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Influence of different 2,4-D concentrations on antioxidant contents and activities in sweet basil leaf-derived callus during proliferation

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Abstract

The potential of sweet basil callus cultures as a source of natural antioxidants was investigated. Sweet basil (*Ocimum basilicum* L.) leaves were cultured on a semi-solid nutrient medium supplemented with 0, 0.5, 1 or 2 mg/l 2,4-D under a light illumination regime (20 µmol m^{-2} s^{-1} for 16 h and 8 h in the dark daily) at 25°C for 4 weeks. All the media used, except the plant growth regulator-free medium, could induce formation of green compact callus. After 4 weeks, callus cultured on the medium supplemented with 0.5 mg/l 2,4-D had the highest fresh weight. Antioxidant content analysis showed that this callus culture had the highest levels of β-carotene, ascorbic acid, phenolics and flavonoids (0.08 mg/gFW, 0.64 mg/gFW, 6.54 mg gallic acid/gFW and 7.38 mg rutin/gFW, respectively) after 1 week of callus proliferation. An evaluation of antioxidant ability suggested that changes in reducing power, ascorbic acid content, DPPH free radical scavenging activity, phenolics and flavonoids contents in sweet basil leaf-derived callus might all be interlinked.

Introduction

In the genus *Ocimum*, sweet basil (*Ocimum basilicum* L.) is one of the most extensively cultivated species all over the world (Carovic´-Stanko *et al*., 2010). This plant belongs to Lamiaceae or the mint family and its generic name, *Ocimum*, is derived from the ancient Greek word, *okimon* (smell) which implies the naturally impressive fragrance while the specific epithet, *basilicum*, is from *basilikon* in Greek which signifies royal or kingly (Meyers, 2003). In Thailand, sweet basil is a well-known herb in the cuisine. The leaves of this aromatic plant have been consumed fresh as salad or cooked in Thai dishes. It is also an ingredient in the famous Thai green curry. In addition to being a food plant, sweet basil has traditionally been used to cure many symptoms such as headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunction. Moreover, the major aroma compounds discovered in volatile extracts of this herb showed an effective antioxidant activity too (Lee *et al*., 2005).

Aseptic culture of sweet basil has been initiated for a variety of objectives including micropropagation (Sahoo *et al*., 1997; Phippen and Simon, 2000; Shahzad *et al*., 2012), physiological investigations (Dalton, 1984) and production of some important secondary metabolic compounds such as essential oils (Purohit and Khanna, 1983), rosmarinic acid (Kintzios *et al*., 2003; Kiferle *et al*., 2011), anthocyanins (Madhavi *et al*., 1995; Strazzer *et al*., 2011) and triterpenes (Marzouk, 2009).

There are some reports showing the influence of different kinds of plant growth regulators on antioxidant content during induction of callus in some plant species (Li and van Staden, 1998; Prasad *et al*., 2006; Rasool *et al*., 2011). However, research on the effect of plant growth regulator concentration on antioxidant production from *in vitro* callus culture during proliferation is still scarce. There is a growing interest in the health benefit of natural antioxidants in herbs such as sweet basil when consumed as food (Wongsen *et al*., 2013) and also in industrial production of the natural antioxidants using callus cultures (El-Baz *et al*., 2010; Stanly *et al*., 2011; Estrada-Zúñiga *et al*., 2012; Sharma and Ramawat, 2013). Therefore, the aim of the present research was to investigate the influence of different 2,4-D concentrations on antioxidant contents and their activities in relation to growth of sweet basil callus.

Materials and Methods

Plant materials and in vitro culture

Sweet basil (*Ocimum basilicum* L.) seeds were purchased from Chia Tai Co., Ltd., Bangkok, Thailand and immersed in clean tap water for 3 h before use. Subsequently, they were surfaced

