Rhizogenesis and Cell Suspension Culture ~~from~~ of Mango Ginger (*Curcuma amada* Roxb.) as Source of Isosorbide and n-Hexadecanoic Acid

Word Count:

~~A~~Summary~~bstract~~

Mango ginger (*Curcuma amada* Roxb.) belongs to the monocotyledonous family Zingiberaceae. It ~~is p~~provides ~~commonly known as mango ginger and used as a spice and~~a valuable medicine, as well as a spice. ~~I~~In this study, adventitious roots of *C. amada* ~~have been~~were successfully established from cell suspension culture. ~~The highest percentage of a~~Adventitious root production was highest from ~~obtained from~~ friable callus-derived cell suspension culture. The optimal culture conditions ~~of~~ for adventitious root production were ~~optimized~~ determined; ~~and~~ ~~the~~ maximum adventitious root production was obtained in half-strength MS liquid medium containing 0.3 mg ~~L~~l indole-3-butyric acid~~IBA along with~~and 3% ~~of~~ sucrose after 5 weeks of culture. ~~Among the different initial inoculum density, t~~The ~~best~~ optimal initial inoculum density ~~culture condition~~ for root growth ~~occurred at~~was 10 g fresh weight~~FW of initial inoculum density~~. ~~GC-MS~~Gas chromatography-mass spectrometry analysis revealed that ~~the *in vitro* raised~~ adventitious roots generated *in vitro* ~~containing~~ contained two valuable bioactive compounds, isosorbide and n-hexadecanoic acid. These results ~~outcome of the present work~~ will be helpful ~~for~~ in advancing the large-scale cultivation of adventitious roots for the production of valuable bioactive compounds.

Keywords: Mango ginger; *Curcuma amada*; callus; adventitious root; *in vitro* culture; Murashige and Skoog medium; sucrose; indole-3-butyric acid; isosorbide; n-hexadecanoic acid

1. Introduction

Curcuma amada Roxb. (mango ginger) belongs to the family of the Zingiberaceae family which is a unique perennial rhizomatous herb, and which morphologically resembles ginger and has a flavour of raw mango (*Mangifera indica*). There are 68 volatile aromas, and more than 130 chemical constituents present in the mango ginger rhizome. The plant's 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](Dutt and Tayal, 1941; Gholap and Bandyopadhyay, 1984; Rao *et al*., 1989; Choudhury *et al*., 1996; Srivastava *et al*., 2001; Singh *et al*., 2003; Mustafa *et al*., 2005; Jatoi *et al*., 2007). 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](Policegoudra and Aradhya, 2007). High amylase activity has been reported for The *C. amada*; has been reported with high amylase activity that this enzyme converts starch into simple metabolizsable sugars, from andwhich, in turn, into several valuable aromatic compounds are synthesized [10](Policegoudra and Aradhya, 2008). Due to this metabolic advantage, the curcumin-free portion is effective in lowering liver cholesterol levels in animals [11](Srinivasan *et al*., 2008). Recently, three terpenoid bioactive terpenoid compounds (difurocumenonol, amadannulen, and amadaldehyde) were isolated from their mango ginger rhizomes. They also exhibit potential actions such as antimicrobial, antioxidant, platelet aggregation inhibitor activities, and anticancer property [12](Policegoudra *et al*., 2010). It also contains antitubercular agents like labdane diterpenoid [13](Singh *et al*., 2010).

