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Effect of Mevalonic Acid (MVA) and Linalool as a Precursor in Enhancement of Limonene in *Citrus grandis* Osbeck Albedo Tissue Culture

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ABSTRACT

The effects of melavonic acid (MVA) and linalool as precursors in the production of limonene and linalool on *Citrus grandis Osbeck* callus tissues were investigated. MVA and linalool were used as precursors to stimulate limonene production in the biosynthetic pathway. This study proved that low concentrations of MVA (0.077 mM to 1.557 mM) and linalool (0.056 mM to 1.117 mM) were able to produce limonene when tested on callus tissues for 7 to 35 days. The aim was to determine the highest accumulation of both limonene and linalool. The highest production of limonene obtained was 0.97 ppm on day 28 when the tissues were treated with linalool and 1.50 ppm on day 35 with the addition of 0.077 mM MVA. On the other hand, linalool concentration reached a maximum of 2.88 ppm on day 7 with tissues treated with 0.077 mM MVA. As the culture period lengthened, the limonene level increased from 0.76 ppm at day 7 to 1.82 ppm on day 28, whereas linalool concentration decreased steadily from 2.88 ppm at day 7 to 1.55 ppm at day 35. This is due to the bioconversion of linalool to limonene. The best result for precursor-treated tissues was at 0.838 mM linalool, where the limonene level achieved was 0.97 ppm on day 28. The production of limonene and linalool using low precursor concentrations within a short period of time is favourable as it has good market value.

Keywords: Mevalonic acid (MVA), linalool, precursor, limonene production, *Citrus grandis Osbeck*, albedo tissue culture

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INTRODUCTION

A precursor is a compound that participates in a chemical reaction that produces another compound. Precursors are naturally-occurring compounds, intermediates originating from biosynthetic pathways or related synthetic compounds (Orlita et al., 2008). Exogenous addition of a precursor to a culture medium may enhance the production of secondary metabolites, especially alkaloid accumulation, where production is limited due to the lack of a particular precursor (Vijaya et al., 2010; Yoshida et al., 1988). This approach is advantageous if the precursors are inexpensive. Precursor feeding at low levels, especially intermediates of the biosynthetic pathway, would prove beneficial to the production of a desired product. Naturally-occurring as well as related synthetic compounds can be used as precursors. However, synthetic compounds are expensive and not safe for consumption; therefore, they should not be used in in food and drinks. Only a few studies have been found to use mevalonic acid (MVA) and linalool as precursors to increase secondary metabolite accumulation (NikNorulaini et al., 2003; Zarina, 2005). Likewise, the use of MVA and linalool as precursors for limonene and linalool production on citrus albedo tissue culture has not been widely published. The manipulation of culture conditions with precursor supplements may potentially promote limonene accumulation in *Citrus grandis* cell cultures.

Evidence from the literature suggests that limonene and linalool are biosynthesised from mevalonic acid (MVA), which is the primary precursor of all terpenoids generated from the acetate-mevalonate pathway predominantly localised in the cytosol (Zarina, 2005). Juice vesicles of Citrus sp. have an enzyme system, which is capable of phosphorylating MVA and forming both isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMPP) (Figure 1) (Mayakrishnan, 2008).

