Production of coloured callus in *Orthosiphon stamineus* Benth and antioxidant properties of the extracted pigments

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Abstract

Purpose – The purpose of the present study is to understand the role of auxin and cytokinin in stimulating the production of pigmented callus in *Orthosiphon stamineus* and to gain correlation between the callus colours with their antioxidant capacity and bioactive constituents.

Design/methodology/approach – In this study, plant tissue culture was used to induce production of callus of various colours from leaf explants of *O. stamineus*, via manipulation of plant hormones (0-2.0 mg L^{-1} indole-3-acetic acid [IAA] and Kinetin [Kin]). The coloured callus was subjected to solvent extraction and used for quantification of its carotenoid, chlorophyll, anthocyanin and phenolic contents. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the extracts was also evaluated, before and after four weeks of storage at −20°C.

Findings – The highest mean (per cent) explants that produced roots (93.33 ± 0.05 per cent) were observed when the cultures were supplemented with 2.0 mg L^{-1} IAA. The colour of the callus changed with time, from green to cream to brown after two and four months of culture, respectively. Optimum production of green callus was achieved with addition of 2.0 mg L^{-1} Kin plus 1.0-2.0 mg L^{-1} IAA to the media, while cream callus in 0.5 mg L^{-1} Kin plus 2.0 mg L^{-1} IAA and brown callus in 0.5 mg L^{-1} Kin plus 1.5 mg L^{-1} IAA. Green callus was found to contain the highest amount of chlorophylls, carotenoid and anthocyanin, while cream callus contained the highest amount of phenolic compounds. The amount of pigments and secondary metabolites in the callus extracts decreased after four weeks of storage, except anthocyanin. The antioxidant potential of the extracts also increased after storage.

Research limitations/implications – The major compounds identified in the methanolic extracts of *O. stamineus*-coloured callus are chlorophylls, carotenoids, flavonoids and phenolic acids. Future research work should include improvements in the extraction and identification methods which may lead to detection of other compounds that could attribute to the antioxidant capacity, to complement the findings of the current study.

Practical implications – This analysis provides valuable information on the application of IAA and Kinetin (Kin) to manipulate the content of major pigments with medicinal benefits in *O. stamineus* by using the plant tissue culture system.

Originality/value – A comparative study on antioxidant capacity and bioactive constituents of pigmented callus from *O. stamineus* leaves is original. To the best of the authors' knowledge, this is the first attempt of comparative evaluation on antioxidant potential of *O. stamineus*-coloured callus produced using IAA and Kin.

Keywords Pigments, Coloured callus, Indole-acetic acid, Kinetin, *Orthosiphon stamineus*

Paper type Research paper

Introduction

Misai Kucing (*Orthosiphon stamineus*) is a well-known medicinal herb that belongs to the *Lamiaceae* family. It is also known as Kumis Kucing and Java Tea. This species can be found throughout Southeast Asia and tropical Australia.

Conflict of interest: The authors declare that they have no competing interests.

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Malaysia and Indonesia have a tropical climate with high temperature and rainfall all year. These favourable conditions enable the plant to flourish extensively (Hossain and Rahman, 2015). This plant can easily be identified by its flowers that resemble cat whiskers. The flowers are either purple or white and have long wispy-shaped stamens (Figure 1) (Almatar *et al.*, 2013). The leaves are green, glabrous with lanceolate leaf blades and serrated margins, and are arranged in opposite pairs. The stem is quadrangular, reddish in colour, erect and with profuse branching (Basheer and Majid, 2010).

This species is usually propagated vegetatively through a mature stem. However, using only the traditional method for commercial plantation is inadequate for a large market demand (Nawi and Samad, 2012). To improve productivity, plant growth regulators are commonly used for a large number of agricultural crops and seed germination (Dhoran and Gudadhe, 2012). This method has been widely used in the mass propagation of plantlets to cope with demand. Moreover, the production of bioactive compounds tends to be inconsistent in field-grown plants (Zobayed *et al.*, 2005), leading to the use of the plant tissue culture system as a method of choice for propagation. In Malaysia, Misai Kucing is used as a traditional remedy for kidney problems, gout and diabetes. The whole plant, with the exception of the root, can be consumed. Traditionally, this plant is harvested before flowering, washed and sundried. It has great potential for commercial cultivation, as it contains a variety of secondary metabolites with potentially beneficial biological activities.

A variety of pigments produced by plants may be beneficial to humans, both for health (de Pascual-Teresa and Sanchez-Ballesta, 2008) and general use, such as dye-sensitized solar cells (Hug *et al.*, 2014). The colour changes of most of these pigments is caused by the production, interaction and breakdown of three classes of compounds: porphyrins, carotenoids and flavonoids (Alkema and Seager, 1982; Madureira and Ferreira, 2016). All colours have different compound composition. Carotenoids are potential antioxidants and have been shown to be useful in the treatment of certain human diseases such as atherosclerosis and cancer (Tanaka *et al.*, 2012; Palozza *et al.*, 2010). Flavonoids possess many pharmacological activities such as anti-ulcer, anti-ageing, anti-bacterial, antioxidant, anti-fungal, anti-inflammatory, anti-diabetic, anti-hepatotoxic, anti-allergic, anti-cancer, anti-tumour and as a vasodilator (Shohaib *et al.*, 2011). To date, various studies have been conducted in trying to devise the best method to enhance production of secondary metabolites in plants (Akula and Ravishankar, 2011; Hussain *et al.*, 2012; Karuppusamy, 2009).

Orthosiphon stamineus has been shown to contain numerous beneficial compounds. To date, 69 compounds have been identified from its essential oils from leaves and stems (Hossain *et al.*, 2008), and more than 20 phenolic compounds have been identified in this species (Akowuah *et al.*, 2004). The antioxidant potency of *O. stamineus* leaf extract has been reported by Yam *et al.* (Yam *et al.*, 2007). The study showed that the leaf extract possessed anti-inflammatory and non-narcotic analgesic activities. The leaf extract also exhibited antioxidant activity against oxygen-free radicals, facilitating the repair of damaged proteins in blood vessel walls (Yam *et al.*, 2007; Oancea and Oprean, 2011). The leaf parts have been shown to contain the highest total phenolic compounds compared to stems and roots (Farhan *et al.*, 2012). Rosmarinic acid has been identified as the major component responsible for the antioxidant activity of *Orthosiphon* plants (Nuengchamnong *et al.*, 2011) and also possibly the main compound exhibiting antibacterial and free radical scavenging activities of *O. stamineus* extracts (Ho *et al.*, 2010). *Orthosiphon stamineus* has also been shown to be effective as an anti-hypertensive agent in spontaneously hypertensive rats (Azizan *et al.*, 2012).

