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Comparative Analysis on the Role of 2,4-dichlorophenoxyacetic Acid in the Expression of Bioactive Compounds in Callus of *Capsicum frutescens*

(Analisis Perbandingan Peranan Asid 2,4-diklorofenoksiasetik dalam Pengekspresan Sebatian Bioaktif dalam Kalus *Capsicum frutescens*)

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ABSTRACT

Plant cell culture technology serves as an effective alternative system for *in vitro* production of bioactive molecules, as it allows for the exploration of valuable compounds under a controlled environment. The present study was conducted to evaluate the effect of plant growth regulator (PGR); 2,4-dichlorophenoxyacetic acid (2,4-D) on the expression of compounds in coloured callus of *Capsicum frutescens*, a vital spice in various cuisines worldwide. The differential accumulation of compounds in the callus was analysed using liquid chromatography-mass spectrometry (LCMS) and the PGR concentration that resulted in the highest accumulation of the valuable compounds was identified. In this study, calli of various colours (cream, yellow and green) were successfully produced from *C. frutescens* through plant tissue culture. The increase in 2,4-D concentrations was found to increase callus growth index (GI) and specific growth rate (S_g), where the highest GI (0.5690) and S_g (0.6348 mg/week) were observed in callus produced in media supplemented with 0.5 mg/L 2,4-D. LCMS data analyses showed that 19 compounds were detected in the callus, with 8 compounds (fatty acids and phenolics) were successfully identified, while the remaining 11 compounds were reported as unknowns. Yellow-coloured callus was observed to contain the highest number of compounds (18 compounds), while green callus contained the least (14 compounds). This analysis provides valuable information on the application of biotechnological tools such as plant tissue culture as an alternative for sustainable production of compounds with high bioactivity in *Capsicum frutescens*.

Keywords: 2,4-dichlorophenoxyacetic acid; *Capsicum frutescens*; compounds; LCMS; plant growth regulator

ABSTRAK

Teknologi kultur sel tumbuhan berfungsi sebagai sistem alternatif yang berkesan untuk menghasilkan molekul bioaktif secara *in vitro* kerana ia membolehkan penerokaan sebatian berharga dijalankan di bawah persekitaran yang terkawal. Kajian ini dijalankan untuk menilai kesan pengatur pertumbuhan tumbuhan (PGR); asid 2,4-diklorofenoksiasetik (2,4-D) ke atas pengekspresan sebatian dalam kalus berwarna *Capsicum frutescens* yang merupakan rempah penting dalam pelbagai masakan di seluruh dunia. Perbezaan kandungan sebatian dalam kalus tersebut dianalisis menggunakan kromatografi cecair spektrometri jisim (LCMS) dan sukatan PGR yang menghasilkan kandungan sebatian yang tertinggi telah dikenal pasti. Dalam kajian ini, kalus pelbagai warna (krim, kuning dan hijau) berjaya dihasilkan daripada *C. frutescens* melalui kultur tisu tumbuhan. Peningkatan sukatan 2,4-D didapati meningkatkan indeks pertumbuhan kalus (GI) dan kadar pertumbuhan khusus (S_g) dengan kalus yang dihasilkan dalam media yang ditambah dengan 0.5 mg/L 2,4-D didapati menunjukkan GI (0.5690) dan S_g (0.6348 mg/minggu) yang paling tinggi. Analisis data LCMS

pula menunjukkan bahawa 19 sebatian telah dikesan dalam kalus tersebut dengan 8 sebatian (asid lemak dan fenolik) berjaya dikenal pasti, manakala 11 sebatian selebihnya dilaporkan sebagai tidak diketahui. Kalus berwarna kuning didapati mengandungi jumlah sebatian tertinggi (18 sebatian), manakala kalus hijau mengandungi jumlah sebatian yang paling sedikit (14 sebatian). Analisis ini telah memberi maklumat berharga tentang aplikasi teknik bioteknologi seperti kultur tisu tumbuhan sebagai alternatif untuk penghasilan sebatian dengan bioaktiviti tinggi dalam *Capsicum frutescens* secara mapan.

Kata kunci: Asid 2,4-diklorofenoksiasetik; *Capsicum frutescens*; LCMS; pengatur pertumbuhan tumbuhan; sebatian

Introduction

Capsicum is a vital spice used as the basic ingredient in a great variety of cuisines worldwide. It is also used as a flavouring agent, colourant, and adds tang and taste to the otherwise insipid food. As a part of a nightshade family, *Capsicum* consists of around 20 to 27 species but only 5 species are used domestically which are *C. annuum*, *C. baccatum*, *C. chinens*e, *C. frutescens,* and *C. pubescens* (Csilléry 2006). One of the most popular species in Malaysia and Southeast Asia is the bird's eye chilli (*C. frutescens*) due to its fruity taste and high spiciness.

