

# Ultrastructural and biochemical alterations during browning of pigeon orchid (*Dendrobium crumenatum* Swartz) callus

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**Abstract** The phenomenon of browning can be a major limitation for orchid callus culture, causing a loss of regenerative capacity and subsequent cell death. This research was conducted to determine the effect of in vitro culture period (1–3 months) on the appearance of tissue browning. Biological alterations at the cellular and subcellular levels, as well as biochemical aspects, were examined. Callus derived from bisected pigeon orchid (*Dendrobium crumenatum* Swartz) protocorms were cultured on modified Vacin and Went solid medium supplemented with 0.5 mg L<sup>-1</sup> 1-naphthaleneacetic acid and 1 mg L<sup>-1</sup> 6-benzyladenine. Callus that was not subcultured was collected at 1-month intervals and examined for structural (using scanning electron microscopy and transmission electron microscopy) and biochemical alterations associated with browning. Three-month-old unincubated callus cells were loosely arranged and major organelles were deformed, exhibiting nuclear envelope breakage, dysfunctional mitochondria, tannin-filled vesicles and swollen chloroplasts, relative to 1-month-old green callus, which served as the control. Ultrastructural

disorganization involving the nucleus, mitochondria and chloroplasts typified enzymatic oxidative browning. Browning 3-month-old callus had significantly higher polyphenol oxidase (PPO) activity and total phenolic content than control callus. The levels of PPO and total phenolics may serve as useful biochemical markers when selecting suitable callus for subsequent regeneration trials or for particle bombardment in genetic transformation experiments.

**Keywords** Browning · Phenolic content · Pigeon orchid · Polyphenol oxidase · Swollen chloroplast · Tannin

## Abbreviations

BA	6-Benzyladenine
Chl	Chlorophyll
CIM	Callus induction medium
NAA	1-Naphthaleneacetic acid
PAL	Phenylalanine ammonia lyase
PAS	Periodic acid-Schiff
PCD	Programmed cell death
POD	Peroxidase
PPO	Polyphenol oxidase
RER	Rough endoplasmic reticulum (ER)
ROS	Reactive oxygen species
SER	Smooth ER
SDS	Sodium dodecyl sulfate
TBO	Toluidine blue O
VW	Vacin and Went medium

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## Introduction

Tissue browning is a serious obstacle in the maintenance of plant tissue cultures, and is believed to be caused by the

accumulation and oxidation of phenolic compounds (Jones and Saxena 2013) resulting in the inhibition of growth and a decrease in the regeneration ability of plant cells (Arnaldos et al. 2001; Ling et al. 2007; Chugh et al. 2009; Mondal et al. 2013). Yoruk and Marshall (2003) revealed many factors responsible for tissue browning, including the presence and action of reactive oxygen species (ROS), phenolic compounds and oxidative enzymes, all of which are involved in the process of phenolic oxidation.

In general, ROS can be generated during normal plant metabolic processes, namely photosynthesis and respiration, and their levels can increase in response to abiotic stresses such as cold, UV light, salinity and drought (Sharma et al. 2012; Tripathy and Oelmüller 2012). Moreover, mechanical damage in vitro, such as wounding during explant preparation, is a key factor attributed to browning during callus induction as a result of an increase in ROS (Wang et al. 2011). Various organelles and cellular structures such as chloroplasts, mitochondria, peroxisomes, plasmalemma, the endoplasmic reticulum (ER) and cell walls are the sites of production and scavenging of ROS in response to stress (Gill and Tuteja 2010). This excessive generation of ROS can induce oxidative damage in various cellular components such as the deformation of chloroplasts, disintegration of the plasmalemma, and breakage of the nuclear membrane, all of which are ultimately caused by the oxidation of proteins, the peroxidation of lipids, and hydroxylation of nucleic acids (Mittler 2002; Gill and Tuteja 2010). The appearance of excess ROS, which is accompanied by organellar disorganization, results in cell death (Kratsch and Wise 2000; Wi et al. 2005; Wahid et al. 2007; Sharma et al. 2012), which can be visualized by the browning colour of cells.

Liu and Chen (2010) reported that the production of ROS in *Phalaenopsis* spp., including the hydroxyl radical ( $\cdot\text{OH}$ ), superoxide anion ( $\text{O}_2\cdot^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) increased significantly during callus induction. This overproduction of ROS indicated the presence of oxidative stress negatively affected plant growth and development (de Pinto and de Gara 2004; Pitzschke et al. 2009). Zhou et al. (2009) had earlier shown that the presence of gibberellic acid repressed *Phalaenopsis* callus browning as a result of repressed phenylalanine ammonia lyase (PAL; EC 4.3.1.5) activity and gene expression.