Figure 1 Orthosiphon stamineus

In a study by Chin *et al.* (2008), the methanolic extract of *O. stamineus* was shown to have varied toxic effects on Sprague–Dawley rats, depending on ingestion dosage. Furthermore, Ho *et al.* (2010) reported that *O. stamineus* extracts have the potential as a natural food preservative. These studies indicate that this species is indeed a valuable plant with potential for commercial propagation and has garnered a lot of interest due to its potential medicinal properties.

In this study, we aimed to manipulate the content of major pigments with medicinal benefits in *O. stamineus* by using plant tissue culture system, with the aid of plant growth regulators. We report the production of callus in various colours from leaf explants of this species via manipulation of plant growth regulators: indole-acetic acid (IAA) and Kinetin (Kin). The pigment content (chlorophyll, carotenoid, phenolic compounds and anthocyanin), as well as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities and total phenolic content of the different coloured callus, is also reported.

Experimental

Preparation of explant and growth media

The leaves from a field-grown *O. stamineus* plant were excised and used as explant source in the production of callus. First, the leaves were surface-sterilized by washing under tap water for 1 h followed by treatment with 50 and 30 per cent (v/v) commercial bleach (Clorox) for 1 min at each concentration. Two drops of Tween 20 were added during the treatment with 50 per cent (v/v) Clorox. This was followed by rinsing with sterile distilled water to remove excess Clorox. The leaves were then submerged in 70 per cent ethanol (v/v) for 1 min and finally rinsed three times with sterile distilled water.

To study the effects of selected plant growth regulators (PGR) on *O. stamineus*, IAA and Kin were added to the media either singly or at different combinations. The MS media was supplemented with different concentrations of IAA and Kinetin (0.5, 1.0, 1.5, 2.0 mg L^{-1}) and 30 g L^{-1} sucrose. The pH of the media was adjusted to 5.7-5.8 using 0.1M NaOH or 0.1M HCl. The media was solidified by adding 2 g L^{-1} Gelrite Gellan Gum (Duchefa Biochemie B.V, 2003, The Netherlands) and autoclaved at 120°C for 20 min.

Production of coloured callus

After surface sterilization, leaves were cut into 1-cm^2 pieces before being cultured on Murashige and Skoog (1962) media with the adaxial part of the explant facing the media. All cultures were maintained in a culture room at 25 ± 1°C, with a 16-h photoperiod and 1,000 lux of light intensity. Callus colour was observed for six months. The coloured callus was then excised from the explants at various time points (green; <2 months, cream; 2-4 months and brown; >6 months) and stored at −80°C for subsequent experiments. The mean percentage (per cent) of explants producing callus and rhizogenesis (formation of roots), mean number of roots per explant and mean fresh weight of callus per explant were recorded after four weeks. Experiments were conducted in triplicates of n = 10 and followed a randomized complete block design (RCBD). Data analysis was conducted through analysis of variance and Duncan's multiple range test (DMRT) at 5 per cent significance level.

Extraction of pigments from coloured callus

Fresh coloured calli of different age (green, cream and brown) were excised from the explants and weighed. Each coloured callus was then freeze-dried separately using a Labconco freeze dryer (Labconco Corporation, MO) at −50°C. Dry weight was recorded for each callus sample. Subsequently, each sample was immersed in pure methanol (10 per cent w/v) and ground gently using chilled mortar and pestle. A small quantity of magnesium carbonate was added to prevent conversion of chlorophyll to pheophytin under acidic condition. This experiment was conducted in low light as pigments are light-sensitive. The solution was then transferred to 50-mL falcon tubes and wrapped with aluminium foil before being incubated at −20°C for 48 h. The incubated solution was then centrifuged using a Universal 32 R centrifuge (Hettich Zentrifugen, D-78532 Germany) at 9,050*g* for 5 min at 4°C. The resulting supernatant was collected, divided into three parts and used in subsequent analysis.

For extraction of carotenoids, 1.0 g of freeze-dried callus samples were rehydrated with 1.0-mL distilled water and soaked overnight in 5 mL of acetone:methanol (7:3) at room temperature (RT). Then, the mixture was vortexed and centrifuged at 13,500*g* for 2 min, where the supernatant was then transferred into a 50-mL graduated polypropylene centrifuge tubes covered with foil. The supernatant was centrifuged again at 13,500*g* for 5 min to remove the fine particulates. To extract the carotenoids, an equal volume of hexane and distilled water was added to the sample mixture, vortexed and centrifuged at 13,500*g* for 1 min. The upper layer containing the carotenoids was collected and dried under a gentle stream of O_2-free nitrogen gas. Then, the vials were immediately capped, sealed with parafilm and stored at −80°C until subsequent analysis.

HPLC quantification of individual carotenoids

Quantification of individual carotenoid content was conducted using HPLC (Agilent 1200 series model) comprising a binary pump with an autosampler injector, micro-vacuum degassers, thermostatted column compartment and a diode array detector. Separation was done using a reverse-phase column ZORBAX SB-C18 end capped (5 μm, 4.6 × 250 mm) (Agilent Technologies). The eluents used were (A) acetonitrile:water (9:1 v/v) and (B) ethyl acetate. The solvent gradient used was: 0-40 per cent Solvent B (0-20 min), 40-60 per cent Solvent B (20-25 min), 60-100 per cent Solvent B (25-25.1 min), 100 per cent Solvent B (25.1-35 min) and 100-0 per cent Solvent B (35-35.1 min) at a flow rate of 1.0 mL min^{-1}. The column was allowed to re-equilibrate in 100 per cent Solvent A for 10 min prior to the next injection. The injection volume was 10 μL, with the temperature of the column kept at 20°C. Carotenoid peaks were detected between the wavelength ranges of 350 to 550 nm. In this study, the carotenoid extracts were screened for eight types of carotenoid; α-carotene, β-carotene, lycopene, lutein, neoxanthin, violaxanthin, zeaxanthin and β-cryptoxanthin.

