Rhizogenesis in Cell Suspension Culture of Mango Ginger (*Curcuma amada* Roxb.): A Source of Isosorbide and n-Hexadecanoic Acid

Abstract

Mango ginger (*Curcuma amada* Roxb.) belongs to the monocotyledonous family Zingiberaceae. It provides a valuable medicine, as well as a spice. In this study, adventitious roots of *C. amada* were successfully established from cell suspension culture. Adventitious root production was highest from friable callus-derived cell suspension culture. The optimal culture conditions for adventitious root production were determined; maximum adventitious root production was obtained in half-strength MS liquid medium containing 0.3 mg l^{-1} indole-3-butyric acid and 3% sucrose after 5 weeks of culture. The optimal initial inoculum density for root growth was 10 g fresh weight. Gas chromatography-mass spectrometry analysis revealed that adventitious roots generated *in vitro* contained two valuable bioactive compounds, isosorbide and n-hexadecanoic acid. These results will be helpful in advancing the large-scale cultivation of adventitious roots for the production of valuable bioactive compounds.

1. Introduction

Curcuma amada Roxb. (mango ginger) of the Zingiberaceae family is a unique perennial rhizomatous herb, which morphologically resembles ginger and has a flavour of raw mango (*Mangifera indica*). There are 68 volatile aromas and more than 130 chemical constituents in the mango ginger rhizome. The plant's aromatic smell is mainly attributed to the presence of car-3-ene and cis-ocimene compounds, which are used in food, beverages, cosmetics, and medicines [1–8]. The rhizome is composed, on a 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 fibre, 0.9% essential oil, and 45.6% starch [9]. High amylase activity has been reported for *C. amada*; this enzyme converts starch into simple metabolizable sugars, from which, in turn, several valuable aromatic compounds are synthesized [10]. Due to this metabolic advantage, the curcumin-free portion is effective in lowering liver cholesterol levels in animals [11]. Recently, three bioactive terpenoid compounds (difurocumenonol, amadannulen, and amadaldehyde) were isolated from mango ginger rhizomes. They also exhibit potential actions such as antimicrobial, antioxidant, platelet aggregation inhibitor activities, and anticancer property [12]. It also contains antitubercular agents like labdane diterpenoid [13].

In plants, secondary metabolites accumulate in specific or specialized cells, tissues, or organs [14]. *In vitro*, tissues need to undergo dedifferentiation (callus formation) and redifferentiation (rhizogenesis and embryogenesis) processes to achieve the biosynthesis and accumulation of secondary metabolites [15, 16]. Adventitious root culture, especially cell suspension culture, is a valuable tool for this purpose, and adventitious root induction is the biomass production process most suitable for automation. The present study is a report of a simple and reliable procedure for *in vitro* adventitious root induction from homogenous cell suspension culture of *C. amada* and an examination of the resultant bioactive compounds using gas chromatography-mass spectrometry (GC-MS) analysis.

3. Results

3.1. *Initiation of Cell Suspension Culture and Induction of Adventitious Roots*

MS medium containing 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 0.25 mg l^{-1} BAP produced friable callus. Medium containing 1.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP was favourable for semi-friable callus formation, and that containing 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP was found to produce nonfriable callus (data not shown). To induce adventitious root formation, all three types of calli were transferred to MS liquid medium containing indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA). Friable callus was suspended easily as single cells (Figure 1(a)), and semi-friable callus formed cell aggregations. Nonfriable callus settled down in the medium and could not proliferate into roots (Table 1). The auxin used also significantly influenced adventitious root formation from the callus cultures. The presence of IBA in the medium resulted in a higher percentage of root induction than that of IAA. Maximum root formation (100%) was obtained from friable callus-derived cell suspension in the media containing 0.2 and 0.3 mg l^{-1} IBA. Maximum root length (7.23 cm) was observed in the medium containing 0.3 mg l^{-1} IBA (Figure 1(b)). When the concentration of IBA was higher or lower than this level, the percentage of adventitious root formation gradually decreased.

3.2. *Optimization of Medium Strength and Sucrose Concentration for Adventitious Root Biomass Production*

MS liquid medium strength and sucrose concentration significantly influenced adventitious root formation. Among the tested medium strengths and concentrations of sucrose, the highest root biomass production (51.60 g fresh weight [FW]) 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 higher or lower than 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 determine the optimal inoculum density for maximum root biomass production. Maximum adventitious root biomass (121 g FW) and growth rate (12.1%) were recorded at 10 g FW of the initial inoculum (Figure 1(c)). Furthermore, any decrease or increase in inoculum density away from this level led to a decrease in biomass production (Table 3).