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disinfected as follows: soaking in 70% (v/v) ethanol for 1 min, rinsing with sterile distilled water for 1 min, soaking again for 15 min with 15% (v/v) Clorox (5.25%, w/w, sodium hypochlorite available as chlorine) and rinsing with sterile distilled water 3 times each for 1 min. After this, seven seeds were placed per jar (4.5 cm X 8.5 cm) containing 10 ml of basal MS medium containing inorganic salts and vitamins as described in Murashige and Skoog, 1962. To initiate callus, leaf explants (8 mm X 8 mm) were excised from 4-week-old seedlings grown under aseptic conditions and transferred to basal MS medium supplemented with 0, 0.5, 1 or 2 mg/l 2,4-D in a glass container (4.0 cm X 6.5 cm). After 4 weeks on callus induction medium, the callus formed was transferred to fresh callus induction medium and allowed to proliferate further for 4 weeks. Fresh weights of callus were determined and appearance of callus was noted at each week throughout both the induction and proliferation periods. All media using in this research were adjusted to pH 5.7, solidified with 0.8% (w/v) agar, and autoclaved for 20 minutes at 121°C and 15 psi. All the culture jars containing seed for germination, or callus for induction and proliferation were kept at 25±2^{0}C in a growth room equipped with daylight fluorescent lamps (20 µmol/m^2/s) to maintain a daily photoperiod of 16 h.

Antioxidative component determination

Freshly harvested sweet basil callus (1 g) from each treatment was homogenized in 5 ml of acetone: hexane (2:3) solution. The pigment contents in the extracts were measured spectrophotometrically at 663, 645, 505 and 453 nm (Nagata and Yamashita, 1992). For the determination of the concentrations of pigments in the callus as mg/100 ml, the following formulae were used:

Chlorophyll a = 0.999Abs.663 - 0.0989Abs.645
Chlorophyll b = -0.328Abs.663 + 1.77Abs.645
β-Carotene = 0.216Abs.663 - 1.22Abs.645 - 0.304Abs.505 + 0.452Abs.453

Extraction and analyses of ascorbic acid, phenolics and flavonoids in freshly harvested sweet basil callus were as described in Wongsen *et al.* (2013).

Antioxidant activity assays

Reducing power assay of sweet callus extracts was slightly modified from the method of Chang *et al.* (2006) as described in Wongsen *et al.* (2013). Ferrous iron chelating ability and DPPH scavenging activity assays of sweet callus extracts were also carried out as described in Wongsen *et al.* (2013).

Statistical analysis

The analysis of variance (ANOVA) for fresh weights of sweet basil callus, antioxidant component and activity values was first conducted at the significance level of $P<0.05$. When some of means were found to be different, then comparison of means by Duncan's multiple range test (DMRT) was performed at $P<0.05$ using SPSS for Windows (SPSS Inc.).

Results and Discussion

Growth of sweet basil callus

There was no callus formation in sweet basil leaf explants on agar-gelled basal MS medium without any plant growth regulator (Figure 1A).

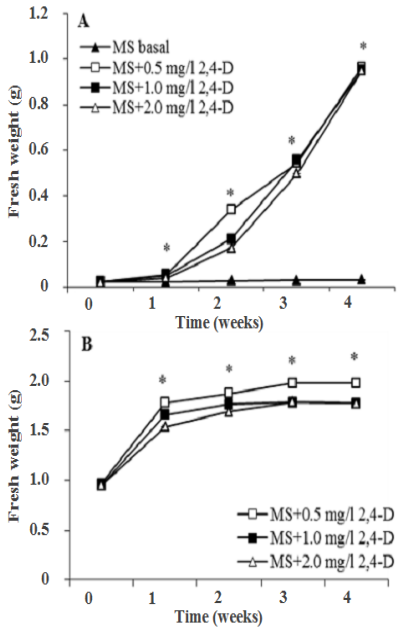

Figure 1. Fresh weight of callus derived from sweet basil leaf explants at induction (A) and proliferation (B) stages. Each data value is an average fresh weight of 14 replicate callus pieces. An asterisk over each week shows statistical difference among the treatments ($P<0.05$)

In response to the MS medium supplemented with different concentrations of 2,4-D (0.5, 1 or 2 mg/l), callus first appeared around the edges of the leaf explants after 1 week of culture and 2 more weeks later the entire explant was covered with light green, compact callus (Figure 2). The effects of the different concentrations of 2,4-D on the increases in the fresh weights of leaf explant+callus during callus induction over 4 weeks were statistically different (Figure 1A). In a previous study, it was found that 0.2 mg/l NAA in combination with high or low levels of BA resulted in the formation of compact or friable callus, respectively. In another study, a friable green callus was induced in sweet basil on MS medium