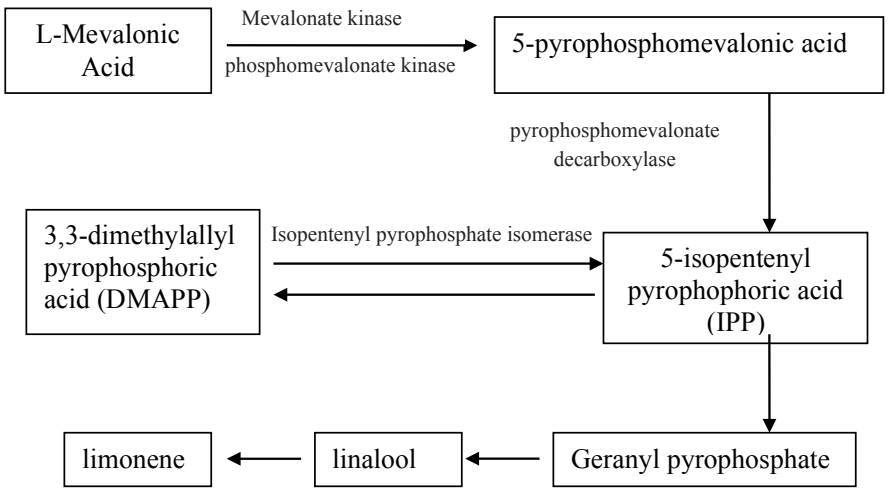

Figure 1. Schematic diagram of mevalonic acid (MVA) pathway in plants

The final product from the phosphorylation and decarboxylation-dehydration of MVA is linalool. Enzyme systems that can be found in intact fruit and leaves are responsible for the conversion of linalool to limonene (Karoui et al., 2010). Linalool thus can be considered as an immediate precursor that is directly converted to limonene. The feeding of MVA and linalool is expected to enhance the production of linalool and limonene, respectively.

Essential oil of citrus species consists of 1.85% of the total volatile oils extracted. Linalool is among the important compounds that are found in the essential oils of *Anibarosae odora* (rosewood), *Coriandrum sativum* L, *Bursera delpechiana, Citrus* spp, *Citrusa urantium sub* sp. *amara* L, *Laurus nobilis* L, *Cinnmamomun camphora, Cinnmamomun verum* L, *Matricaria chamomilla* L, *Salvia sclarea* L., *Lavandula officinalis Chaix* and *Ocimum basilicum* (Maia et al., 2006). The linalool content in the peel oils of oranges and tangerines decrease markedly in relative concentration as the fruits mature (Tounsi et al., 2011).

Limonene is the main compound in the essential oils of citrus fruits, where it occurs in concentrations of more than 90% and in pure form. It produces a pleasant aroma, which results in the essential oil being in high demand for the manufacture of perfumes and soaps, among other scented and flavoured products. Natural sources such as plants are the best alternatives for producing limonene in order to fulfil the global demand and at the same time reduce the cost of limonene production. Limonene production using citrus fruit cell cultures can be enhanced by applying a precursor in the culture media. The present study was conducted to determine the effect of precursor (MVA and linalool) concentrations on limonene and linalool accumulation in tissue culture at a certain culture period.

METHODOLOGY

Chemicals and Standards

Chemicals (analytical grade) for the Murashige and Skoog (MS) medium preparation were purchased from Sigma Chemicals, St. Louis, the United States; Koch-light Laboratory, Colbrook, Bucks, England; Fluka, Japan and Merck, Darmstadt, German. Agar was bought from Bacto Difco Laboratories, Detroit, USA. Plant growth regulators such as 2, 4-dichlorophenoxyacetic acid (2, 4-D), 6-furfurylaminopurine (kinetin), abscisic acid (ABA) and standards such as limonene and linalool (liquid form with ~97 % purity) were obtained from Fluka, Japan. Yeast extract was purchased from Becton Dickinson & Co, USA.

Cell Culture

The fruit, pomelo, (*Citrus grandis*) was obtained from a plantation in Sungai Gedong, Perak. Young fruits 4-7 cm in diameter were chosen. The spongy white rind surrounding the juice vesicle, called albedo, was used in all the studies conducted. The albedo was soaked in Clorox® (5.75%

sodium hypochlorite) in a sterile Petri dish and then cut into pieces measuring 1 cm × 1 cm × 0.5 cm before being placed in Petri dishes containing MS medium.

Precursor Preparation

Effect of linalool on tissue growth and limonene accumulation. The amount of linalool used was 10, 50, 100, 150 and 200 µL. Each sample was added to another 1 L, modified MS medium (Zarina, 2005) to obtain media with different final concentrations of linalool (0.056 mM, 0.279 mM, 0.559 mM, 0.838 mM and 1.117 mM). The linalool-added MS modified media were autoclaved at 121°C, 15 psi for 15 min after pH adjustment (pH 5.7). The media were poured into Petri plates. Albedo tissues were placed on the modified MS media once the media had cooled and hardened.

Each albedo tissue was cut into 1 cm × 1 cm × 1.5 cm, and five pieces were planted onto the modified MS medium with different linalool concentrations. Cultures were maintained in the dark at room temperature (25 ± 2°C). The five albedo tissues were pooled and analysed as a single sample.