Due to its importance in the food industry, most of the chemical compounds in *Capsicum* have already been characterised. It is rich in esters, carotenoids, steam-volatile oil, fatty oils, capsaicinoids, vitamins, protein, fibre, and mineral elements which contributed to its bright colours and distinct taste (Bosland et al. 2012). In particular, it contains high vitamin C (as much as seven times than orange), A, B-complex and E as well as other minerals like molybdenum, manganese, folate, potassium, and thiamine (Chuah et al. 2008; Kantar et al. 2016; Martínez et al. 2005; Materska & Perucka 2005). Not only that, the beta-carotenoids and vitamins C and A in chillies are also powerful antioxidants that annihilate free radicals (Alam et al. 2018). These compositions could confer many health benefits, including anti-mutagenic, and anti-tumour-promoting activities (Aboul-Enein et al. 2012). Additionally, it has been used for inducing weight loss (Varghese et al. 2017), treating low-grade inflammation (Qin et al. 2017), controlling blood cholesterol (Dubey 2017) and improving the function of the digestive system (Sachan et al. 2018). Topical capsaicin has been shown to have a safe analgesic effect against many painful conditions such as burning mouth syndrome (Jørgensen & Pedersen 2017), idiopathic trigeminal neuralgia (Fusco & Alessandri 1992), neuropathic pain (Raber et al. 2015) and osteoarthritis (Guedes et al. 2018).

Plants serve as one of the prominent sources of natural drugs, with around 80% of the global population still depending on herbal or traditional medicine to treat various health conditions (Parsaeimehr et al. 2017; Okoye et al. 2014; Veeresham 2012). Plant-derived compounds are often preferred due to their safety, stability and affordability (Gupta et al. 2017). In order to cater to its increasing demand, plant cell culture technology has served as an effective alternative system for *in vitro* production of bioactive molecules, as it allows for the exploration of valuable bioactive compounds under a controlled environment. In terms of product harvest, the *in vitro* method is more rapid, efficient, continuous and purer as it reduces interfering compounds, compared to extraction from field-grown plants.

Plant growth regulators (PGR) function to alter plant growth, trigger cell differentiation and modulate the plant's physiological response (Khan et al. 2020; Luczkiewicz et al. 2014). One of the important PGRs; auxin, plays an essential role in plants' injury response, growth and development, apical dominance, flowering and ethylene biosynthesis (Balla et al. 2016; Higashitani 2013; Reid & Ross 2011; Zemlyanskaya et al. 2018). At high concentrations, auxin was reported to induce callus, while low concentration stimulates root initiation. Synthetic auxins such as α- naphthaleneacetic acid (NAA), 2,4-dicholorophenoxyacetic acid (2,4-D) and para-chlorophenoxyacetic acid (p-CPA) are usually used in plant tissue culture in combination with cytokinin to induce the growth of callus (Bhojwani & Dantu 2013). Although there are multiple studies dealing with *in vitro* regeneration of the capsicum species (do Rêgo et al. 2016; Hegde et al. 2017; Renfiyeni & Trisno 2017), very few reports were found on the effects of a synthetic auxin, 2,4-D, on the induction of callus and its impact on bioactive metabolites in *C. frutescens*. For example, Johnson et al. (1991) reported that elicitation with curdlan and xanthan yielded significantly higher amounts (by 1.8 – 2-fold) of capsaicin in immobilized callus cells of *C.*

frutescens supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin. Therefore, in this study, different concentrations of 2, 4-D were used to induce the formation of coloured callus from *C. frutescens* and its effects on the production of certain bioactive metabolites of interest were also evaluated. This study would provide valuable insight into the effect of 2, 4-D on *in vitro* callogenesis of *C. frutescens* and its potential use for the production of valuable bioactive metabolites.

Materials and Methods

Preparation of samples and tissue culture media

The seeds of *Capsicum frutescens* (bird's eye variety) were purchased from a local supplier (Bijih Benih Serbajadi). The seeds were sterilized by soaking in these solutions in the following order: tap water (1 h), sterile distilled water added with Tween-20 (3 min), 70% Chlorox with Tween-20 (3 min), sterile distilled water (3 min), 50% Chlorox (3 min), sterile distilled water (3 min), 30% Chlorox (3 min), sterile distilled water (3 min), 10% Chlorox (3 min) and sterile distilled water (3 min). Then, the seeds were transferred into new sterile tubes and were soaked with 70% alcohol for 1 min and in sterile distilled water for 1 min (3 times), followed by drying using sterile tissues. Then, the seeds were cultured in glass jars containing MS (Murashige & Skoog 1962) media without hormones and kept in the incubation room at 25 °C under 16/8 h photoperiod of 1000 lux light intensity provided by white fluorescent tubes with 55% relative humidity. The resulting *C. frutescens* plantlets were harvested and used as explants for callus initiation. The explants; 0.8 cm × 0.8 cm leaves (adaxial-side-up) and 1.0 cm stem were excised and cultured on the MS media supplemented with 0.1, 0.3 or 0.5 mg/L 2,4-D to induce production of callus. The cultures were also kept in the incubation room at 25 °C under 16/8 h photoperiod of 1000 lux light intensity provided by white fluorescent tubes with 55% relative humidity.