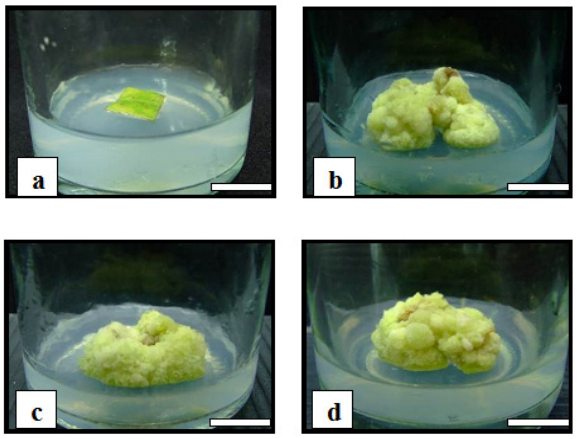

Figure 2. Callus induction at week 4 on MS medium supplemented with 0 (a), 0.5 (b), 1 (c) and 2 (d) mg/l 2,4-D (Scale bar = 1 cm)

supplemented with 2 mg/l 2,4-D and 2 mg/l NAA (Kintzios *et al*., 2003). In the present study, we also found that BA was not necessary for callus induction.

There is a paucity of data on sweet basil callus growth through the proliferation stage. In this work, callus initiated on media supplemented with 0.5, 1 or 2 mg/l 2,4-D was transferred onto the same respective media for 4 weeks, the fresh weights of the callus increased during the first week in all three treatments (Figure 1B) but there was a minimum change in the callus fresh weights in the next 3 weeks. After 4 weeks, the callus cultured on the medium supplemented with 0.5 mg/l 2,4-D had the highest fresh weight.

Antioxidative components and activity

During callus proliferation, there was a slight increase in chlorophyll a content after 1 week of culture on media supplemented with 0.5, 1 or 2 mg/l 2,4-D ((Figure 3A). At week 2, the chlorophyll a content decreased in callus cultured on the medium containing 2 mg/l 2,4-D but increased in callus cultured on the medium with 0.5 or 1 mg/l 2,4-D. The callus cultured on the callus induction medium supplemented with 1 mg/l 2,4-D had the highest level of chlorophyll b in comparison to callus kept on medium than callus with 0.5 or 2 mg/l 2,4-D (Figure 3B). During callus proliferation on the medium supplemented with 1 mg/l 2,4-D, the level of chlorophyll b fluctuated slightly over 4 weeks. Chlorophyll b content almost duplicated after 1 week of culture on the medium supplemented with 2 mg/l 2,4-D and then remained relatively unchanged in the next 3 weeks. The increase in chlorophyll b content in callus cultured on the medium supplemented with 0.5 mg/l 2,4-D in the first week was much less than that in 2 mg/l 2,4-D treatment. However, after 2 weeks the chlorophyll b contents in callus cultured on 0.5, 1 and 2 mg/l 2,4-D were not different.