Several oxidative enzymes are involved in enzymatic browning, namely PAL, peroxidase (POD, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.10.3.1) (Xu and Li 2006; Yingsanga et al. 2008). In particular, PPO plays a more crucial role than other enzymes (He et al. 2009). PPO is a copper-containing enzyme implicated in two oxidative reactions associated with the oxidation of phenolic compounds, including the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones

(Mayer 2006). These highly reactive quinones can then polymerize with other quinones or amino acids to form brown melanin-like pigments which are the major cause of enzymatic browning and are extremely harmful to plant cells (Gacche et al. 2006; Oren-Shamir 2009). These pigments cause the visual symptoms commonly termed “browning” in plant tissue culture. PPO is localized on the thylakoid membrane of chloroplasts while phenolic substrates are present in the vacuole (Toivonen and Brummell 2008). This enzyme-substrate compartmentation can prevent the occurrence of in vivo oxidation. However, the latent form of PPO can be activated when it is released from plastids after degradation of the thylakoid membrane followed by chloroplast breakdown caused by oxidative damage (Dai 1993; Wang et al. 2010). In addition, physical damage such as explant preparation or wounding can break down this compartmentation, increasing oxidation and thus browning (North et al. 2012). Incidentally, root growth of tree peony in vitro also increased the activity of PPO (Fu et al. 2011). This active PPO then mixes with the vacuolar substrates (i.e., phenolic compounds) inducing the formation of toxic *o*-quinone which leads to the production of a brown complex pigment (Ahmad et al. 2013).

Tang and Newton (2004) reported that maximum PPO activity, which was observed in a non-browning callus line of *Pinus virginiana* Mill., gradually declined over a 3-week culture period. However, PPO activity increased dramatically in the first 3 weeks and increased continuously during culture of a browning callus line. It might, in the latter case, have been due to the presence of oxidative stress during the extended culture period, but ultimately proving, through a simple experimental design, that PPO activity is unequivocally associated with browning.

In various plant species, embryogenic callus has totipotency and is thus usually utilized as the target material for studying somaclonal variation, somatic hybridization and genetic transformation (Belarmino and Mii 2000; Hossain et al. 2013). In particular, indirect somatic embryogenesis via an intervening callus phase can provide a high rate of formation and conversion of somatic embryos into plantlets (Zhao et al. 2008). This callus-mediated regeneration is also one of the favourite techniques for rapid in vitro propagation and for biotechnological applications that are widely used in many plants, including orchids (Tokuhara and Mill 2001; Debnath et al. 2006). Embryogenic callus is in fact, in *Cymbidium*, a cluster of immature protocorm-like bodies, or somatic embryos (Teixeira da Silva and Tanaka 2006). Successful callus culture is based on its ability to be maintained for an extended period of time—ideally without changes in regenerative ability—and to regenerate whole plants when needed. Unfortunately, in certain orchids, callus culture is still limited due to its slow growth, difficulties in

maintaining callus cultures and their tendency to brown and become necrotic (Begum et al. 1994; Chang and Chang 1998; Khosravi et al. 2008). Although callus can be maintained by frequent subcultures onto fresh media, this technique can give rise to somaclonal variation (Bairu et al. 2011). Furthermore, culturing callus without subculture can increase the accumulation of free radicals, lipid peroxidation and also arrest the morphogenic transition of plant cells (Benson 2000; George 2008). Therefore, efficient callus culture without signs of browning is a key requirement in orchid tissue culture, and thus genetic transformation. To better understand the phenomenon of browning during orchid tissue culture, the incubation period and how it affects the biological response at the biochemical and cellular and/or subcellular levels must be determined. This is important since the timing of sampling can influence the interpretation of results (Teixeira da Silva and Dobránszki 2013), and thus negative aspects that hinder the in vitro development of callus will also thus reflect themselves in the quantitative output of that study.

*Dendrobium crumenatum* Swartz (pigeon orchid), a common epiphytic orchid widely distributed in South East Asia such as India, Sri Lanka, Myanmar, Indo-China, Malay Peninsula, Philippines and Taiwan (Leong and Wee 2013), has a very unique and attractive white and fragrant flower. This orchid also exhibits synchronous flowering which is triggered by a sudden drop in temperature (about 10 °C), usually after heavy rainfall and then blooms 9 days later (Meesawat and Kanchanapoom 2007). This orchid species can be a good choice for studying the phenomenon of browning because it displays rapid growth and provides a high rate of callus induction and proliferation (Meesawat and Kanchanapoom 2002). Meesawat and Kanchanapoom (2002) reported that axillary bud-derived callus could be induced on a modified Vacin and Went (VW) solid medium (Vacin and Went 1949) containing 0.1 mg L<sup>-1</sup> 1-naphthaleneacetic acid (NAA) and 1 mg L<sup>-1</sup> 6-benzyladenine (BA) within 4 weeks of culture. Other than these studies, the in vitro culture of this orchid has not been described.