The MS media were prepared as follows (per litre): 4.4 g MS with vitamins powder, 30 g of sucrose as the carbon source and 8 g of agar as the gelling agent. For callus induction media, 0.1, 0.3 or 0.5 mg/L 2,4-D was also added. The pH of the media was adjusted to 5.8 using 0.1M NaOH or 0.1M HCl before being autoclaved at 121 °C for 20 min. After sterilization, the media were transferred into glass jar for seed culture and to sterile tube for callus induction.

Callus scoring and callus induction efficiency

Observation of the proliferation rate of callus was recorded using the scoring method of: '-' no callus, '+' meagre (0.5-1.0 cm width), '++' moderate (1.0 - 1.5 cm width of callus) and '+++' intense (> 1.5 width of callus). The colour of the callus was also recorded. After 30 days of culture, the callus was sub-cultured and the induction rate was calculated based on the formula described below (Sahraroo et al. 2014):

$$\text{Callus induction rate (\%)} = \frac{\text{number of explants produced callus}}{\text{total number of explants cultured}} \times 100\%$$

While the growth index (GI) of the callus was calculated after 9 weeks of culture based on the formula described below (Sahraroo et al. 2014):

$$\text{Growth index, GI} = \frac{F_{\text{m}} - I_{\text{m}}}{I_{\text{m}}}$$

where, F_m is the final mass of callus and I_m is the initial mass of callus.

The specific growth rate (S_g) of callus production in this study was also calculated. It is defined as the rate of increase of biomass of a cell population per unit of biomass concentration, usually following a sigmoidal curve where it occurs between the lag and stationary phases (where cell growth would follow a straight line). The weight of the callus before and after freeze-drying was measured and used to determine the specific growth rate (S_g) by using the equation as described below (Bhatia 2015):

$$\ln F_m = S_g t + \ln I_m$$

$$S_g = (\ln F_m - \ln I_m)/t$$

where, S_g is the specific growth rate, F_m is the biomass at time t and I_m is the initial biomass.

LCMS-based determination of bioactive compounds

Bioactive compounds from freeze-dried stem-derived callus samples were extracted with a 1:1 ratio of water:methanol, where the samples were soaked in the solution and shaken for 10 min before being filtered

with a 0.22 µM nylon filter. The LCMS used in this study consisted of AB Sciex 3200QTrap LCMS/MS. In short, 20 µL of the extract was injected into an Agilent Zorbax C18 column (4.6 mm × 15 cm, 5 µm) with two buffer systems: (A) 0.1% formic acid and 5 mM ammonium formate and (B) acetonitrile with 0.1% formic acid and 5 mM ammonium formate, for a total of 15 min running time. The system was ran using a gradient setting of 10% B to 90% B from 0.01 min to 10 min, hold for 2 min and back to 10% B in 0.1 min and re-equilibrated for 3 min. For MS settings, negative ionisation mode was used to detect a mass range from m/z 100 to m/z 1200, with an ionisation voltage of -4500 V, drying temperature at 500 °C, nebulization gas at 40 psi, collision voltage at 35 eV +/- 15 eV and declustering potential at 40 V. Identification of the compounds was made by comparing the MS/MS spectrum with that in the in-house database.

STATISTICAL ANALYSIS

Data was analysed using ANOVA in IBM SPSS Statistics version 24 and the post-hoc analysis (Duncan's multiple range test or DMRT) was conducted to determine the significant difference between the group means.

RESULTS

The initial observation indicated that the *C. frutescens* seeds started to produce shoots and roots after 14-15 days of culture on MS media without hormones. After 21 days of culture, 85% of the seeds were found to produce shoots while 88% produced roots. The leaf and stem of the resulting seedlings were excised and used to initiate the production of callus on the MS media added with 0.1 - 0.5 mg/L 2,4-D. It was observed that the use of *C. frutescens* leaves as the explant source resulted in poor callus formation (data not shown) while the use of stem explants resulted in the production of callus of various colours (Figure 1). Initiation of callus production was observed to occur on the stem explants after about 27-28 days of culture (Table 1) and the addition of 0.3 mg/L 2,4-D to the media was observed to result in the highest callus induction rate (67 ± 0.8%) (Table 1). This is in contrast to the control (MS basal), which resulted in no production of callus.