Measurement of total chlorophyll and carotenoid content

The first part of the extract was used in the measurement of chlorophylls (*a* and *b*) and carotenoid content. Absorbance was read using a Sunrise microplate reader (Tecan, Austria) and used to determine the content of chlorophylls a and b and

carotenoid, based on the formula by Lichtenthaler and Buschmann (2001):

$$\mathbf{C_a}\,(\mu\mathbf{g/mL}) = \mathbf{16.72\,A_{665.2} - 9.16\,A_{652.4}}$$

$$\mathbf{C_b}\,(\mu\mathbf{g/mL}) = \mathbf{34.09\,A_{652.4} - 15.28\,A_{665.2}}$$

$$\mathbf{C_{(x+c)}}(\mu\mathbf{g/mL}) = (\mathbf{1000\,A_{470} - 1.63\,C_a - 104.96\,C_b})/\mathbf{221}$$

The extract was stored at −20°C for four weeks and the procedure was repeated to determine pigment degradation rates.

Measurement of total anthocyanin content

The second part of the extract was used to measure total anthocyanin content. The pH of the extract was adjusted to pH 1 and pH 4.5, and pH-differential method (Giusti and Wrolstad, 2001) was used to determine the total anthocyanin content in green, cream and brown calli. The absorbance at 510 nm and 700 nm was read using a Sunrise microplate reader. The extract was also stored at **−20°C** for four weeks and the procedure was repeated to determine pigment degradation rates. Monomeric anthocyanin pigment concentration (cyanidin-3-glucoside) was calculated using formula described by Giusti and Wrolstad (2001), (Ponmozhi *et al.*, 2011):

$$\text{Anthocyanin pigment content}\left(\text{mg L}^{-1}\right) = \frac{(\text{A} \times \text{MW} \times \text{DF} \times 1000)}{(\varepsilon \times 1)}$$

$$\text{Where, A} = (\text{Abs}_{\lambda\,\text{vis}-\text{max}} - \text{Abs}_{700})\text{pH1} - (\text{Abs}_{\lambda\,\text{vis}-\text{max}} - \text{Abs}_{700})\text{pH4.5}$$

$$\text{MW (cyanidin} - 3 - \text{glucoside)} = 449$$

$$\text{DF} = \text{dilution factor}$$

$$\varepsilon\ (\text{molar absorptivity of cyanidin} - 3 - \text{glucoside}) = 26,900$$

$$\lambda_{\text{vis}-\text{max}}\ (\text{of cyanidin} - 3 - \text{glucoside, in aquous } \textit{buffer at pH}1) = 510\text{nm}$$

Measurement of total phenolic content

For measurement of total phenolic content, the third part of the extract was transferred into the evaporating flask of a Rotavapor R-3 (BÜCHI Labortechnik, Switzerland) and the extract was concentrated under vacuum at 60°C. The resulting solvent-free extract was then weighed repeatedly until a constant weight was achieved and subsequently diluted to 20 mg L^{-1} in methanol.

Total phenolic content in the sample was determined using the spectrophotometric method described by Singleton *et al.* (1999) and Stanković (2011), with minor modifications. Briefly, 2.5 mL of 10 per cent Folin–Ciocalteu reagent and 2.5 mL of 7.5 per cent $NaHCO_3$ were added to 0.5 mL of extract and mixed thoroughly. A blank was prepared in a similar fashion, with the extract replaced with methanol. All samples were incubated in the dark at room temperature for 30 min. Absorbance at 765 nm was then determined using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, HP9 1QA, UK). The protocol was repeated using gallic acid (0.001, 0.002, 0.003, 0.004, 0.005 and 0.006 mg mL^{-1}) as standard. Total phenolic content in the callus extracts was expressed in terms of gallic acid-equivalent (microgram of GA per gram of extract). The extract was also stored at −20°C for four weeks to determine any phenolic degradation.

DPPH-free radicals scavenging assay

DPPH-free radicals scavenging assay was performed as described by Gawron-Gzella *et al.* (2012) and Khalaf *et al.* (2008), with minor modifications. Extracts were first diluted to a series of concentrations (10, 50, 100, 200, 300, 500, 700 and 1,000 μg mL^{-1}). The diluted extracts (1 mL) were mixed with 1 mL of DPPH in methanol (0.0062 g/100 mL of methanol) and incubated in the dark at room temperature for 30 min. The absorbance was then read at 517 nm using a Sunrise microplate reader. The extract was replaced with solvent (methanol) in the negative control, while ascorbic acid was used as the positive control. DPPH-free radicals scavenging activity of the callus extract was evaluated by comparing the results with the control. The percentage of DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH free radicals scavenging activity}\,(\%) = \frac{\text{A}_0 - \text{A}_1}{\text{A}_0} \times 100.$$

$$\text{Where, } \text{A}_0 = \text{absorbance of control, } \text{A}_1 = \text{absorbance of sample}$$

The graph of percentage of DPPH radical scavenging activity against extract concentration was plotted by performing non-linear regression (third-degree polynomial) as described by Abourashed (2005) and Samarakoon *et al.* (2011). The results were presented as 50 per cent inhibition concentration (IC_{50}) in micrograms per millilitre.

Statistical analysis

All analyses were carried out in triplicate and the data were expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Duncan's multiple-range test was used to determine the significance of the difference between means. Correlations were obtained by Pearson's correlation coefficient (r) in bivariate linear correlation.

Results and discussion

Rhizogenesis in vitro

The range of PGR concentrations used in this study was between 0.00 and 2.00 mg/L, which is the adequate concentrations of hormones in plant tissue culture (Bhojwani and Razdan, 1996). Moreover, the use of high cytokinin concentration may lead to morphological abnormalities and

cause hyperhydration (Bhojwani and Razdan, 1996). Initiation of roots can be detected after one week in proliferation medium. In most cases, the roots were formed by direct organogenesis (rhizogenesis). After four weeks, the samples were observed and weighed. Mean percentage (per cent) of explants producing roots and mean number of roots per explant are presented in Tables I and II. No formation of shoots was observed in all hormone treatments. In general, a greater number of roots were produced in proliferation media consisting of a single hormone (either IAA or Kin) compared to media with a combination of hormones.