In plants, secondary metabolites accumulate in specific or specialized cells, tissues, or organs [14](Flores-Sanchez *et al*., 2009). *In vitro*, tissues need to undergo dedifferentiation (callus formation) and redifferentiation (rhizogenesis and embryogenesis) processes for to achieve the biosynthesis and accumulation of secondary metabolites [15, 16] (Laurain-Mattar *et al*., 1999; Ramawat and Mathur, 2007). Adventitious root culture, especially cell suspension culture, is one of thea valuable tools for this purpose,, especially cell suspension culture, and adventitious root induction is the best biomass production automation process most suitable for automation biomass production. 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 (Sivakumar, 2006; Sivanandhan *et al*., 2012). The present work study is a report ofs a simple and reliable procedure for *in vitro* adventitious root induction from homogenous cell suspension culture of *C. amada* and an examination ofes 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*

As the result of the present study, MS medium containing 1.0 mg lL^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-D in combination with 0.25 mg lL^{-1} BAP was found to produced friable callus. Medium containing 1.0 mg Ll^{-1} 2,4-D and 0.5 mg Ll^{-1} BAP was favourable for semi-friable callus formation, and that containing 2.0 mg Ll^{-1} 2,4-D and 0.5 mg Ll^{-1} BAP were was found to produce nonfriable callus (data not shown). To induce adventitious root formation, all three types of callius were transferred to MS liquid medium containing indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA)IBA or IAA. Friable callus was suspended easily in as single cells manner (Figure 1(a)1(a)), and semi-friable callus formed cell aggregations. 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. The presence of IBA in the medium resulted in a showed higher percentage of root induction than that of IAA. Maximum root formation um percentage (100%) of root formation was obtained from friable callus- derived cell suspension in the mediaum containing 0.2 and– 0.3 mg Ll^{-1} IBA. However, maximum root length (7.23 cm) was observed observed in the medium containing 0.3 mg Ll^{-1} IBA (Figure 1(b)1(b)). When increasing or decreasing the concentration of IBA was higher or lower than to this level, the 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 the tested medium strengths and concentrations of sucrose, the highest root biomass (51.60 g FW) production (51.60 g fresh weight [FW]) was observed in half- strength MS medium supplemented with 3.0% sucrose (Table 22). In contrast, root growth was inhibited when the medium strength or sucrose concentration was was increased or decreased to 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 ml Erlenmeyer flasks containing 50 mL ml medium were used to determine the optimal optimize the inoculum density for achieving maximum root biomass production. On the differentM initial inoculum density, 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)1(c)). Furthermore, any decrease or increase in inoculum density away from this level led to a decrease in the biomass production (Table 33).

3.4. *GC-MS Analysis*

The essential oil components were found to be variedvary between the rhizomes of field- grown plants and *in vitro* -raised adventitious roots (Tables 44 and 55). Out of 29 peaks which were detected from in the rhizome samples, 14 peaks were identified in the cultured root samples (Figure 2(a)2(a)), and out of 21 peaks detected from in the adventitious roots, 3 peaks were identified in the rhizome samples (Figure 2(b)2(b)), with their respective compounds. Interestingly, the *in vitro* -raised adventitious root samples showed only three compounds within the detectable relative percentage zone of the peak area. This was not the case with for the rhizome, where in which additional other compounds were also found in detectably larger proportions. Among those three compounds detected in the adventitious root 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 larger peak areas than in the when compared to samples of rhizome samples. The Rrelative peak area of n-hexadecanoic acid was less smaller in samples from adventitious root samples than in rhizome samples.

4. Discussion

Adventitious root culture is one of the valuable biological tools for feasible production of bioactive compounds without 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, fFriable callus respondsed more favourably for in terms of adventitious root formation when compared withthan semi-friable and nonfriable callus. Prakash *et al*. [22](2004) also reported that the friable callus seems to be one of the most suitable starting materials for the induction of organogenesis in *C. amada*. It This ismay be probably due to the presence of more physiologically active cells, which are more