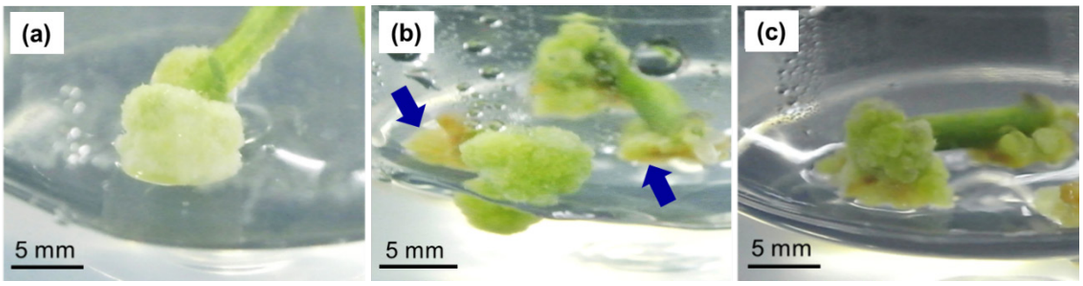

FIGURE 1. (a) Cream callus produced from cultures supplemented with 0.1 mg/L 2,4-D, (b) mixture of yellow and green callus produced from cultures supplemented with 0.3 mg/L 2,4-D and (c) green callus produced from cultures supplemented with 0.5 mg/L 2,4-D

TABLE 1. Effects of 2,4-D on induction of callus from stem explants of *C. frutescens*

Concentration of 2,4-D (mg/L)	No of days taken to initiate callus production	Callus induction rate (%)	Observation
0.0	N/A	ND	N/A
0.1	27 ± 1[a]	58 ± 0.6[b]	Cream coloured callus
0.3	28 ± 1[a]	67 ± 0.8[c]	Yellow + green callus
0.5	27 ± 1[a]	50 ± 0.2[a]	Green callus

*Mean values with different letters within a column are significantly different at $p < 0.05$. N/A: not available, ND: not detected

The GI and S_g of the callus were also determined to evaluate the efficiency of 2,4-D as a callus-inducing plant growth regulator. Based on the results (Table 2), it can clearly be observed that supplementation of 0.5 mg/L 2,4-D to the media resulted in the highest value of callus fresh and dry weights. The increase in 2,4-D concentrations was also observed to result in the increase in callus GI and S_g, where the highest GI (0.5690) and S_g (0.6348 mg/week) was observed in callus produced in media supplemented with 0.5 mg/L 2,4-D (Table 2). This indicates that the use of higher 2,4-D concentration is beneficial for the production of callus cells from *C. frutescens*.

TABLE 2. Effects of 2,4-D on callus yield (fresh weight, dry weight, growth index and specific growth rate) from stem explants of *C. frutescens*

Concentration of 2,4-D (mg/L)	Mean fresh weight (mg)	Mean dry weight (mg)	Growth index (GI)	Specific growth rate, S_g (mg/week)
0.0	ND	ND	N/A	N/A
0.1	393.2 ± 5.2 [a]	0.0325 ± 0.01[a]	0.4150	0.0983
0.3	418.1 ± 0.5 [a]	0.0335 ± 0.01[a]	0.4480	0.1051
0.5	449.9 ± 2.6 [a]	0.0406 ± 0.01[a]	0.5690	0.6348

*Mean values with different letters within a column are significantly different at $p < 0.05$. N/A: not available, ND: not detected

The freeze-dried coloured callus was extracted using methanol and subjected to an LCMS analysis to determine its metabolite composition. Figure 2 shows the LCMS chromatograms of the samples whereas Table 3 shows the major chemical compound constituents present in the extracts. The extracts were found to contain several significant chemical compounds, of which, many were found to be present in all treatments (Figure 3). However, no presence of caffeoyl glucose conjugate was observed in the extract from callus produced with 0.5 mg/L 2,4-D (Figure 2 & Table 3). There were also 11 unknown compounds detected in the samples, whereby 4 out of 11 compounds were not present in the green callus produced on MS media supplemented with 0.5 mg/L 2,4-D (Table 3 & Figure 3).