IAA was found to be a better hormone for direct rhizogenesis from leaf explants of *O. stamineus* compared to Kin. Ling *et al.* (2009) reported that leaf was the best explant type for induction of adventitious root and showed the best rooting ability when the cultures were supplemented with 2.0 mg L^{-1} IAA. A similar observation was recorded in the current study where it was found that the addition of IAA to the media is advantageous for induction of rhizogenesis in *O. stamineus* leaves, especially when the MS media was supplemented with only IAA (Tables I and II). Occurrence of rhizogenesis was observed to increase with increasing concentrations of IAA. The highest percentage of explants producing roots was achieved in MS media supplemented with either 2.0 mg L^{-1} IAA (93.33 per cent) or 1.5 mg L^{-1} IAA (86.67 per cent); 2.0 mg L^{-1} and 1.5 mg L^{-1} IAA also produced the highest number of roots per explant, with 7.00 ± 1.06 roots and 6.00 ± 0.85 roots per explant, respectively.

On the other hand, addition of Kin to the media only resulted in the production of roots at high Kin concentration (2.0 mg L^{-1}), where 3.33 per cent of the leaf explants produced roots, while no response was observed at lower Kin concentrations. In a study on *Agapanthus praecox*, rooting induction from leaf

Table I Effects of different concentrations of single hormones on mean (%) explants producing roots and callus, mean number of roots per explant and mean fresh weight of callus per explant

MS + Hormone (mg L^{-1})	Mean (%) explants produced roots	Mean no. of roots per explant	Mean (%) explants produced callus	Mean fresh weight of callus per explant (g)
Control	NR	NR	100.00 ± 0.00^{d}	$0.02 \pm 0.01^{a,b}$
0.5 Kin	NR	NR	100.00 ± 0.00^{d}	$0.05 \pm 0.03^{b,c}$
1.0 Kin	NR	NR	100.00 ± 0.00^{d}	0.12 ± 0.07^{d}
1.5 Kin	NR	NR	100.00 ± 0.00^{d}	0.06 ± 0.05^{c}
2.0 Kin	3.33 ± 0.03^{a}	3.00 ± 0.10^{a}	96.67 ± 0.03^{d}	0.07 ± 0.58^{c}
0.5 IAA	73.33 ± 0.08^{c}	4.00 ± 0.69^{b}	26.67 ± 0.82^{b}	$0.02 \pm 0.00^{a,b}$
1.0 IAA	53.33 ± 0.09^{b}	$5.00 \pm 1.35^{b,c}$	46.67 ± 0.93^{c}	$0.03 \pm 0.01^{a,b}$
1.5 IAA	86.67 ± 0.06^{cd}	$6.00 \pm 0.85^{b,c}$	$13.33 \pm 0.63^{a,b}$	$0.02 \pm 0.00^{a,b}$
2.0 IAA	93.33 ± 0.05^{d}	7.00 ± 1.06^{c}	6.67 ± 0.46^{a}	0.00 ± 0.00^{a}

Notes: Data represent mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters within the same column are significantly different at $p < 0.05$ according to Duncan's multiple-range test (DMRT). Kin: Kinetin, IAA: indole-acetic acid, NR: no response

Table II Effect of different concentrations and combinations of hormones on mean (%) explants producing roots and callus, mean number of roots per explant and mean fresh weight of callus per explant

MS + hormone (mg L^{-1})	Mean (%) explants produced roots	Mean number of roots per explant	Mean (%) explants produced callus	Mean fresh weight of callus per explant (g)
Control	NR	NR	100.00 ± 0.00^{b}	0.02 ± 0.00^{a}
0.5 Kin + 0.5 IAA	NR	NR	100.00 ± 0.00^{b}	$0.20 \pm 0.03_{d}$
0.5 Kin + 1.0 IAA	NR	NR	100.00 ± 0.00^{b}	$0.17 \pm 0.03^{c,d}$
0.5 Kin + 1.5 IAA	6.67 ± 0.05^{a}	2.33 ± 0.18^{a}	93.33 ± 0.05^{b}	0.11 ± 0.02^{b}
0.5 Kin + 2.0 IAA	16.67 ± 0.07^{b}	4.00 ± 0.18^{b}	83.33 ± 0.07^{a}	0.11 ± 0.01^{b}
1.0 Kin + 0.5 IAA	NR	NR	100.00 ± 0.00^{b}	0.09 ± 0.01^{b}
1.0 Kin + 1.0 IAA	NR	NR	100.00 ± 0.00^{b}	0.09 ± 0.01^{b}
1.0 Kin + 1.5 IAA	NR	NR	100.00 ± 0.00^{b}	0.10 ± 0.01^{b}
1.0 Kin + 2.0 IAA	NR	NR	100.00 ± 0.00^{b}	0.20 ± 0.03^{d}
1.5 Kin + 0.5 IAA	NR	NR	100.00 ± 0.00^{b}	0.12 ± 0.01^{b}
1.5 Kin + 1.0 IAA	NR	NR	100.00 ± 0.00^{b}	0.10 ± 0.01^{b}
1.5 Kin + 1.5 IAA	NR	NR	100.00 ± 0.00^{b}	0.10 ± 0.01^{b}
1.5 Kin + 2.0 IAA	NR	NR	100.00 ± 0.00^{b}	0.10 ± 0.01^{b}
2.0 Kin + 0.5IAA	NR	NR	100.00 ± 0.00^{b}	0.10 ± 0.01^{b}
2.0 Kin + 1.0 IAA	NR	NR	100.00 ± 0.00^{b}	0.09 ± 0.01^{b}
2.0 Kin + 1.5 IAA	NR	NR	100.00 ± 0.00^{b}	$0.13 \pm 0.01^{b,c}$
2.0 Kin + 2.0 IAA	NR	NR	100.00 ± 0.00^{b}	0.08 ± 0.02^{b}

Notes: Data represent mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters within the same column are significantly different at $p < 0.05$ according to Duncan's multiple-range test (DMRT). Kin: Kinetin, IAA: indole-acetic acid, NR: no response

explant was reported to be unsuccessful with addition of Kin to the media, even when used in combination with an auxin (Yaacob *et al.*, 2014). In the present study, poor rooting induction was observed when IAA is used in combination with Kin. No rhizogenesis was observed from the leaf explants, except when a low concentration of Kin was combined with a high concentration of IAA. As shown in Table II, 16.67 per cent of explants supplemented with 0.5 mg L^{-1} Kin combined with 2.0 mg L^{-1} IAA produced the highest number of roots (four roots per explant). Combinations between IAA and other hormones had also been found to result in poor rooting in other species, such as *Labisa pumila* (Hussein and Ibrahim, 2014) and *Vanilla planifolia* (Palama *et al.*, 2010).

