medium [18] with 3.0% sucrose and different concentration of 2,4-D (1.0, 2.0, and 3.0 mg L^{-1}) alone or in combination with BA or Kn (0.25, 0.5 mg L^{-1}). The medium was solidified with 0.8% agar and the pH of the media was adjusted to 5.7 ± 0.1 before solidification. Media were autoclaved at 121°C and 104 kPa for 15 min. Cultures were maintained at 25 ± 2°C for 16 hrs photoperiod with 40 μmol m^{-2} s^{-1} light intensity provided by white fluorescent tubes and a relative humidity of 55–65%.

2.2. Initiation of Cell Suspension Culture and Induction of Adventitious Roots. For induction of adventitious roots, ~250 mg fresh mass of different types of callus (nonfriable, semifriable, and friable callus) was transferred to a 150 mL Erlenmeyer flask (each in separate flask). Each flask containing MS liquid medium was supplemented with different concentration of auxins (0.1, 0.2, 0.3, 0.4, and 0.5 mg L^{-1} IBA or IAA), and then they were placed on orbital shaker at 100 rpm in continuous darkness. MS medium without auxin was used as a control. After one week of culture period, callus responding with root induction (%) was calculated using the following equation:

$$\begin{aligned}&\text{Callus responding with root induction (\%)}\\&\quad=\text{Number of callus culture which}\\&\qquad\text{reponded with root induction}\\&\qquad\cdot(\text{Total number of callus culture})^{-1}\times 100.\end{aligned}\tag{1}$$

For biomass production, adventitious roots (~0.5 cm; 35 roots/flask) were transferred in the same media composition and harvested during the 5th week of culture when the biomass reached a maximum level. The suitable auxin was selected for further studies based on the comparison of root length.

2.3. Optimization of Medium Strength, Sucrose Concentration, and Initial Inoculum Density for Adventitious Root Culture. The culture medium was optimized by transferring initial inoculum (~2.5 g FW adventitious roots) to various strengths of MS liquid medium (1/4, 1/2, 3/4, and full strength) and different concentrations of sucrose (1.0, 3.0, 4.5, and 6.0%) for biomass production. For improving adventitious root biomass, optimal inoculum density was standardized using various levels (2.5, 5.0, 10.0, 15.0, and 20.0 g FW) of initial inoculum. Each treatment was carried out three times with seven flasks. Growth ratio (GR) was calculated using the following equation [19]:

$$\text{Growth ratio}=\frac{\text{FW of the harvested biomass (g)}}{\text{FW of the initial inoculum (g)}}.\tag{2}$$

2.4. GC-MS Analysis. The adventitious root mass (1.0 g FW) harvested from suspension culture and rhizome of field grown plants was subsequently air-dried for 1 hour and completely ground using pestle and mortar. Extraction was carried out with methanol (10 mL) until ground root changed into white color by sonication. After centrifugation at 8,000 rpm for 15 min, the upper aqueous layer was collected and filtered through a nylon membrane filter and injected into the GC-MS equipment for analysis.

2.4.1. GC-MS Programme. Consider the following:

column: Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) (Perkin Elmer) 30 m × 0.25 mm × 0.25 μm, film thickness 0.25 μm,

equipment: GC Clarus 500 Perkin Elmer, California, USA,

carrier gas: 1 mL per min., split: 10 : 1,

detector: mass detector Turbomass gold-Perkin Elmer,

software: Turbomass 5.2,

sample injected: 2.0 μL.

2.4.2. Oven Temperature Programme. Consider the following:

110°C – 2.0 min: hold,

up to 200°C at the rate of 10°C/min: no hold,

up to 280°C at the rate of 5°C/min: 9.0 min hold,

injector temperature: 250°C,

total GC running time: 36 min.

2.4.3. MS Programme. Library used NIST version 2005:

inlet line temperature: 200°C,

source temperature: 200°C,

electron energy: 70 Ev,

mass scan (m/z): 45–450,

solvent delay: 0–2.0 min,

total MS running time: 36 min.

2.5. Statistical Analysis. All experimental data were subjected to one way ANOVA followed by statistical significance test. Data were presented as mean, mean ± SE. The mean separations were analyzed by using Duncan's multiple range test with significance level of $P < 0.05$ (IBM SPSS statistics 19).