As demonstrated by the chromatograms in Figure 2 and frequency map in Figure 3, there were four peaks of interest at retention times between 3.012 - 3.140 min (U_9; m/z of 529), 5.350 - 5.502 min (U_{11}; m/z of 723), 6.553 min (U_{10}; m/z of 714) and 10.897 - 11.155 min (C_6; m/z of 325). Each of the peaks of interest was chosen based on their presence or shift in the intensity as different concentrations of 2,4-D were used. The first peak (3 min) was an unknown compound that could be found abundantly when the callus was supplemented with 0.3 & 0.5 mg/L 2,4-D, which might be responsible for the yellow callus observed in 0.3 mg/L of 2,4-D. The second peak (5.4 min) was also an unknown compound and was significantly abundant in the cream-coloured callus (produced with 0.1 mg/L 2,4-D) (Figure 2). The third peak (6.553 min) was only present in the green callus extract produced with 0.5 mg/L 2,4-D (Figure 3). Additionally, treatment with 0.5 mg/L 2,4-D also caused a significant presence of a compound at 11.15 min, which was identified as octadecadienoic acid.

DISCUSSION

PGRs play a vital role in callus initiation and development. In this study, callus was found to be induced using stem explants of *C. frutescens* in all different concentrations of 2,4-D. However, the callus induction rate, as well as the callus colour, differed with the concentrations of 2,4-D. Similarly, the addition of 0.5 mg/L 2,4-D in the MS media was found to result in the highest callus induction in *Garcinia schomburgkiana*, with a callus induction rate of 50% from stem explants and 79.17% from leaf explants (Suwanseree et al. 2019). However, the callus colour remains unchanged at different 2,4-D concentrations, unlike in the current study. According to Adil et al. (2019), the change of colours in the calli indicated cell activity during cell division. In another