Effect of mevalonic acid (MVA) on tissue growth and limonene accumulation. Aqueous solution of MVA was prepared by dissolving mevalonic acid lactone (Fluka, Japan) in distilled water. The solutions were prepared in concentrations of 5, 10, 50, 100, 150 and 200 mg/L and were filter-sterilised in Nalgene® disposable sterile filterware and kept as stock stored in the refrigerator at 4 ºC. Final concentrations of MVA were calculated as 0.038, 0.077, 0.384, 0.768, 1.152 and 1.537 mM. These solutions were prepared by adding the appropriate amount of sterile stock solution into different flasks of sterilised modified MS medium before the medium solidified. Each flask was swirled to mix the solution without creating any bubbles and the solution was carefully poured into Petri plates in a sterile laminar flow hood.

This study was conducted using a factorial combination of different concentrations of MVA at 0.077, 0.384, 0.768, 1.152 and 1.537 mM and culture periods of week 1 to week 5. Albedo tissue was cut into 1 cm × 1 cm × 1.5 cm pieces and planted in the medium containing MVA at the various concentrations mentioned above. Cultures were maintained in the dark at room temperature (25 ± 2°C). Cultures were pooled from five tissues and analysed as a single sample.

Tissue Growth Determination

The growth of the tissue was determined by subtracting the final weight and the initial weight of tissue grown on media within the studied incubation period. The initial weight was measured once calli were removed from the parent tissue prior transferring to modified MS media and incubated for 7, 14, 21, 28 and 35 days. After the respective incubation period, the calli were removed from the media and cleaned of excess media using tissue paper before weighing for the final weight. Five calli were measured as replicates for each incubation period.

Extraction of Limonene and Linalool

After recording the wet weight, the same tissues were used to extract limonene and linalool. A mass of 2 g of tissue was cut and chopped in preparation for Soxhlet extraction. Extraction was carried out for 2 h using methanol as the solvent. The liquid sample obtained was concentrated to 2 mL using a rotary evaporator and was also used for limonene and linalool determination.

Analysis of Limonene and Linalool

Limonene and linalool concentration was determined using gas chromatography (GC) (Brand Shimadzu, GC-17A, Japan). The operating system of the gas chromatography was carried out under several conditions. The carrier gas used was helium. The column used for chromatographic separation was Phase –HP-5 (30 m × 320 µm × 0.25 µm). The system was left to reach equilibrium state for 30 min before the samples were injected. The temperature of the transfer line and ion source was maintained at 280ºC and 250ºC, respectively. Injector temperature was set at 250ºC. The column temperature was programmed at between 60ºC and 104ºC at 4 °C/min, while the rate was changed to 6°C/min for 6 min when it reached 104°C up to 182°C.

Statistical Analysis

The statistical analysis was carried out using the Analysis of Variance (ANOVA) using the MINITAB 17 statistical software.

RESULTS AND DISCUSSION

Effect of Linalool on Tissue Growth

The change in fresh weight of the callus tissue grown in culture treated with varying concentrations of linalool can be observed in Table 1 below.

Table 1
Effect of tissue fresh weight when added with different concentrations of linalool as precursor. mean ± s.d, n=3. (No limonene was detected in control)

Culture Period (days)	Linalool Concentration in mM					
	Control	0.056	0.279	0.559	0.838	1.117
	Wet Weight of Tissue (g)					
7	0.23 ± 0.05	0.21 ± 0.08	0.11 ± 0.05	0.15 ± 0.06	0.10 ± 0.08	0.07 ± 0.04
14	0.12 ± 0.04	0.21 ± 0.04	0.18 ± 0.05	0.18 ± 0.05	0.18 ± 0.01	0.24 ± 0.04
21	0.18 ± 0.01	0.23 ± 0.06	0.20 ±0.03	0.20 ± 0.02	0.19 ± 0.06	0.28 ± 0.03
28	0.91 ± 0.03	0.25 ± 0.05	0.22 ± 0.05	0.16 ± 0.07	0.27 ± 0.05	0.22 ± 0.05
35	1.12 ± 0.02	0.21 ± 0.01	0.25 ± 0.05	0.20 ± 0.02	0.28 ± 0.01	0.14 ± 0.04