Production of coloured callus in vitro

Leaf was chosen as the explant type in this study, as it has been reported that leaves were the most responsive explant type followed by the petiole and stem in induction of callus in *O. stamineus* (Wai-Leng and Lai-Keng, 2004). This was confirmed in our study where the leaf explants yielded callus in most treatments. Induction of callus formation was observed to be initiated at the cut surfaces of the leaf explants. In contrast to the results obtained for rhizogenesis from leaf explants, the leaf explants were found to be more responsive towards Kin than IAA in the production of callus (Table I). Further, 100 per cent of explants cultured in the presence of only Kin yielded formation of callus. On the other hand, the mean percentage of explants producing callus was decreased with increasing IAA concentration, with IAA at 1.0 mg L^{-1} being the optimum concentration. Overall, in treatments with a single hormone, 1.0 mg L^{-1} Kin was identified as the best hormone in inducing formation of callus from leaf explants of *O. stamineus*, yielding 0.12 ± 0.07 g of fresh callus per explant.

On the other hand, when Kin was added to the media with IAA, the induction of callus formation was observed to be improved in all cultures (Table II). This suggested that although Kin is a poor rhizogenesis inducer, Kin played an important role in callus formation. It has been well established that Kin is able to induce cell division in plant tissue culture, if it is used in combination with an auxin (Mishra, 2009). Data analysis showed that the best combinations of hormones for callus induction from leaf explants of *O. stamineus* were 0.5 mg L^{-1} Kin combined with 0.5-1.0 mg L^{-1} IAA and 1.0 mg L^{-1} Kin combined with 2.0 mg L^{-1} IAA. At this juncture, it was apparent that callus formation was highest when the concentration of IAA was twofold higher than that of Kin. These findings were supported by previous reports in literature, where addition of Kin together with other auxins (either IAA or NAA) was found to yield maximum biomass accumulation in *Nigella sativa* cultures (Chaudhry *et al.*, 2014). Similarly, usage of both Kin and IAA combined also leads to callus formation in *Nicotiana* species (Schaeffer *et al.*, 1963). However, contrasting results were reported by Wai-Leng and Lai-Keng, where insignificant callus growth was observed from *O. stamineus* leaves when Kin or other cytokinins were added to the media (Wai-Leng and Lai-Keng, 2004).

The colour of the callus was found to change with age. Green callus formed within two months. It changed to cream after two months of culture and to brown after four months of culture (Figure 2). A similar observation was recorded by Sheena and Jothi (2015), where callus of *O. stamineus* produced using 2,4-D and NAA hormones changed colour from light creamy greenish to light brown and eventually to dark brown (Sheena and Jothi, 2015). However, the colour change occurred earlier, where the callus colour became dark brown after eight weeks (Sheena and Jothi, 2015). The mean percentage of explants producing the different coloured calli is shown in Tables III and IV. Analysis of the results suggested that the addition of hormones to the media (either applied singly or in combinations) had a profound effect on the formation of coloured callus, especially in producing cream and brown callus. The results also indicate that a combination of hormones is better at inducing the formation of coloured callus compared to a single hormone. The highest rate of green callus production was observed in cultures supplemented with a combination of 2.0 mg L^{-1} Kin and 1.0-2.0 mg L^{-1} IAA. Production of cream callus was optimum in media containing 0.5 mg L^{-1} Kin and 2.0 mg L^{-1} IAA, while brown callus was best produced in media with 0.5 mg L^{-1} Kin and 1.5 mg L^{-1} IAA.

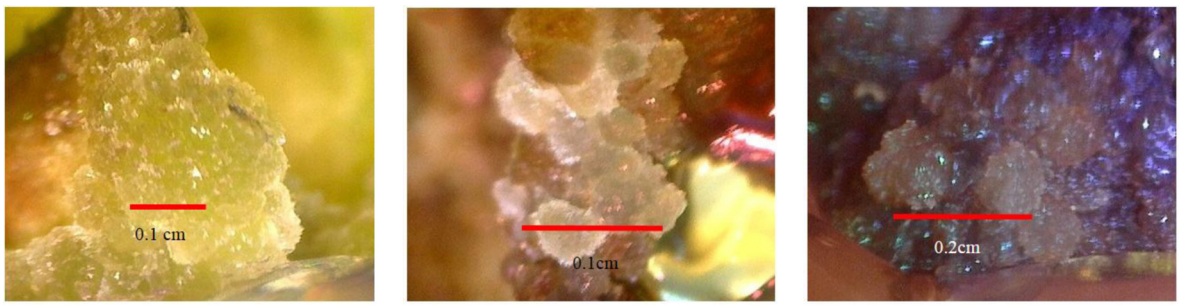
Detection of pigments

The extracts of the coloured calli of *O. stamineus* were found to contain three natural pigments, namely, chlorophyll, carotenoid and anthocyanin (Tables V-VIII). A comparison of chlorophyll *a*, chlorophyll *b*, carotenoid and total chlorophyll and carotenoid in the samples, measured before and after four weeks of storage, is presented in Table V. In general, the results showed that pigment content was highest in green callus (callus age, <2 months), followed by cream (callus age between 2 and 4 months) and brown callus (callus age, >4 months). The highest total chlorophyll plus carotenoid pigment content was obtained from green callus after four weeks of storage (4.52 ± 0.17 g kg^{-1} dry weight), while the lowest was obtained from brown callus after four weeks of storage (2.01 ± 0.41 g kg^{-1} dry weight). All samples showed degradation of total pigments (chlorophyll plus carotenoid) after four weeks of storage at −20°C. Furthermore, carotenoid content was found to have increased after storage, while total chlorophyll decreased after storage, in all samples. This suggested that carotenoids were less prone to degradation than chlorophyll when stored at low temperature.

Table VI shows the ratio of chlorophyll *a* to chlorophyll *b* (C*a*/C*b*) and total chlorophylls to total carotenoids C*a* + C*b*/C (x + c), measured before and after storage at −20°C for four weeks. According to Lichtenthaler *et al.* (1981), measurement of both parameters is important as an indicator of functional pigment equipment, plant's greenness and light adaptation of the photosynthetic apparatus. The results showed that the C*a*/C*b* ratio of the extracts increased after storage, except the extract of brown callus which showed that the C*a*/C*b* ratio decreased significantly after four weeks (Table VI). Moreover, the ratio of chlorophylls *a* and *b* to total carotenoids (a + b)/(x + c) was also observed to decrease after storage (Table VI).