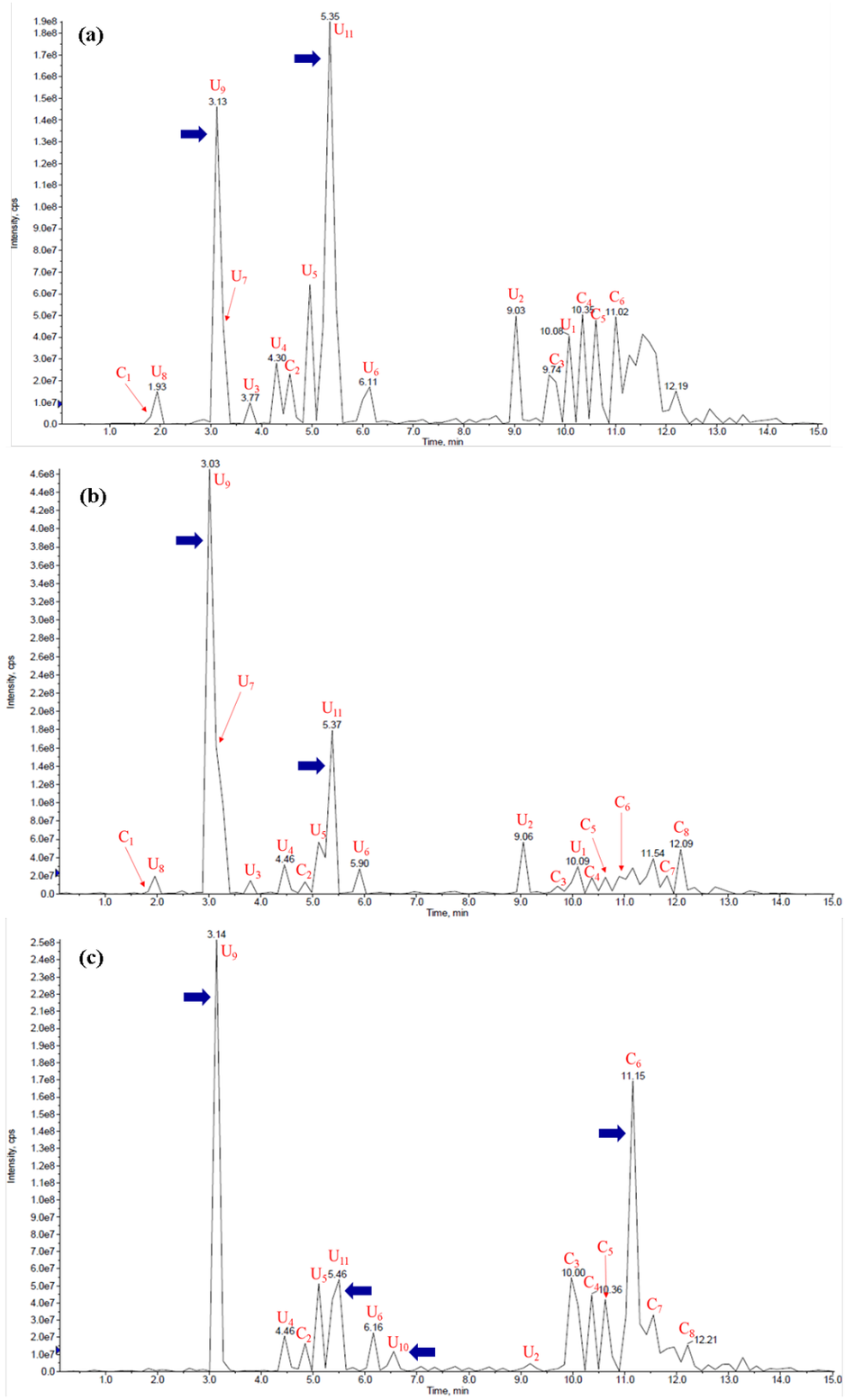

FIGURE 2. LCMS chromatograms of callus extracts from cultures supplemented with (a) 0.1 mg/L, (b) 0.3 mg/L and (c) 0.5 mg/L 2,4-D. Blue arrows indicate the three peaks of interest, which show differential expression of compounds resulted from the different PGR treatments

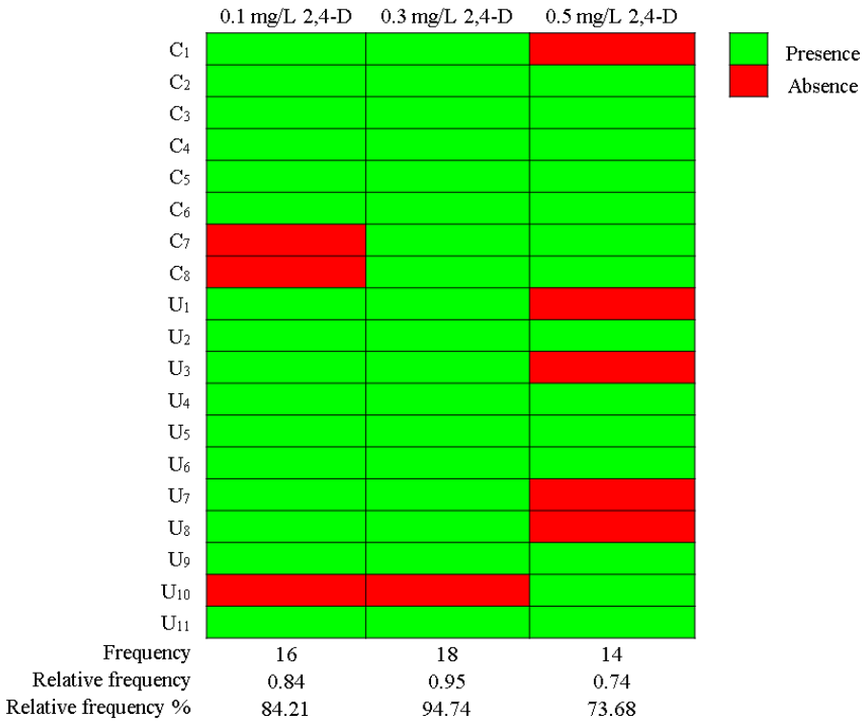

FIGURE 3. Frequency map of the compounds present in the extracts from cultures supplemented with 0.1 mg/L, 0.3 mg/L and 0.5 mg/L 2,4-D. *C – compound; U – unknown

study, it was observed that all of the callus induced from hypocotyl segments of *C. frutescens* treated with 0.5 - 5.0 mg/L 2,4-D resulted in more than 70% of callus induction rate (Kumar et al. 2017). The highest callus induction rate of 87% was obtained with the addition of 2.5 mg/L 2,4-D to the media. However, in the study, the callus colour (creamy white) remains unchanged at different concentrations of 2,4-D (Kumar et al. 2017). Similar findings were obtained by Abbas et al. (2018), who observed that the addition of different concentrations of 2,4-D (ranging from 0.5-1.5 mg/L) has a stimulative effect on callus formation of *Parkia biglobosa,* with the highest callus induction rate of 100% observed from stem explants cultured on MS media supplemented with 1.0 mg/L 2,4-D.

In this study, the increase in 2,4-D concentration was observed to result in higher callus biomass (dry weight), possibly due to enhanced cellular activity and higher frequency of cell division in the callus cells (Adil et al. 2018; Budisantoso et al. 2017). Parallel to the results of this study, induction of callus using endosperm as the explant source in *Barringtonia racemosa* cultured on MS media added with 1.0 - 2.5 mg/L 2,4-D has shown increment in the mean fresh weight and mean dry weight of the species, as the concentration of 2,4-D was increased (Osman et al. 2016). In another study, induction of callus from cotyledon as the explant source in *Fagonia indica* has shown increment in the mean dry weight of callus when the MS media was supplemented with 1.0, 2.5 and 5.0 µM 2,4-D (Ebrahimi & Payan 2013). Therefore, at this juncture, it can be concluded that the callus induction rate for any plant type, is dependent on several factors such as the explant type and concentrations of PGRs used.