3.4. *GC-MS Analysis*

The essential oil components were found to vary between the rhizomes of field-grown plants and *in vitro*-raised adventitious roots (Tables 4 and 5). Out of 29 peaks detected in the rhizome samples, 14 were identified in the cultured root samples (Figure 2(a)), and out of 21 peaks detected in the adventitious root samples, 3 were identified in the rhizome samples (Figure 2(b)), with their respective compounds. Interestingly, the *in vitro*-raised adventitious roots showed only three compounds within the detectable relative percentage zone of the peak area. This was not the case for the rhizome, in which additional compounds were found in detectably large proportions. Among the three compounds detected in the adventitious root samples, isosorbide and 1-buten-1-ol, 2-methyl-4-(2,6,6,-trimethyl-1-cyclohexen-1-yl)-, formate, (E)- exhibited larger peak areas than in the rhizome samples. The relative peak area of n-hexadecanoic acid was smaller in adventitious root samples than in rhizome samples.

4. Discussion

Adventitious root culture is a valuable biological tool capable of producing bioactive compounds without depending on field-grown parent plants and not subject to outdoor 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. Friable callus responded more favourably in terms of adventitious root formation than semi-friable and nonfriable callus. Prakash *et al*. [22] also reported that friable callus seems to be one of the most suitable starting materials for the induction of organogenesis in *C. amada*. This is probably due to the presence of more physiologically active cells, which are more powerful than the cells in semi-friable and nonfriable callus [21]. Our exogenous auxin treatment results indicated that 0.3 mg l^{-1} IBA was optimal for adventitious root formation, outperforming IAA. Similar phenomena have also been reported in *Withania somnifera* [21], *Morinda citrifolia* [23], and *Periploca sepium* [24]. Adventitious root culture is not season-dependent and could solve the problem of seasonal availability of mango ginger.

In plant cell/organ culture, sucrose is an important balanced carbon source, acting as a substrate to provide energy for cell growth and thus plays a vital role in the synthesis of cell constituents (Baque *et al*., 2012). It promotes cell growth via the hydrolysis of invertase, sucrose synthase generates building blocks, and sugars regulate osmotic potential [26, 27]. In the present study, 3% sucrose was suitable for adventitious root growth in terms of biomass production. Lower concentrations did not provide enough energy, and high concentrations negatively affected root primordium induction.

The concentration of salts in the MS medium is an important contributor to biomass production and phytochemical accumulation in cultured cells and tissues [28]. Wu *et al*. [29] proposed that interactions among nutritional salts enhance the availability of ions to the roots and thereby promote root growth and phytochemical production. In the present study, it was confirmed that the optimization of MS salt concentration is essential for adventitious root production and that half-strength MS medium results in optimal root primordium induction and growth in *C. amada*. The same phenomenon was documented when culturing roots of *Alpinia galanga*, also belonging to the Zingiberaceae family [30]. Furthermore, we observed that increasing the MS salt strength in the medium resulted in reduced root biomass production. This suggests that high MS salt concentration produced stress, thereby reducing the growth of adventitious roots. Determination of the optimal inoculum density is a prerequisite for enhanced production of secondary metabolites from *in vitro*-grown root biomass [19, 31, 32]. In *W. somnifera*, the optimal initial inoculum density is 15 g FW. Higher or lower inoculum densities inhibit root biomass production [21]. In the present study, maximum root biomass production in *C. amada* was obtained when the inoculum density was 10 g FW.

In vitro-raised adventitious roots contained higher proportions of two compounds and a similar proportion of a third, compared to the proportions in the field-grown rhizome. This offers a new avenue for scaling up production of two of the identified compounds, namely isosorbide and n-hexadecanoic acid [33, 34]. Isosorbide, a valuable derivative of glucose, can be used as the chemical basis for the production of green solvents, fuels, fuel additives, and so forth [33]. Likewise, n-hexadecanoic acid is a component in the production of cetyl alcohol, which is used in the food and cosmetics industries [34]. In the present study, the levels of two useful bioactive compounds produced by field-grown plants were successfully reproduced *in vitro*. A study in a related species (*Cucurma longa*) has achieved similar results; this study compared *ex vitro* plants and *in vitro*-raised plants subsequently established *ex vitro* [35].