In the current study, the colour of the callus was observed to change from green to cream and then brown after two and four months, respectively. The colour transformation of the calli was due to the presence of pigments in the callus, occurring at different concentrations. Our results showed that the concentration of chlorophyll *b* was higher in all samples than chlorophyll *a*, similar to findings by Ling *et al.* (2010) and

Figure 2 Production of coloured callus from leaf explant of *O. stamineus* in vitro



(a) **(b)** **(c)**

Notes: (a) Green callus obtained from MS media plus 1.0 mg L−1 Kin + 1.0 mg L−1 IAA within 2 months;(b) Cream callus obtained from MS media plus 1.5 mg L−1 Kin + 1.5 mg L−1 IAA after 2 months;(c) Brown callus obtained from MS media plus 2.0 mg L−1 Kin after 4 months

Table III Effects of different concentrations of single hormones on mean percentage of explants producing coloured callus

MS + hormone (mg L^{-1})	Green Mean (%) explants produced callus	Cream Mean (%) explants produced callus	Brown Mean (%) explants produced callus
Callus age	<2 months	2-4 months	>4 months
Control	96.67 ± 0.03 ^{d}	NR	3.33 ± 0.33 ^{a}
0.5 Kin	40.00 ± 0.09 ^{a}	13.33 ± 0.06 ^{a}	50.00 ± 0.09 ^{c}
1.0 Kin	NR	43.33 ± 0.09 ^{bcd}	86.67 ± 0.06 ^{d}
1.5 Kin	53.33 ± 0.09 ^{abc}	20.00 ± 0.07 ^{ab}	40.00 ± 0.09 ^{bc}
2.0 Kin	70.00 ± 0.09 ^{bc}	43.33 ± 0.09 ^{bcd}	20.00 ± 0.07 ^{ab}
0.5 IAA	66.67 ± 0.09 ^{bc}	13.33 ± 0.06 ^{a}	33.33 ± 0.09 ^{bc}
1.0 IAA	50.00 ± 0.09 ^{ab}	30.00 ± 0.09 ^{abc}	50.00 ± 0.09 ^{c}
1.5 IAA	40.00 ± 0.09 ^{a}	50.00 ± 0.09 ^{cd}	73.33 ± 0.08 ^{d}
2.0 IAA	76.67 ± 0.08 ^{cd}	56.67 ± 0.09 ^{d}	10.00 ± 0.06 ^{a}

Notes: Data represent mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters within the same column are significantly different at $p < 0.05$ according to Duncan's multiple-range test (DMRT). Kin: Kinetin, IAA: indole-acetic acid, NR: no response

Kiong *et al.* (Ling *et al.*, 2008). It has been suggested that this may be due to chlorophyll *a* deteriorating more rapidly than chlorophyll *b* (Wolf, 1956). According to Lichtenthaler and Buschmann (2001), chlorophyll *b* is exclusively located in the pigment antenna system, while chlorophyll *a* can be found in both the pigment antenna and in the reaction centres of both Photosystems I and II. The level of light-harvesting pigment protein LHC-II of Photosystem II varies, depending on adaptation to surrounding light, for example, shade plants will have a significantly higher amounts of LHC-II (therefore, lower C*a*/C*b* ratios) than sun-exposed plants (Lichtenthaler and Buschmann, 2001). Our results showed that C*a*/C*b* ratio in all samples was low, indicative of an enlargement of the pigment antenna system of Photosystem II (Lichtenthaler and Buschmann, 2001). In the current study, the weight ratio of chlorophylls *a* and *b* to total carotenoid (a + b)/(x + c) was also measured, to indicate the greenness of the samples (Lichtenthaler and Buschmann, 2001). According to Lichtenthaler and Buschmann (2001), lower (a + b)/(x + c) ratio values indicate stress, senescence and damage of the plant photosynthetic apparatus, as a result of faster degradation of chlorophylls than carotenoids. Moreover, the continuous decrease of (a + b)/(x + c) ratio may also be due to the development of chromoplast, which can be clearly observed via the change of plant colour (e.g. fruit) from green to yellow to orange or red (Giusti and Wrolstad, 2001). In our study, the colour of the callus was observed to change with time, from green to cream to brown. The weight ratio of chlorophylls *a* and *b* to total carotenoid [C*a* + C*b*/C(x + c)] was also observed to decrease following the colour change, which implied development of chromoplast in the callus. The C*a* + C*b*/C(x + c) ratio also decreased after storage, possibly due to stress and a faster degradation of chlorophyll than carotenoids. Furthermore, analysis of results also showed that anthocyanin content in the green callus increased significantly after four weeks of storage (Table VII). This may be due to the continued biosynthesis of phenolic compounds after harvest, related to the ripening process (Miguel *et al.*, 2004).

The carotenoid extract was also separated and analysed using HPLC, where it was screened against eight types of carotenoids: neoxanthin, violaxanthin, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene and lutein. Based on the results, only four types of carotenoids were found in the callus, i.e. zeaxanthin, violaxanthin, lutein and β-carotene (Figure 3). Green callus contained the highest amount of violaxanthin, lutein and β-carotene, while cream callus contained the least (Table VII).

Anthocyanin and total phenolic content of the coloured callus extracts were also measured and the results are presented

Table IV Effects of different concentrations and combinations of hormones on mean (%) explants producing coloured callus