Along with these two factors, the type of PGRs (auxin and cytokinin) used, either singly or in combination, has also been reported to influence the callus induction rate. In a study reported by Kumar et al. (2017), the formation of *C. frutescens* callus was noticeable in all MS media supplemented with either auxin, cytokinins or auxin combined with cytokinins, with the highest callus induction rate (100%) observed in MS media supplemented with just cytokinin (1 mg/L 6-benzylaminopurine; BAP) and MS media

TABLE 3. List of compounds detected in callus of *C. frutescens*

2,4-D (mg/L)	No.	Compound ID	Retention time, t_R (min)	MW (m/z)	Compound name
0.1	1	C1	1.810	377	Caffeoyl glucose conjugate
	2	C2	4.562	497	Polyphenol acid
	3	C3	9.819	297	Oxooctadecanoic acid
	4	C4	10.345	311	15,16-dihydroxy- 9Z,12Z-octadecadienoic acid
	5	C5	10.609	312	15,16-dihydroxy- 9Z,12Z-octadecadienoic acid isomer
	6	C6	11.005	325	Octadecadienoic acid conjugate
	7	U1	10.082	265	Unknown 1
	8	U2	9.030	293	Unknown 2
	9	U3	3.775	472	Unknown 3
	10	U4	4.229	514	Unknown 4
	11	U5	4.955	514	Unknown 5 (isomer of Unknown 4)
	12	U6	6.138	514	Unknown 6 (isomer of Unknown 4)
	13	U7	3.250	527	Unknown 7
	14	U8	1.941	529	Unknown 8
	15	U9	3.119	529	Unknown 9 (isomer of Unknown 8)
	16	U11	5.350	723	Unknown 11
0.3	1	C1	1.831	377	Caffeoyl glucose conjugate
	2	C2	4.851	497	Polyphenol acid
	3	C3	9.713	297	Oxooctadecanoic acid
	4	C4	10.371	311	15,16-dihydroxy- 9Z,12Z-octadecadienoic acid
	5	C5	10.634	312	15,16-dihydroxy- 9Z,12Z-octadecadienoic acid isomer
	6	C6	10.897	325	Octadecadienoic acid conjugate
	7	C7	11.820	340	Octadecadienoic acid conjugate isomer
	8	C8	12.084	339	Octadecadienoic acid conjugate isomer 2
	9	U1	10.108	265	Unknown 1
	10	U2	9.055	293	Unknown 2
	11	U3	3.800	472	Unknown 3
	12	U4	4.457	514	Unknown 4
	13	U5	5.114	514	Unknown 5 (isomer of Unknown 4)
	14	U6	5.902	514	Unknown 6 (isomer of Unknown 4)
	15	U7	3.275	527	Unknown 7
	16	U8	1.963	529	Unknown 8
	17	U9	3.012	529	Unknown 9 (isomer of Unknown 8)
	18	U11	5.377	723	Unknown 11
0.5	1	C2	4.846	497	Polyphenol acid
	2	C3	9.969	297	Oxooctadecanoic acid
	3	C4	10.363	311	15,16-dihydroxy- 9Z,12Z-octadecadienoic acid
	4	C5	10.627	312	15,16-dihydroxy- 9Z,12Z-octadecadienoic acid isomer
	5	C6	11.155	325	Octadecadienoic acid conjugate
	6	C7	11.550	340	Octadecadienoic acid conjugate isomer
	7	C8	12.212	339	Octadecadienoic acid conjugate isomer 2
	8	U2	9.180	293	Unknown 2
	9	U4	4.452	514	Unknown 4
	10	U5	5.108	514	Unknown 5 (isomer of Unknown 4)
	11	U6	6.159	514	Unknown 6 (isomer of Unknown 4)
	12	U9	3.140	529	Unknown 9 (isomer of Unknown 8)
	13	U10	6.553	714	Unknown 10
	14	U11	5.502	723	Unknown 11