powerful with the cells in semi-friable ~~callus~~ and nonfriable callus ~~[21]~~(Sivanandhan *et al*., 2012). ~~The results of~~Our exogenous auxin treatment results indicated that 0.3 mg ~~L~~l^{-1} IBA was ~~the~~ optimalum for adventitious root formation, outperforming ~~more than~~ IAA. ~~A s~~Similar phenomenaon ~~was~~ have also been reported~~found~~ inn *Withania*~~.~~ *somnifera* ~~[21]~~(Sivanandhan *et al*., 2012), *Morinda citrifolia* ~~[23]~~(Baque *et al*., 2010), and *Periploca sepium* ~~[24]~~(Zhang *et al*., 2012). ~~The year round availability of a~~Adventitious root culture is not season-dependent and ~~can~~ 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 ~~as substrate to provide energy for cell growth~~ ~~[25]~~(Baque *et al*., 2012). It promotes cell growth ~~by~~via the hydrolysis of invertase, sucrose synthase ~~acts as~~generates building blocks, and sugars regulate~~s~~ osmotic potential ~~[26, 27]~~(Stepan-Sarkissian and Fowler, 1986; Calamar and de Klerk, 2002). In the present study, 3% sucrose was suitable for adventitious root growth in terms of biomass production. Lower concentrations ~~cannot~~ did not provide enough energy, and high ~~sucrose~~ concentrations ~~exhibited~~ negatively affected ~~in~~ root primordium~~al~~ induction.

The concentration of salts in the MS medium is an important contributor ~~significantly contributes~~ to biomass production and phytochemical accumulation in cultured cells and tissues ~~[28]~~(Rajesh *et al*., 2014). Wu *et al*. (2006)~~[29]~~ proposed that ~~the~~ interactions among ~~the~~ nutritional salts enhance the availability of ions to the roots and thereby promote~~ing~~ ~~the~~ root growth and phytochemical production. ~~The~~ In the present study, it was confirmed that the optimization of MS salt concentration is ~~very~~ essential for adventitious root production and that half-strength MS medium ~~is the best for~~results in optimal root primordia~~l~~um induction and growth in *C. amada*. The same phenomenon was ~~also~~ documented ~~in root~~when culturing roots ~~e~~ of ~~Zingiberaceae member~~ *Alpinia galanga*, also belonging to the Zingiberaceae family ~~[30]~~(Rao *et al*., 2012). Furthermore, ~~it was~~we observed ~~that when~~ increasing the MS salt strength in the medium, resulted in reduced root biomass production ~~was reduced~~. ~~It~~ This ~~indicate~~suggests that high MS salt concentration ~~promoted~~ produced ~~a~~ stress ~~condition, and~~ thereby reducing~~ed~~ 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]~~ (Dörnenburg and Knorr, 1995; Jeong *et al*., 2009; Praveen and Murthy, 2010). In *W. somnifera*, the optimal ~~level of~~ initial inoculum density is 15 g FW. ~~The increase or decrease level of~~Higher or lower inoculum densities~~y~~ inhibit~~s~~ root biomass production ~~[21]~~(Sivanandhan *et al*., 2012). In the present study, maximum~~um~~ root biomass production in *C. amada* was obtained when the inoculum density was ~~at~~ 10 g FW.

~~The i~~*In vitro*-raised adventitious roots contained higher proportions of two compounds ~~in higher proportion~~, and a similar proportion of a third compared to the proportions in the ~~one on par with~~ field-grown rhizome. This offers a new avenue for scaling up production of ~~the~~ two of the identified compounds, ~~such as~~namely isosorbide and n-hexadecaonoic acid ~~[33, 34]~~ (Rose and Palkovits, 2012; [ref 34] Isosorbide, ~~being~~ a valuable derivative of glucose, can be used as the chemical basis for the production of ~~further conversions into several chemicals like~~ green solvents, fuels, fuel additives, and so forth ~~[33]~~(Rose and Palkovits, 2012). Likewise, n-h~~H~~exadecaonoic acid is ~~also very useful~~a component in the production of cetyl alcohol, which is used in the food and cosmetics industries~~y~~ ~~[34]~~([ref 34] ~~Similar attempts have been made by other investigators [35].~~ In the present study, ~~successfully mimics~~ the levels of two useful bioactive compounds produced by field-grown ~~rhizome~~plants were successfully reproduced *in vitro*. A study in a ~~Reports~~ related species (*Cucurma*~~.~~ *longa*) ~~have~~ has achieved ~~this~~ similar~~ity~~ results; this study compared ~~between~~ *ex*

vitro plants and *in vitro* raised plants that aresubsequently established *ex vitro* [35](Singh *et al*., 2011).