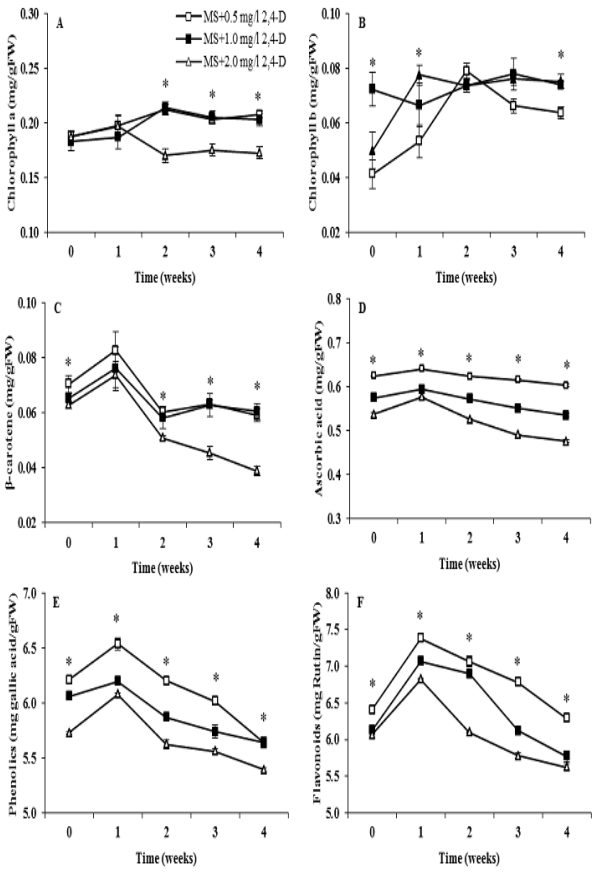

Figure 3. Changes in contents of chlorophyll a (A), chlorophyll b (B), β-carotene (C), ascorbic acid (D), phenolics (E) and flavonoids (F) during proliferation of callus derived from sweet basil leaf explants. Data are means of 6 replicates. An asterisk over each week shows statistical difference among the treatments ($P<0.05$)

The levels of β-carotene peaked similarly to chlorophyll b after 1 week of culture on the media supplemented with three different concentrations of 2,4-D before declining to lower levels (Figure 3C). Then the β-carotene content in callus cultured on the medium supplemented with 2 mg/l 2,4-D continued declining throughout the experiment, while that in callus cultured on 0.5 or 1 mg/l 2,4-D remained relatively unchanged till week 4. In contrast, the levels of ascorbic acid exhibited slight changes over 4 weeks of callus proliferation on the medium supplemented with 0.5 or 1 mg/l 2,4-D (Figure 3D). The ascorbic acid content in callus in the 2 mg/l 2,4-D treatment peaked at week 1 and then exhibited a continuous decline. Callus in the treatment with 0.5 mg/l 2,4-D had the highest level of ascorbic acid (0.64 mg/gFW) compared to the other two treatments.

The levels of phenolics and flavonoids during callus proliferation on the media supplemented with 0.5, 1 or 2 mg/l 2,4-D increased (6.54 mg gallic acid/gFW and 7.38 mg rutin/gFW, respectively) after 1 week of culture and then they declined to lower levels (Figure 3E and F). Callus grown on the

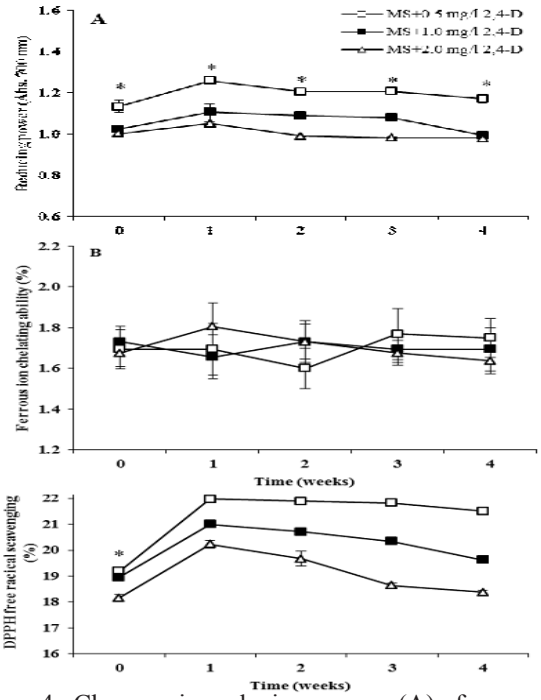

Figure 4. Changes in reducing power (A), ferrous ion chelating (B) and DPPH free radical scavenging (C) activities in sweet basil leaf-derived callus over 4 weeks of culture. Data are means of 6 replicates. An asterisk over each week shows statistical difference among the treatments ($P<0.05$)

medium supplemented with 0.5 mg/l 2,4-D had the highest levels of phenolics and flavonoids. Overall, the medium supplemented with 0.5 mg/l 2,4-D resulted in the highest levels of the four antioxidants (β-carotene, ascorbic acid phenolics and flavonoids) mainly after 1 week of culture except ascorbic acid. The levels of the four antioxidants also appeared to be lower later on during callus proliferation.