supplemented with auxin and cytokinin (2.5 mg/L NAA plus 2.5 mg/L BAP). These results were in direct contrast to that observed in MS basal media (0%). These clearly indicated that the use of PGRs is essential for callus induction. In another study, the highest callus induction rate of 98.33 ± 1.66% was obtained using leaf explants of *Salacia macrosperma* on MS media added with 2.5 mg/L 2,4-D and 1.5 mg/L BAP (Mahendra et al. 2020). The influence of different concentrations of PGRs on callus induction was observed in hypocotyl cultures of *Lycium barbarum*, where maximum callus induction frequency (100%) was observed in MS medium added with 0.25 mg/L thidiazuron (TDZ) plus 0.1 mg/L indole-3-acetic acid (IAA) and 0.25 mg/L BAP plus 0.5 mg/L NAA (Karakas 2020). In addition, the effect of both explant types and the use of different PGRs at different concentrations on callus induction rate from hypocotyls of okra was reported (Rizwan et al. 2020). In the study, the highest callus induction frequency (71.0%) was observed from the hypocotyl explants when the explants were cultured on MS media supplemented with 1.0 mg/L BAP and 1.5 mg/L 2,4-D. On the other hand, the use of cotyledon explant only resulted in as high as 58% of callus induction frequency when cultured on MS media supplemented with 1.5 mg/L NAA plus 0.5 mg/L BAP. Meanwhile, the use of leaf explants of *Nilgirianthus ciliatus* resulted in the highest callus induction rate of 89.5% when cultured on MS media supplemented with 4.0 mg/L of 2,4-D plus 0.4 mg/L of BAP (Rameshkumar et al. 2018). Taken together, these observations clearly implied that the ability of PGRs (either used singly or in combination) to enhance callus induction is species-dependent. Thus, it is important for further optimizations to be done, in order to determine the optimum concentration and type of PGRs to result in efficient callus induction in any species.

Apart from that, the utilization of PGRs in the culture media for induction of callus also has a profound influence on the callus proliferation rate and metabolite production (Ptak et al. 2013; Raj et al. 2015). In *Stevia rebaudiana* Bertoni, a significant increase in phenols and flavonoids content was observed in the *in vitro* grown plantlets cultured on MS media supplemented with a combination of BAP and gibberellic acid (GA3) or IAA compared to when the PGRs were applied singly (Radić et al. 2016). Meanwhile, Tarigholizadeh et al. (2021) reported that treatments with 2 mg/L 2,4-D combined with 2 mg/L kinetin were found to yield the highest amounts of phenolics and flavonoids in the callus of *Satureja sahendica* Bornm. Similar observations were also recorded in this study, where different auxin (2,4-D) concentrations were found to affect the metabolites profile and influence the total number of chemical compounds present in the callus extracts. It was postulated that the exogenous application of 2,4-D had resulted in the induction of oxidative stress and generation of reactive oxygen species (ROS) that acted as the signaling molecules to activate the ethylene, jasmonate (JA) and abscisic acid (ABA) signaling pathways (McCarthy-Suárez 2017). It was the activation of these pathways that led to the differential accumulation of the secondary metabolites in the callus. For example, Song (2014) reported that the activation of ethylene and JA signaling pathways due to 2,4-D applications had resulted in increased trypsin proteinase inhibitor activity and production of volatile compounds in rice.

In a previous study, a total number of 29 compounds of various classes were identified in n-hexane and chloroform extracts of *C. frutescens* seeds through GC-MS analysis (Gurnani et al. 2016). Among all of the detected compounds, compounds belonging to the same class (octadecadienoic acid) were the only compound that was also found to be present in the current study. However, in the current study, with the use of different concentrations of 2,4-D, other metabolites in *C. frutescens* were also discovered. Data analysis showed that the identified compounds present in the extracts of callus grown under different 2,4-D concentrations were fatty acids and phenolics. These identified compounds have already been reported to be pharmacologically active. For example, compounds belonging to the same classes of oxooctadecanoic acid are known to exhibit antioxidant potential (Furumoto et al. 2016) and showed anti-inflammatory activities (Othman et al. 2019). Meanwhile, 15,16-dihydroxy-9Z,12Z-octadecadienoic acid has been reported to possess anti-inflammatory effects (Othman et al. 2019), while polyphenol acid has been shown to also exhibit anti- inflammatory activity (Sobhani et al. 2020), antifungal (da Rocha Neto et al. 2015), antioxidant (Zhao et al. 2021) and cardioprotective properties (Priscilla & Prince 2009). Phenolic acid compounds such as gallic acid, vanillic acid, syringic acid, coumaric acid, and caffeic acid are found in fruits, vegetables, and spices. These compounds are said to be responsible for the colour and flavour of food sources. The presence of such bioactive compounds in the callus of *C. frutescens* along with their multiple bioactive properties that have been reported in this study further contributes to the potential use of *C. frutescens* callus as a functional source of food colourants.

Conclusion

In this study, callus of various colours (cream, yellow, and green) has been successfully produced from *Capsicum frutescens* through plant tissue culture. The supplementation of auxin (2,4-D) to the media has resulted in the accumulation of several valuable bioactive compounds in the callus, indicating that PGRs played a role in the expression of secondary metabolites in plants. The increase or decrease in the concentration of these growth regulators may have triggered a change in the secondary metabolite biosynthesis pathways, thus causing differential expression of the compounds in this plant. The use of different concentrations of 2,4-D has resulted in newly reported metabolites in *C. frutescens*. The presence of such bioactive compounds in the callus of *C. frutescens* further implies its potential contribution to be used as a source of functional food colourants.

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