*Control - Without Additional Linalool

The change in tissue wet weight with relation to linalool concentration in the media depends on the number of days in culture. Early culture period, at 7 days, showed that the control culture promoted growth, while linalool inhibition increased with concentration. With progressive days in culture up to 21 days, the tissue samples without linalool experienced a decrease in wet weight; this was in contrast to the increase in weight that was evident in cultures with linalool. However, feeding the callus with a high concentration of linalool did not induce any significant tissue growth ($p>0.05$). It was apparent that the presence of high linalool (1.117 mM) exerted a negative effect on tissue growth as the culture days extended beyond 21 days. The wet weight of the control culture was more than triple that of the linalool-treated cultures at 28 and 35 days in culture, after a drop at 14 days that later rose again. Tissue in cultures with 0.279, 0.559 and 0.838 mM linalool saw its wet weight steadily increase with more days in culture but the net weight did not meet the higher weight of the control tissue (without linalool). Adding linalool as a precursor led to an inhibition of tissue growth. The inhibition of tissue growth in the presence of linalool was consistent for all the tested concentrations. Statistically, no significant ($p>0.05$) difference in tissue growth was observed for the cultures added with different concentrations of linalool (Appendix 1A).

Slow growth of precursor-treated tissue may reflect an unadapted culture prone to necrosis, leading to stressed conditions suitable for the onset of secondary metabolism (Oswald et al., 2007). Although no necrosis was observed in the studies, the albedo tissue samples showed slow or retarded growth rates indicating stressed conditions suitable for a switch from primary to secondary metabolism.

Linalool in this experiment was shown to be a tissue growth suppressor or a cell division suppressor. The treated albedo tissue samples appeared to be under a prolonged lag phase, unlike untreated or control tissue. During lag phase, little or no cell division takes place. However, this does not mean that the cells are dormant, as the cells are undergoing a period of intense metabolic activity involving DNA and enzyme synthesis (Tortora et al., 1995).

The control tissue growth followed a typical lag phase up to 21 days in culture, followed by a 7-day log or exponential growth from 21 to 25 days; after that, the growth slowed down. Cellular production is most active during the log phase, when cells are metabolically active. Since only the control tissue reached this stage, it can be concluded that only untreated tissue undergoes primary metabolism or cell division.

After the 28th day, cell growth slowed as it reached the stationary phase due to nutrient exhaustion, accumulation of waste products and harmful changes including pH of the culture media. Removing tissue from spent media and providing fresh media can overcome this problem. Thus,

the albedo tissue samples were transferred to fresh media after 28 days to move past the stationary phase in this study.

The findings on hampered growth of linalool-treated albedo tissue were consistent with a study conducted by Engels et al. (2008) on anthraquinones production, where high levels of secondary metabolite production inhibited culture growth.

Effect of Linalool on Limonene Accumulation

Linalool is an immediate precursor to limonene and its presence in a medium is expected to trigger the conversion of linalool to limonene under favourable conditions as reported by NikNorulaini et al. (2003). As evident from Table 2 below, the presence of linalool influenced the production of limonene in the tissue samples.

Table 2
Effect of various concentrations of linalool on the accumulation of limonene in albedo tissue culture. mean ± s.d, n=3. (No limonene was detected in control)

Culture Period (days)	Linalool Concentration in mM				
	0.056	0.279	0.559	0.838	1.117
	Limonene Concentration in mM				
7	0.42 ± 0.06	0.49 ± 0.05	0.34 ± 0.01	0.36 ± 0.03	0.54 ± 0.04
14	0.48 ± 0.09	0.59 ± 0.06	0.45 ± 0.02	0.47 ± 0.03	0.62 ± 0.01
21	0.68 ± 0.10	0.64 ± 0.02	0.63 ± 0.05	0.68 ± 0.04	0.72 ± 0.05
28	0.81 ± 0.10	0.83 ± 0.05	0.72 ± 0.05	0.97 ± 0.05	0.66 ± 0.02
35	0.70 ± 0.11	0.88 ± 0.01	0.65 ± 0.04	0.72 ± 0.05	0.43 ± 0.06