MS + hormone (mg L^{-1})	Green Mean (%) explants produced callus	Cream Mean (%) explants produced callus	Brown Mean (%) explants produced callus
Callus age	<2 months	2-4 months	>4 months
Control	96.67 ± 0.03^{g}	NR	3.33 ± 0.03^{a}
0.5 Kin + 0.5 IAA	30.00 ± 0.09^{bc}	70.00 ± 0.09^{ef}	90.00 ± 0.06^{e}
0.5 Kin + 1.0 IAA	33.33 ± 0.09^{cd}	43.33 ± 0.09^{bcde}	76.67 ± 0.08^{de}
0.5 Kin + 1.5 IAA	3.33 ± 0.03^{a}	60.00 ± 0.09^{def}	96.67 ± 0.03^{e}
0.5 Kin + 2.0 IAA	66.67 ± 0.09^{ef}	80.00 ± 0.07^{f}	26.67 ± 0.08^{bc}
1.0 Kin + 0.5 IAA	60.00 ± 0.09^{ef}	56.67 ± 0.09^{cdef}	36.67 ± 0.09^{c}
1.0 Kin + 1.0 IAA	43.33 ± 0.09^{cde}	56.67 ± 0.09^{cdef}	33.33 ± 0.09^{c}
1.0 Kin + 1.5 IAA	53.33 ± 0.09^{def}	46.67 ± 0.09^{bcde}	30.00 ± 0.09^{c}
1.0 Kin + 2.0 IAA	100.00 ± 0.00^{g}	6.67 ± 0.05^{a}	6.67 ± 0.05^{ab}
1.5 Kin + 0.5 IAA	46.67 ± 0.09^{cde}	36.67 ± 0.09^{bcd}	26.67 ± 0.08^{bc}
1.5 Kin + 1.0 IAA	10.00 ± 0.06^{ab}	66.67 ± 0.09^{ef}	93.33 ± 0.05^{e}
1.5 Kin + 1.5 IAA	3.33 ± 0.03^{a}	43.33 ± 0.09^{bcde}	60.00 ± 0.09^{d}
1.5 Kin + 2.0 IAA	66.67 ± 0.09^{ef}	23.33 ± 0.08^{ab}	23.33 ± 0.08^{abc}
2.0 Kin + 0.5IAA	73.33 ± 0.08^{f}	26.67 ± 0.08^{ab}	33.33 ± 0.09^{c}
2.0 Kin + 1.0 IAA	73.33 ± 0.08^{f}	30.00 ± 0.09^{abc}	20.00 ± 0.07^{abc}
2.0 Kin + 1.5 IAA	53.33 ± 0.09^{def}	23.33 ± 0.08^{ab}	36.67 ± 0.09^{c}
2.0 Kin + 2.0 IAA	100.00 ± 0.00^{g}	NR	3.33 ± 0.03^{a}

Notes: Data represent mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters within the same column are significantly different at $p < 0.05$ according to Duncan's multiple-range test (DMRT). Kin: Kinetin, IAA: indole-acetic acid, NR: no response

Table V Comparison of chlorophyll, carotenoid and total pigment contents (g kg^{-1} dry weight) of *O. stamineus* callus before and after four weeks of storage at −20°C

Callus age	Coloured callus	Pigments content (g kg^{-1} dry weight) *Ca*	*Cb*	*Ca* + *Cb*	C (x + c)	Total
<2 months	Green (A)	1.18 ± 0.20^{a}	3.68 ± 0.14^{d}	4.86 ± 0.12^{d}	N/A	N/A
	Green (B)	1.16 ± 0.21^{a}	2.89 ± 0.48^{cd}	4.05 ± 0.42^{c}	0.47 ± 0.25	4.52 ± 0.17
2-4 months	Cream (A)	1.12 ± 0.04^{a}	2.53 ± 0.08^{c}	3.65 ± 0.12^{bc}	0.19 ± 0.04	3.84 ± 0.14
	Cream (B)	0.99 ± 0.04^{a}	2.13 ± 0.30^{bc}	3.11 ± 0.31^{b}	0.43 ± 0.05	3.55 ± 0.36
>4 months	Brown (A)	1.00 ± 0.01^{a}	1.24 ± 0.02^{ab}	2.24 ± 0.03^{a}	0.21 ± 0.02	2.45 ± 0.04
	Brown (B)	0.69 ± 0.32^{a}	1.08 ± 0.51^{a}	1.77 ± 0.31^{a}	0.24 ± 0.12	2.01 ± 0.41

Notes: Means with different letters within the same column are significantly different at $p < 0.05$ according to Duncan's multiple-range test (DMRT). *Ca*: chlorophyll *a*, *Cb*: chlorophyll *b*, *Ca* + *Cb*: total chlorophyll *a* and *b*, C(x + c): total carotenoid (xanthophyll and carotene), A: before, B: after, N/A: not available

Table VI Ratio of pigments content (g kg^{-1} dry weight) of *O. stamineus* callus measured before and after four weeks of storage at −20°C

Callus age	Coloured callus	Pigments content (g kg^{-1} dry weight) *Ca*/*Cb* ratio	*Ca* + *Cb*/C(x + c) ratio
<2 months	Green (A)	0.32	N/A
	Green (B)	0.40	8.62
2-4 months	Cream (A)	0.44	19.21
	Cream (B)	0.46	7.23
>4 months	Brown (A)	0.81	10.67
	Brown (B)	0.64	7.38

Notes: Means with different letters within the same column are significantly different at $p < 0.05$ according to Duncan's multiple-range test (DMRT). *Ca*: chlorophyll *a*, *Cb*: chlorophyll *b*, *Ca* + *Cb*: total chlorophyll *a* and *b*, C(x + c): total carotenoid (xanthophyll and carotene), A: before, B: after, N/A: not available

in Table VIII. In general, it was observed that the anthocyanin content of the coloured callus appeared constant and stable with the exception of the green callus, even after four weeks of storage. The green callus extract showed an approximately twofold increase in anthocyanin content after storage (Table VIII). On the other hand, the total phenolic content of the extracts was observed to reduce after storage, albeit the decrease was not significant in the brown callus extract. The highest anthocyanin content was found in green callus extract after four weeks of storage (24.12 ± 4.87 g kg^{-1} dry weight), while cream callus extract before storage exhibited the highest total phenolic content (546.61 ± 93.49 μg GA g^{-1} of extract). Total phenolic contents in the callus extracts ranged from 7.95 to 546.61 μg GA/g extract (Table VIII), lower than the amount recorded in in vivo grown *O. stamineus* plants (Ibrahim and Jaafar, 2013). However, synthesis of secondary metabolites

Table VII Distribution and amount of individual carotenoids present in coloured callus of *O. stamineus*

Callus age	Coloured callus	Individual carotenoid (μg/g DW) Violaxanthin	Zeaxanthin	Lutein	β-carotene
<2 months	Green	6.320 ± 0.942 [a]	17.993 ± 0.899 [c]	103.617 ± 16.100 [b]	185.823 ± 47.734 [b]
2-4 months	Cream	3.493 ± 2.153 [a]	10.230 ± 0.178 [a]	22.920 ± 9.440 [a]	ND
>4 months	Brown	6.503 ± 0.715 [a]	13.833 ± 1.140 [b]	34.817 ± 12.392 [a]	0.250 ± 0.029 [a]

Note: Means with different letters within the same column are significantly different at $p < 0.05$ according to Duncan's multiple-range test (DMRT). ND: not detected

Figure 3 HPLC chromatograms of green, cream and brown callus, showing presence of violaxanthin, zeaxanthin, lutein and β-carotenoid.