In vitro culture techniques have been applied for the production of antioxidants from a lot of plants (Matkowski, 2008). Many types of antioxidants can be found in sweet basil plants grown in soil or under in vitro culture conditions. Jayasinghe *et al*. (2003) found that the main antioxidative compound in sweet basil leaves from plants grown in soil was rosmarinic acid. Cell suspension culture of sweet basil was used to produce rosmarinic acid (Kintzios *et al*., 2003) and it was found that the highest level of rosmarinic acid accumulation occurred early on during culture. In sweet basil plantlets grown in vitro, the highest level of this antioxidant was found during multiplication rather than the initiation and rooting stages (Kiferle *et al*., 2011). The present study of antioxidative components (and their abilities as studied in the next section) during proliferation of sweet basil callus is rare.

The reducing power and DPPH free radical scavenging activity in callus grown on the medium supplemented with the three different concentrations of 2,4-D increased after 1 week of callus proliferation (Figure 4A and C), while there was no significant difference in ferrous ion chelating activity among all three treatments over 4 weeks (Figure 4B). Callus grown on the medium supplemented with 0.5 mg/l 2,4-D had the highest levels of reducing power and DPPH free radical scavenging activity than callus grown on the medium supplemented with 1 or 2 mg/l 2,4-D. Moreover, the patterns in reducing power seemed to be related to those in ascorbic acid (compare Figures 3D and 4A) whereas DPPH free radical scavenging activity pattern appeared to similar to the alterations in phenolic or flavonoid content (compare Figures 3E, 3F and 4C). The ferrous ion chelating activity ability did not resemble the patterns of changes in the antioxidaive substances studied here and might therefore be associated with other antioxidants (Figure 3).

Lee *et al*. (2005) identified the volatile components and its antioxidant properties in sweet basil leaves from plants grown in soil and found that eugenol, thymol, carvacrol, and 4-allylphenol exhibited effective antioxidant activities by the aldehyde/carboxylic acid assay. Hinneburg *et al.* (2006) showed that sweet basil extracts had the highest antioxidant ability associated with the DPPH radical scavenging activity but not with iron(II) chelation activity. In the present research, it was found that the concentration of 2,4-D in the medium had an influence on the antioxidant capability of sweet basil callus which was associated with reducing power and DPPH free radical scavenging activity but not ferrous ion chelating ability. This finding was similar to that in another study showing that callus of Rosmarinus officinalis grown on a medium supplemented with 1 mg/l NAA had a higher yield of phenolic content and superior antioxidant activity than callus grown in medium with higher NAA levels (Yesil-Celiktas *et al*., 2007). Also it was reported that there was an excellent relationship between DPPH free radical scavenging ability and total phenolic content in Zingiber zerumbet callus cultured on MS medium supplemented with 0.5 mg/l 2,4-D (Stanly *et al*., 2011).

The presence of different 2,4-D concentrations affected both antioxidant components and activity. The higher concentrations of 2,4-D (1 and 2 mg/L) seemed to have a greater effect than the lower one. Thus, callus cultured on the medium supplemented with 0.5 mg/l 2,4-D had the highest levels of β-carotene, ascorbic acid, phenolics and flavonoids than the other two concentrations. Besides, reducing power and DPPH free radical scavenging activity

from callus cultured on medium containing 0.5 mg/L 2,4-D were also higher than callus on the other two 2,4-D concentrations.

In conclusion, the increases in fresh weights of sweet basil callus during proliferation did not always correlate with the changes in antioxidant quantity and capacity. The concentration of 2,4-D, particularly 0.5 mg/l, in the medium and culture time seemed to be important in influencing changes in the antioxidant contents and activities of sweet basil callus during proliferation. Higher doses of 2,4-D and three or more weeks of callus proliferation seemed to be unfavorable for antioxidant production and activities. To increase the antioxidant content in sweet basil callus further, mutation induction following irradiation with gamma rays or somaclonal variation might be a possibility (Guirgis *et al.*, 2007; Homhuan *et al*., 2008).

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