*Control - Without Additional Linaloo1

Different concentrations of linalool acting as precursor to limonene showed an increase of limonene concentration in all the tissues except for the control, which did not produce any limonene at all. The increase was steady for all the samples up to day 21. On day 28, further increase was obtained for all the cultures, except for the culture with 1.117 mM linalool added. The highest limonene accumulation, 0.97 ppm, was obtained on the 28th day of the tissue samples cultured in 0.838 mM linalool. Tissue samples induced with 0.838 mM linalool showed an increase from 0.68 ppm (day 21) to 0.97 ppm (day 28) before decreasing slightly to 0.72 ppm on day 35. The difference in linalool concentration feeding showed a statistically significant ($p<0.05$) value in the limonene accumulation (Appendix 1B). Adding a precursor to the MS media induced the production and accumulation of limonene up to a certain period before the amount showed a decline. Table 2 also shows the presence of limonene in all the tissue samples grown in media supplemented with linalool for all the concentrations used.

The fresh tissue weight of the albedo tissue did not increase in linalool-added media as much as in the control. Conversely, the limonene concentration rose steadily, while no limonene was detected in the control tissue samples (Table 2).

Zarina (2005) demonstrated that adding linalool at 0.838 mM to callus cultures of *C. grandis* increased limonene production rate from less than 5 x 10^{-5} mg/g/day at day 21 to more than 80 x 10-^{5} mg/g/day at day 49. The rate increased rapidly around the 45th day of the culture period. However, there was no significant increase in callus fresh weight within the same culture period. Supplementing metabolic precursors has proven to be effective in increasing concentrations of desired secondary metabolites. For example, an immediate precursor of vanillin, ferulic acid, was known to affect accumulation of flavour compounds of cultured *Vanilla planifolia* cells (Karoui & Marzouk, 2013). In contrast, Sarfaraj et al. (2012) found that *C. grandis* callus cultures that were not supplemented with any precursor produced limonene only after 10 months of the culture period; as this study revealed, no limonene was detected in untreated albedo tissue up to day 35.

Secondary metabolites, such as limonene in this study, are not products of single genes but are the by-product of metabolism due to multistep and multi-enzyme processes. Changes in the activity of one enzyme or intermediate like exogenous linalool often results in the simultaneous up-regulation of some enzymes and down-regulation of other enzymes in the same and parallel pathways. The production of other metabolites may be enhanced or reduced and thus may alter the overall product profile (Federica, 2012).

Effect of MVA on Tissue Growth

Table 3 compares the growth of tissue grown on modified MS media using different concentrations of MVA. Comparison of tissue fresh weight was determined every 7 days from day 7 till day 35. Exogenous MVA caused a decrease in tissue growth for all the tissues studied. As the days increased, tissue weight also increased steadily. The highest tissue weight of 0.64 g was achieved without the addition of MVA at day 35. The highest tissue fresh weight added with MVA was obtained with 0.077 mM of MVA after 35 days. Addition of 1.537 mM of MVA increased the tissue fresh weight slowly for 2 weeks before the weight surged drastically. The feeding of different MVA concentrations did not give any significant ($p>0.05$) change to tissue wet weight (Appendix 1C).

Table 3
Effect of tissue fresh weight when added with different concentrations of mva as a precursor. mean ± sd, n=3

Culture Period (days)	MVA Concentration in mM					
	Control	0.077	0.384	0.768	1.152	1.537
	Wet Weight of Tissue (g)					
7	0.09 ± 0.02	0.12 ± 0.04	0.09 ± 0.02	0.12 ± 0.07	0.13 ± 0.07	0.07 ± 0.03
14	0.25 ± 0.05	0.14 ± 0.05	0.16 ± 0.02	0.19 ± 0.03	0.24 ± 0.09	0.11 ± 0.02
21	0.38 ± 0.06	0.24 ± 0.08	0.23 ± 0.03	0.24 ± 0.05	0.32 ± 0.12	0.36 ± 0.13
28	0.51 ± 0.08	0.31 ± 0.07	0.32 ± 0.05	0.33 ± 0.08	0.38 ± 0.05	0.39 ± 0.14
35	0.64 ± 0.08	0.46 ± 0.04	0.44 ± 0.04	0.45 ± 0.08	0.39 ± 0.13	0.39 ± 0.12