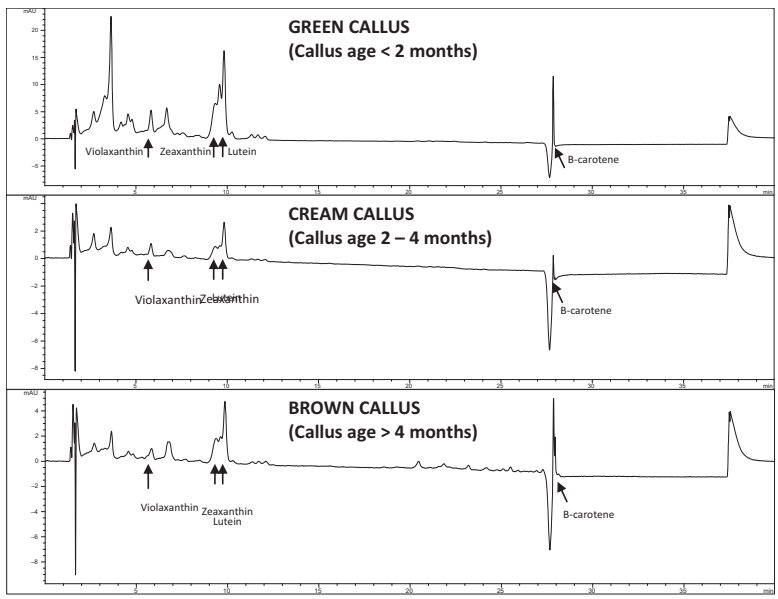


Table VIII Comparison of anthocyanin (g kg^{-1} dry weight) and total phenolic content (μg GA/g of extract) of *O. stamineus* callus before and after four weeks of storage at −20°C

Callus age	Coloured callus	Anthocyanin (g kg^{-1} dry weight)	Total phenolic content (μg GA/g of extract)
<2 months	Green (A)	10.33 ± 0.36 [a]	347.21 ± 66.93 [c]
	Green (B)	24.12 ± 4.87 [b]	7.95 ± 2.07 [a]
2-4 months	Cream (A)	3.73 ± 0.91 [a]	546.61 ± 93.49 [d]
	Cream (B)	3.28 ± 1.99 [a]	303.15± 1.90 [bc]
>4 months	Brown (A)	5.23 ± 1.79 [a]	180.36 ± 22.03 [b]
	Brown (B)	6.90 ± 2.56 [a]	170.93 ± 10.64 [b]

Notes: Means with different letters within the same column are significantly different at $p < 0.05$ according to Duncan's multiple-range test (DMRT). A: before, B: after

(such as phenolic compounds) through tissue culture would still prove to be beneficial, especially through callus induction, as mass production of callus containing these valuable secondary metabolites can easily be achieved through the use of bioreactors.

DPPH radical scavenging assay

All callus extracts exhibited DPPH scavenging activity (Figure 4). DPPH radical scavenging activity (indicated by lowest IC_{50} value) was observed in the following order: green callus > brown callus > cream callus. Interestingly, the antioxidant activities of the extracts were observed to increase after four weeks of storage at −20°C. The highest increase in antioxidant potential was exhibited by the brown callus extract, which showed a more than 50 per cent reduction in its IC_{50}

Figure 4 Antioxidant potential (IC_{50}) of *O. stamineus*-coloured callus extract

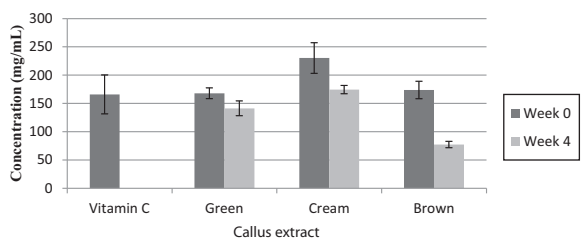


Table IX Pearson's correlation coefficients between the variables

Variables	TPC	TAC	Carotenoid content
DPPH, IC_{50}	0.701*	−0.288	−0.165

Note: *Correlation is significant at the 0.05 level

value. The antioxidant potential of the callus extract was more likely to be due to its anthocyanin and carotenoid content, as shown by the negative correlation observed (Table IX). The negative correlations indicate that the DPPH IC_{50} values decreased with increasing anthocyanin and carotenoid content. However, these correlations were weak.

The increase in antioxidant potential may also be due to the presence of other phenolic compounds in the samples, as some phenols will exhibit higher antioxidant potential after storage. Dourtoglou *et al.* (2006) suggested that not all phenolics are affected in the same manner during storage, as they presented a different evolution pattern throughout the storage period. Phenolic compounds are also susceptible to decomposition as they are sensitive to temperature, oxygen and UV-light (Arnnok *et al.*, 2012). Apart from that, based on the correlation analysis (Table IX), it can be deduced that the antioxidant potential exhibited by the callus extracts could also be partly due to the presence of phenolic compounds. This is in agreement with the findings by Khamsah *et al.* (2006) which stated that antioxidant activities exhibited by the methanolic extracts of *O. stamineus* collected from different geographical origins are not solely due to the presence of phenolic compounds.

Conclusions

Addition of Kin with IAA to the media improved callus formation, compared to when Kin was singly applied. Optimum production of green callus was achieved with addition of 2.0 mg L^{-1} Kin plus 1.0-2.0 mg L^{-1} IAA to the media, while cream callus in 0.5 mg L^{-1} Kin plus 2.0 mg L^{-1} IAA and brown callus in 0.5 mg L^{-1} Kin plus 1.5 mg L^{-1} IAA. Green callus contained the highest amount of chlorophylls, carotenoid and anthocyanin, while cream callus contained the highest amount of phenolic compounds. The amount of pigments and secondary metabolites in the callus extracts decreased after four weeks of storage at −20°C, except anthocyanin. The antioxidant potential of the extracts also increased after storage.

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