3. Results

3.1. Initiation of Cell Suspension Culture and Induction of Adventitious Roots. As the result of the present study, MS medium containing 1.0 mg L^{-1} 2,4-D in combination with 0.25 mg L^{-1} BA was found to produce friable callus. Medium containing 1.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA was favorable for semifriable callus formation, 2.0 mg L^{-1} 2,4-D and

Table 1: Effect of auxins on adventitious root formation from callus via cell suspension culture.

PGRs (mg L^{-1})		Callus responding with root induction (%)			Root length of FC induced (cm)*
IBA	IAA	NFC	SFC	FC	
0.1		—	79.10	88.10	4.46 ± 0.03^{i}
0.2		—	66.66	100	6.84 ± 0.04^{b}
0.3		—	45.88	100	7.23 ± 0.07^{a}
0.4		—	0	91.66	6.37 ± 0.04^{c}
0.5		—	0	83.33	5.42 ± 0.06^{e}
	0.1	—	63.88	91.66	4.51 ± 0.04^{gh}
	0.2	—	41.66	100	6.46 ± 0.05^{c}
	0.3	—	0	88.10	5.66 ± 0.05^{d}
	0.4	—	0	72.22	5.07 ± 0.05^{f}
	0.5	—	0	61.10	4.63 ± 0.05^{g}

Data was recorded from 5-week-old adventitious root cultures.
All experiments were carried out three times with at least 15 cultures.
*Data represented mean ± SE, mean within a column followed by same letter is not significantly different according to Duncan's multiple range (DMRT) test, $P < 0.05$.
NFC: nonfriable callus; SFC: semifriable callus; FC: friable callus.

Table 2: Effect of medium strength and sucrose concentration on adventitious root formation.

Sucrose (%)	Root biomass (g FW)*				Growth ratio			
	1/4 MS	1/2 MS	3/4 MS	Full MS	1/4 MS	1/2 MS	3/4 MS	Full MS
1.5	18.60 ± 0.33^{c}	27.26 ± 0.28^{c}	22.73 ± 0.40^{c}	14.67 ± 0.29^{c}	7.40	10.91	9.09	5.87
3.0	35.73 ± 0.22^{a}	51.60 ± 0.50^{a}	46.73 ± 0.30^{a}	31.47 ± 0.24^{a}	14.29	20.64	18.69	12.59
4.5	25.93 ± 0.36^{b}	39.00 ± 0.43^{b}	24.00 ± 0.50^{b}	19.53 ± 0.43^{b}	10.37	15.60	9.60	7.81
6.0	13.47 ± 0.27^{d}	23.33 ± 0.57^{d}	17.67 ± 0.27^{d}	10.87 ± 0.35^{d}	5.39	9.33	7.09	4.35

Data was recorded from 5-week-old adventitious root cultures.
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*Data represented mean ± SE, mean within a column followed by same letter is not significantly different according to Duncan's multiple range (DMRT) test, $P < 0.05$.

0.5 mg L^{-1} BA were found to produce nonfriable callus (data not shown). To induce adventitious root formation, all three types of callus were transferred to MS liquid medium containing IBA or IAA. Friable callus was suspended easily in single cell manner (Figure 1(a)) and semifriable callus formed cell aggregation. Nonfriable callus settled down in the medium and could not be proliferated into roots (Table 1). Auxins also significantly influenced the adventitious root formation from callus culture. IBA showed higher percentage of root induction than IAA. Maximum percentage (100%) of root formation was obtained from friable callus derived cell suspension in the medium containing 0.2–0.3 mg L^{-1} IBA. However, maximum root length (7.23 cm) was observed in the medium containing 0.3 mg L^{-1} IBA (Figure 1(b)). When increasing or decreasing the concentration of IBA to this level, percentage of adventitious root formation gradually decreased.

3.2. Optimization of Medium Strength and Sucrose Concentration for Adventitious Root Biomass Production. The present study reveals that MS liquid medium strength and gradient sucrose concentration significantly influenced adventitious root formation. Among various medium strengths and concentrations of sucrose, the highest root biomass (51.60 g FW) production was observed in half strength MS medium supplemented with 3.0% sucrose (Table 2). In contrast, root growth was inhibited when the medium strength or sucrose concentration was increased or decreased to this optimum level.