*Control - Without Additional MVA

The highest increase in tissue wet weight was observed after 28 days of culture for all the tissues. The greatest increase was observed in tissue cultured in 0.077 mM MVA, which was 0.02 g/day during the last 7 days in culture (from day 28 to day 35). However, this was equivalent to reduced wet weight by 18% in tissue cultured without MVA (control). All the tissue cultured in the presence of MVA was subject to slower growth and the least increase of weight was in tissue cultured with the highest MVA concentration, 1.537 mM. Primary metabolism occurred during the whole culture period for MVA-added tissue. A study by Vanisree et al. (2004) claimed that protein synthesis took place during primary metabolism, thus increasing tissue growth. Imbault et al. (1996) also found that addition of MVA led to cytokinin synthesis, which functions to promote cell division and elongation. They treated *Catharanthus roseus* with 1 mM MVA and found that this concentration improved culture growth prior to inhibition with pravastatin.

Effect of MVA on Limonene and Linalool Accumulation

The effect of MVA on limonene production was measured by the accumulation of limonene in callus tissue grown in media supplemented with different concentrations of MVA. The data are shown in Table 4. Concentration of MVA played a defined role in the final accumulation of limonene. The concentration of 0.077 mM MVA produced the highest accumulation of limonene throughout the culture periods, achieving 1.50 ppm after 35 days in culture. The second most abundant accumulation of limonene was seen in tissue grown with the addition of 0.384 mM of MVA at day 35, which was 1.27 ppm. Statistical analysis using the Analysis of Variance (ANOVA) test revealed that there was statistically significant ($p<0.05$) accumulation of limonene in cultures (Appendix 1D), with 0.077 mM of MVA producing the highest limonene accumulation compared to the other concentrations used.

Table 4
Effect of various concentrations of mva on the accumulation of limonene in tissue culture. mean ± sd, n=3. (No limonene was detected in control)

Culture Period (days)	MVA Concentration in mM				
	0.077	0.384	0.768	1.152	1.537
	Limonene Concentration in ppm				
7	0.59 ± 0.08	0.50 ± 0.05	0.32 ± 0.04	0.32 ± 0.03	0.17 ± 0.07
14	0.83 ± 0.04	0.79 ± 0.05	0.30 ± 0.06	0.34 ± 0.07	0.30 ± 0.06
21	1.12 ± 0.12	0.90 ± 0.06	0.48 ± 0.06	0.35 ± 0.08	0.38 ± 0.05
28	1.23 ± 0.09	1.12 ± 0.09	0.64 ± 0.03	0.5 ± 0.09	0.45 ± 0.07
35	1.5l ± 0.07	1.27 ± 0.09	0.7 ± 0.07	0.66 ± 0.14	0.71 ± 0.06

*Control - Without Additional MVA

Generally, limonene was detected from day 7 in tissue culture and it increased in production for all the MVA concentrations studied. Cultures without exogenous MVA did not produce any detectable amount of limonene.

Table 5 shows linalool concentrations gradually decreased in all the cultures after 7 days added with MVA. The results demonstrated that linalool production in *C. grandis* was triggered by the introduction of MVA at low concentration. This finding was supported by that of Zarina et al. (2005), who reported that precursor feeding had triggered limonene production in *C. grandis* cultures earlier than in the non-fed cultures. In comparison, the linalool concentrations slowly increased to about 1.25 ppm after a month in the control culture (not supplemented with MVA). The presence of MVA in the medium was able to first trigger a hike in the amount of linalool compared to in the untreated media as early as day 7. However, the amount was reduced for all MVA treatments a week in culture; this was in contrast to the limonene profile (Table 4). Using 0.077 mM of MVA resulted in the highest linalool accumulation, showing statistically significant ($p<0.05$) (Appendix 1E) readings throughout the culture period even though the linalool concentration was seen to decrease from day 7 to day 35 for all the MVA concentrations studied. Tissue samples without the addition of exogenous MVA (control) showed a different trend, where linalool was observed to increase from 0.55 ppm on day 7 to 1.25 ppm on day 35. The rest of the tissue samples all seemed to decline in linalool production for all the MVA concentrations added.