In conclusion, the present investigation opens up a new avenue for the large-scale production of two active compounds, isosorbide and n-hexadecanoic 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-hexadecanoic acid production from adventitious root cultures. Furthermore, the results obtained in the present study could be

useful in further research on biotransformation and production of these secondary metabolites of *C. amada* on a large scale.

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] containing 3.0% sucrose and either 1.0, 2.0, or 3.0 mg l^{-1} 2,4-D, alone or in combination with BAP or one of 0.25 or 0.5 mg l^{-1} kinetin. In all cases, the medium was solidified with 0.8% agar and its pH was adjusted to before solidification. The media were autoclaved at 121 °C and 104 kPa for 15 min. Cultures were maintained at °C for a 16 h photoperiod under 40 µmol m^{-2} s^{-1} light intensity, provided by white fluorescent tubes, and at a relative humidity of 55–65%.

2.2. *Initiation of Cell Suspension Culture and Induction of Adventitious Roots*

For the induction of adventitious roots, ~250 mg fresh masses of nonfriable, semi-friable, and friable callus were transferred to separate 150 ml Erlenmeyer flasks containing MS liquid medium. Each flask was supplemented with one of the following concentrations of auxins: 0.1, 0.2, 0.3, 0.4, or 0.5 mg l^{-1} IBA or 0.1, 0.2, 0.3, 0.4, or 0.5 mg l^{-1} IAA; the flasks were then placed on an orbital shaker at 100 rpm in continuous darkness. MS medium without auxin was used as a control. After one week of culture, the proportion of callus responding with root induction (%) was calculated using the following equation:

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

Optimization of Medium Strength, Sucrose Concentration, and Initial Inoculum Density for Adventitious Root Culture

The optimal culture medium for adventitious root biomass production was identified by transferring the 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%). The optimal inoculum density for adventitious root biomass production was identified using various levels (2.5, 5.0, 10.0, 15.0, and 20.0 g FW) of the initial inoculum. Each treatment was carried out three times with seven flasks. The growth ratio was calculated using the following equation [19]:

2.4. GC-MS Analysis

The adventitious root masses (1.0 g FW) harvested from suspension culture and the rhizomes of field-grown plants were subsequently air-dried for 1 h and completely ground using pestle and mortar. Extraction was carried out by sonication with methanol (10 ml) until the ground root colour changed to white. 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*

The GC apparatus used was an Elite-5MS (5% diphenyl/95% dimethyl polysiloxane) column, 30 m × 0.25 mm × 0.25 µm, with a film thickness of 0.25 µm, with a GC Clarus 500 chromatograph (both Perkin Elmer, California, USA). The carrier gas rate was 1 ml min^{-1}, split: 10:1. The mass detector was a Turbomass Gold (Perkin Elmer, California, USA) running Turbomass v5.2 software. Each injected sample was 2.0 µl.

2.4.2. *Oven Temperature Programme*

The oven temperature programme used was as follows: 110 °C for 2.0 min; hold; 10 °C min^{-1} increase up to 200 °C; immediate further increase up to 280 °C at the rate of 5 °C

min^{-1}; and hold for 9.0 min. The injector temperature was 250 °C, and the total GC running time was 36 min.

2.4.3. *MS Programme*

The MS conditions were as follows: 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; and total MS running time, 36 min. The library used was NIST version 2005.

2.5. ***Statistical Analysis***

All experimental data were subjected to one-way ANOVA followed by statistical significance testing. Data are presented as means ± SE. The mean separations were analysed using Duncan's multiple range test, with $P < [x]$ considered significant (IBM SPSS statistics).

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Table captions

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

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

Table 3: Effect of the initial inoculum density on *Curcuma amada* adventitious root formation.

Table 4: Phytochemical profile of the field-grown rhizome of *Curcuma amada.*

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

Figure captions

Figure 1: Adventitious root culture of *Curcuma 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} indole-3-butyric acid (IBA). (b) Adventitious root growth in MS liquid medium supplemented with 0.3 mg l^{-1} IBA after 5 weeks of culture. (c) Vigorous growth following inoculation with an initial inoculum mass of 10 g FW. Scale bars: (a–c) 0.5 cm.

Figure 2: Gas chromatography-mass spectrometry spectra from methanol extracts of *Curcuma amada*. (a) Field-grown rhizome. (b) Cell suspension of adventitious root material derived from friable callus and cultured in liquid half-strength MS medium supplemented with 3.0% sucrose.