3.3. Optimization of Inoculum Density for Adventitious Root Biomass Production. Inoculum density depends on the volume of culture medium and vessel. In the present study, 250 mL Erlenmeyer flasks containing 50 mL medium were used to optimize the inoculum density for achieving maximum root biomass production. On the different initial inoculum density, maximum adventitious root biomass (121 g FW) and growth rate (12.1%) were recorded at 10 g FW of initial inoculum (Figure 1(c)). Further, decrease or increase in inoculum density led to decrease in the biomass production (Table 3).

3.4. GC-MS Analysis. The essential oil components were found to be varied between rhizome of field grown plants and *in vitro* raised adventitious roots (Tables 4 and 5). Out of 29 peaks which were detected from rhizome, 14 peaks were identified (Figure 2(a)) and out of 21 peaks detected from adventitious roots, 3 peaks were identified

(a) (b) (c)

Figure 1: Adventitious root culture of *C. amada* via cell suspension culture. (a) Adventitious root induction from friable callus derived cell suspension in MS liquid medium supplemented with 0.3 mg L^{-1} IBA. (b) Adventitious roots growth in MS liquid medium supplemented with 0.3 mg L^{-1} IBA after 5 weeks of culture period. (c) Vigorous growth using initial inoculum mass 10 g FW. *Scale bars:* (a–c) 0.5 cm.

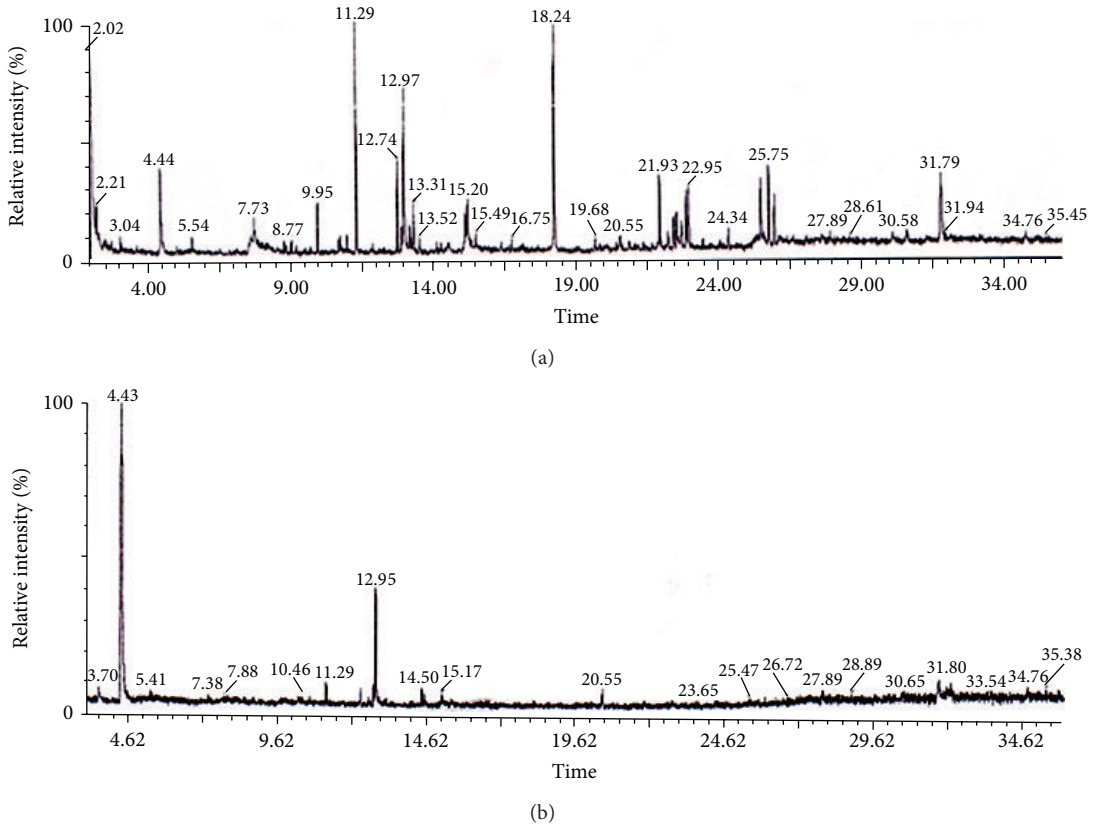

Figure 2: GC-MS spectrum from methanol extract of *C. amada*. (a) Field grown rhizome. (b) Cell suspension induced adventitious roots.