In conclusion, the present investigation opens up a new avenue route for the large-scale production of two active compounds, isosorbide and n-hexanedecaonoic 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-hexadecaonoic acid production from adventitious root cultures. Furthermore, the results obtained in the present study might could be useful in further research on biotransformation and production of these secondary metabolites of *C. amada* on a large scale.

2. Material and MethodsExperimental Procedures

2.1. *Callus Induction*

Microrhizome segments were excised from 3-month-old *in vitro*-grown plants [17](Raju *et al*., 2013). For callus induction, these segments were placed on MS medium [18](Murashige and Skoog, 1962) with containing 3.0% sucrose and different either concentration of 2,4-D (1.0, 2.0, and or 3.0 mg l^{-1}) 2,4-dichlorophenoxyacetic acid (2,4-D), alone or in combination with BAP one of Kn (0.25, or 0.5 mg l^{-1}) kinetin. In all cases, Tthe medium was solidified with 0.8% agar and the its pH of the media was adjusted to 5.8 before solidification. The Mmedia were autoclaved at 121 °C and 104 kPa for 15 min. Cultures were maintained at 25 °C in a 16 hrs photoperiod with 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 different types of callus (nonfriable, semi-friable, and friable callus) was were transferred to a separate 150 ml Erlenmeyer flasks containing MS liquid medium (each in separate flask). Each flask containing MS liquid medium was supplemented with one of the following different concentrations of auxins: (0.1, 0.2, 0.3, 0.4, and or 0.5 mg l^{-1} IBA or 0.1, 0.2, 0.3, 0.4, or 0.5 mg l^{-1} NAA);, and then they were 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 period, the proportion of callus responding with root induction (%) was calculated using the following equation:

For biomass production, adventitious roots (~3.5 cm; 35 roots/flask) were transferred, in using the same mediuma composition, cultured, and and harvested during the 5th week of culture, when the biomass reached a maximum level. Based on the comparison of root length, tThe most 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 optimal culture medium for adventitious root biomass production was identified optimized 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%) for biomass production;. 250 ml Erlenmeyer flasks containing 50 ml medium were used to determine the optimal inoculum density for maximum root biomass production. For improving adventitious root biomass, The optimal inoculum density was standardized 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 Ggrowth ratio (GR) was calculated using the following equation [19](Praveen and Murthy, 2010):

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 separately subsequently air-dried for 1 hour and completely ground using pestle and mortar. Extraction was carried out by sonication with methanol (10 mL ml) until the ground root colour changed into white color by sonication. After centrifugation at 5,000 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: The GC apparatus columnused was an: Elite-5MS (5% diphenyl/95% dimethyl poly-siloxane) (Perkin Elmer)column, 30 m × 0.25 mm × 0.25 µm, with a film thickness of 0.25 µm, with a equipment: GC Clarus 500 chromatograph (both Perkin Elmer, California, USA).- The carrier gas rate was: 1 mlL per min^{-1}, split: 10-:-1. The mass- detector was a: mass detector Turbomass Ggold- (Perkin Elmer, California, USA) running software: Turbomass v5.2 software. Each injected -sample wasinjected: 2.0 µLl.

2.4.2. *Oven Temperature Programme*

Consider the following: The oven temperature programme used was as follows: 110 °C —for 2.0 min;: hold;- up to 200°C at the rate of 10 °C/ min^{-1}: increase up to 200 °C; immediate further increase no hold, up to 280 °C at the rate of 5 °C /min^{-1}:; and hold for 9.0 min. hold, 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 followsLibrary 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;- 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 were are presented as mean, means ± SE. The mean separations were analyszed by using Duncan's multiple range test, with P < [x] considered significantce level of (IBM SPSS statistics).

Author Contributions

Acknowledgements

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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~~C.~~ amada*.

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

Figure captions

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

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