Table 5
Effect of various concentrations of mva on the accumulation of linalool in tissue culture. mean ± sd, n=3

Culture Period (days)	MVA Concentration in mM					
	Control	0.077	0.384	0.768	1.152	1.537
	Linalool Concentration in ppm					
7	0.55 ± 0.12	2.88 ± 0.23	2.44 ± 0.31	2.23 ± 0.31	1.97 ± 0.24	1.53 ± 0.13
14	0.7 ± 0.06	2.32 ± 0.27	2.12 ± 0.23	1.90 ± 0.18	1.55 ± 0.10	1.15 ± 0.12
21	0.85 ± 0.09	2.07 ± 0.24	1.87 ± 0.24	1.76 ± 0.28	1.52 ± 0.13	0.86 ± 0.10
28	0.9 ± 0.11	1.97 ± 0.12	1.58 ± 0.31	1.56 ± 0.21	1.21 ± 0.43	0.94 ± 0.08
35	1.25 ± 0.08	1.55 ± 0.23	1.31 ± 0.12	1.46 ±0.13	0.83 ± 0.10	0.79 ± 0.14

*Control - Without Additional MVA

Linalool accumulation in tissue grown in cultures with MVA gradually decreased after day 7 (Table 5). The decrease of linalool happened when MVA concentration was increased because only low concentrations of MVA feeding promoted linalool accumulation. High concentrations of MVA caused linalool accumulation in cells, and this increased toxicity and led to cell fatality (Brown et al., 1987; Charlwood & Brown, 1987). A small amount of secondary metabolite formation in the cultures despite the presence of MVA may have been due to alternative biosynthetic pathways present, as found by Oswald et al. (2007). They also noted that a complete absence of or low enzyme activity before MVA (for example HMG-coenzyme A reductase) was added might have ceased the biosynthetic pathway for limonene/linalool production. For both treatments, it could be concluded that limonene production was not dependent on the amount of precursors added. It showed that only a small amount of MVA and linalool was needed as a stimulant for limonene production.

CONCLUSION

Mevalonic acid (MVA) and linalool proved to be good precursors in triggering limonene production in the callus tissues of *Citrus grandis Osbeck*. However, tissue culture cell growth prevailed over the secondary metabolite production in untreated tissue. Tissue cultured on linalool-added media displayed increased production of the secondary metabolite limonene, despite lower gains in wet weight that was indicative of slower cellular growth. A similar observation was made on tissue cultured on exogenous MVA-added media. Adding MVA to the media raised the concentration of MVA in the tissue cells, and this triggered the subsequent series of reactions in the pathway, leading to higher linalool concentration and eventually, limonene synthesis. The outcome of precursor addition on linalool or limonene accumulation depended on the age of the culture, which is inadvertently linked to the precursor uptake period and assimilation into the metabolic pathway. Accumulation of linalool precedes limonene production since

linalool is the intermediate to limonene. As the culture ages, limonene has a tendency to be converted to other substances, and this reduces the limonene concentration in the tissue. The highest linalool production in culture for limonene, 2.88 ppm and 1.5 ppm, were obtained using 0.077 mM of MVA at day 7 and day 35, respectively. Manipulation of the medium by addition of exogenous precursors to stimulate relatively high productivity of the secondary metabolites showed a successful example of the plant cell culture technique. Limonene and linalool have good market value, so reducing their production by addition of a low concentration of precursor is very beneficial. In addition, monoterpene hydrocarbon limonene has been widely used as a starting product for bioconversions into flavour and scented compounds, thus increasing its demand.

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APPENDICES

Appendix 1A
Tissues wet weight versus linalool concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
Linalool Conc.	5	0.4185	0.08369	2.15	0.094
Error	24	0.9360	0.03900		
Total	29	1.3545			

Appendix 1B
Limonene accumulation versus linalool concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
Linalool Conc.	5	1.6439	0.32878	13.42	0.000
Error	24	0.5878	0.02449		
Total	29	2.2317			

Appendix 1C
Tissues wet weight versus MVA concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
MVA conc.	5	0.05539	0.01108	0.48	0.787
Error	24	0.55224	0.02301		
Total	29	0.60763			

Appendix 1D
Limonene accumulation versus MVA concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
MVA conc.	5	3.648	0.72967	14.19	0.000
Error 0	24	1.234	0.05140		
Total	29	4.882			

Appendix 1E
Linalool accumulation versus MVA concentration
Analysis of Variance

Source	DF	SS Adj	Adj MS	F-Value	P-Value
MVA conc.	5	6.354	1.2709	8.80	0.000
Error	24	3.466	0.1444		
Total	29	9.820			