(Figure 2(b)) with their respective compounds. Interestingly, the *in vitro* raised adventitious roots showed only three compounds in detectable relative percentage of peak area. This was not the case with rhizome, where other compounds were also found in larger proportion. Among those three compounds detected in samples of adventitious roots, the isosorbide and 1-buten-1-ol, 2-methyl-4-(2,6,6,-trimethyl-1-cyclohexen-1-yl)-, formate, (E)- exhibited higher peak area when compared to samples of rhizome. Relative peak area of n-hexadecanoic acid was less in samples from adventitious roots than rhizome.

4. Discussion

Adventitious root culture is one of the valuable biological tools for feasible production of bioactive compounds without

Table 3: Effect of initial inoculum density on adventitious root formation.

Initial inoculum density (g FW)	Root biomass (g FW)*	Growth ratio
2.5	51.60 ± 0.50^{e}	20.64
5.0	87.20 ± 0.34^{b}	17.44
10.0	121.00 ± 0.78^{a}	12.10
15.0	78.87 ± 0.52^{c}	5.20
20.0	64.27 ± 0.54^{d}	3.21

Data was recorded from 5-week-old adventitious root cultures.
All experiments were carried out three times with at least 15 cultures.
*Data represented mean ± SE, mean within a column followed by same letter is not significantly different according to Duncan's multiple range (DMRT) test, $P < 0.05$.

depending on field grown parent plants and abiotic and biotic factor effects [20, 21]. In the present study, a promising adventitious root induction system was successfully developed for mango ginger, which is an important aromatic rhizomatous plant. Among the different qualities of callus, friable callus responds more favorably for adventitious root formation when compared with semifriable and nonfriable callus. Prakash et al. [22] also reported that the friable callus seems to be one of the most suitable starting materials for induction of organogenesis in *C. amada*. It may be probably due to the presence of more physiological active cells which are more powerful than the cells in semifriable callus and nonfriable callus [21]. The results of exogenous auxin treatment indicate that $0.3\,mg\,L^{-1}$ IBA was the optimum for adventitious root formation more than IAA. A similar phenomenon was also found in *W. somnifera* [21], *Morinda citrifolia* [23], and *Periploca sepium* [24]. The year round availability of adventitious root culture can solve the problem of seasonal availability of mango ginger.

In plant cell/organ culture, sucrose is an important balanced carbon source and plays a vital role in the synthesis of cell constituents as substrate to provide energy for cell growth [25]. It promotes cell growth by hydrolysis of invertase and sucrose synthase acts as building blocks and regulates osmotic potential [26, 27]. In the present study, 3% sucrose was suitable for adventitious root growth in terms of biomass production. Lower concentration cannot provide enough energy and high sucrose concentration exhibited negative effect in root primordial induction.

The concentration of salts in the MS medium significantly contributes to biomass production and phytochemical accumulation in cultured cells and tissues [28]. Wu et al. [29] proposed that the interactions among the nutritional salts enhance the availability of ions to the roots and thereby promoting the root growth and phytochemical production. The present study confirmed that the optimization of MS salt concentration is very essential for adventitious root production and half strength MS medium is the best for optimal root primordial induction and growth in *C. amada*. The same phenomenon was also documented in root culture of Zingiberaceae member *Alpinia galanga* [30]. Further it was observed that when increasing the MS salt strength in the medium, root biomass production was reduced. It indicated that high MS salt concentration promoted a stress condition and reduced the growth of adventitious roots. Determination of optimal inoculum density is a prerequisite for enhanced production of secondary metabolite from *in vitro* root biomass [19, 31, 32]. In *W. somnifera*, optimal level of initial inoculum density is 15 g FW. The increase or decrease level of inoculum density inhibits root biomass production [21]. In the present study, maximum root biomass production in *C. amada* was obtained when inoculum density was at 10 g FW.

The *in vitro* raised adventitious roots contained two compounds in higher proportion and one on par with field grown rhizome. This offers new avenue for scaling up of the two identified compounds such as isosorbide and n-hexadeconoic acid [33, 34]. Isosorbide, being valuable derivative of glucose, can be used for further conversions into several chemicals like green solvents, fuels, fuel additives, and so forth [33]. n-Hexadeconoic acid is also very useful in the production of cetyl alcohol which is used in food and cosmetic industry [34]. Similar attempts have been made by other investigators [35]. The present study successfully mimics the levels of two bioactive compounds produced by field grown rhizome. Reports in related species (*C. longa*) have achieved this similarity between *ex vitro* plants and *in vitro* raised plants that are established *ex vitro* [35].

In conclusion the present investigation opens up a new route for large scale production of active compounds, isosorbide and n-hexandeconoic acid, from homogenous cell suspension mediated adventitious root culture of *C. amada*. To the best of our knowledge, this is the first report of *in vitro* isosorbide and n-hexadeconoic acid production from adventitious root cultures. Further, the results obtained in the present study might be useful in further research on biotransformation and production of these secondary metabolites of *C. amada* in large scale.

Abbreviations

MS: Murashige and Skoog medium
2,4-D: 2,4-Dichlorophenoxy acetic acid
BA: 6-Benzyladenine
Kn: Kinetin
IBA: Indole-3-butyric acid
IAA: Indole-3-acetic acid
GC-MS: Gas chromatography-Mass spectrometry
FW: Fresh weight.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this research paper.

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Table 4: Phytochemical profile of field grown rhizome of *C. amada*.

Number	RT	Name of the compound	Molecular formula	Molecular weight	Peak area (%)*
1	4.44	Isosorbide	$C_6H_{10}O_4$	146	9.52
2	9.95	Cyclopropanementaol, 2-methyl-2-(4-methyl-3-pentenyl)	$C_{11}H_{20}O$	168	2.86
3	11.29	1-Buten-1-ol, 2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, formate, (E)-	$C_{15}H_{24}O_2$	236	11.90
4	12.74	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)-	$C_{15}H_{24}$	204	4.29
5	12.97	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	14.29
6	13.17	2,6,10,14-Hexadecatetraenoic acid, 3,7,11,15-tetramethyl-, methyl ester, (E,E,E)-	$C_{21}H_{34}O_2$	318	0.95
7	13.31	Hydroxydehydrostevic acid	$C_{20}H_{30}O_3$	318	2.86
8	18.24	4-Hexen-1-0l, 6-(2,6,6-trimethyl-1-cyclohexenyl)-4-methyl-, (E)-	$C_{16}H_{28}O$	236	18.57
9	21.95	1-Heptadec-1-ynyl-cyclopentanal	$C_{22}H_{40}O$	320	6.19
10	22.53	Acetic acid, 1-[2-(2,2,6-trimethyl-bicyclo [4,1.0]hept-1-yl)-ethyl]-vinyl ester	$C_{16}H_{26}O_2$	250	2.86
11	22.87	2H-Pyran, 2-(7-heptadecyyloxy) tetrahydro-	$C_{22}H_{40}O$	336	3.81
12	25.47	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	$C_{16}H_{26}O$	410	6.19
13	25.75	Squalene	$C_{22}H_{40}O_2$	410	5.71
14	31.79	Dihydrotachysterol	$C_{28}H_{46}O$	398	10.00

*Relative intensity.

Table 5: Phytochemical profile of *in vitro* raised adventitious roots of *C. amada*.

Number	RT	Name of the compound	Molecular formula	Molecular weight	Peak area (%)*
1	4.43	Isosorbide	$C_6H_{10}O_4$	146	65.71
2	11.29	1-Buten-1-ol, 2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, formate, (E)-	$C_{15}H_{24}O_2$	236	20.00
3	12.95	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	12.86

*Relative intensity.

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