The effects of oxidative stress induced by an extended period of culture in vitro at the biochemical level have been revealed (Xu and Li 2006; Zhao et al. 2010; Shi 2011), to a very limited extent in orchids (*Phalaenopsis* and *Cymbidium*), but alteration at the subcellular level associated with browning phenomenon are still poorly characterized even though the brown tissue is easy to visualize.

Therefore, the aim of this study was to determine the level of browning in callus cultures of a potential model orchid in response to subculture period during callus culture to better understand the ultrastructural dynamics underlying callus browning. This process was assessed by ultrastructural and biochemical analyses. A clearer understanding of

the phenomenon of browning provides valuable insight about the browning process and could be used in a practical way to prevent or reduce tissue browning during callus culture of *D. crumenatum* or other important orchids that show similar browning of callus or other tissues in vitro. Knowledge of the biochemical nature of browning callus would also allow for the selection of the most suitable callus for genetic transformation experiments, for example, particle bombardment.

## Materials and methods

### Plant material

*Dendrobium crumenatum* Swartz plants were cultivated in a shaded greenhouse at a photosynthetic photon flux density (PPFD) of 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  under natural conditions (approximately 28  $\pm$  2 °C) at the Department of Biology, Faculty of Science, Prince of Songkla University, Thailand. Blooming flowers of more than five-year-old mother plants were hand cross-pollinated and 6-week-old capsules were surface-sterilized in 1.2 % sodium hypochlorite (NaOCl) solution (1.14 % active chlorine) containing 1–2 drops of Tween 20 for 20 min and rinsed three times with sterile distilled water. Each capsule was then cut longitudinally and seeds were scooped out and placed into 30 mL of Vacin and Went (VW) (Vacin and Went 1949) liquid medium containing 2 % sucrose and 2 % peptone. The pH of the media was adjusted to 5.3 with 1 N NaOH or HCl prior to autoclaving at 121 °C for 20 min. Seeds, which were subcultured monthly, were maintained under a 16 h photoperiod provided by cool daylight fluorescent lamps (36 Watts, Philips, Bangkok, Thailand) at 23  $\mu\text{mol m}^{-2} \text{s}^{-1}$  on an orbital shaker (120 rpm) at 25  $\pm$  2 °C for 3 months. The protocorms that formed were used as explants for callus induction.

### Callus induction

For callus induction, 3-month-old protocorms (approximately 2–3 mm in diameter) at stage 3 of developmental growth (Stewart and Zettler 2002) were transversely bisected without excising the shoot tip and placed on 10 mL of modified VW solid medium consisting of VW macro salts (full strength), Murashige and Skoog (1962; MS) micro salts (half strength), 2 % sucrose, 2 % peptone, 0.5 mg L<sup>-1</sup> 1-naphthaleneacetic acid (NAA) (Fluka Chemie GmbH, Buchs, Switzerland) and 1 mg L<sup>-1</sup> 6-benzyladenine (BA) (Sigma-Aldrich Co., St. Louis, MO, USA). The medium was solidified with 0.2 % Phytigel (Sigma-Aldrich Co.) and is referred to hereafter as callus induction medium (CIM). The pH of the media was adjusted to 5.3

with 1 N NaOH or HCl prior to autoclaving at 121 °C for 20 min. The transversely bisected protocorms (4 segments per 60 mL glass bottle) were then cultured under conditions of *in vitro* seed culture as described previously. Green compact callus derived from bisected protocorms obtained after 8 weeks of culture and subcultured at 1-month intervals served as the material for subsequent analyses. All CIM-related media constituents were based on optimized in-house trials.

#### Callus culture

In order to determine the effect of culture period on callus culture, callus [100 mg, fresh weight (FW)] was transferred onto 60 mL glass bottle contained 10 mL of CIM. Callus was maintained continually on the same CIM without subculturing under the same culture conditions indicated above. One-month-old proliferated callus served as the control. Samples in which callus was predominant (>80 % of the explant) were collected at monthly intervals for 3 months and subjected to ultrastructural and biochemical analyses.

#### Histological and histochemical analyses

Ten pieces of callus at the end of each culture period were fixed in FAA II [formaldehyde (Ajax Finechem, Taren Point, Australia); glacial acetic acid (J.T. Baker, Phillipsburg, NJ, USA); 70 % ethyl alcohol (Merck, Billerica, MA, USA); 5:5:90 v/v/v] for 48 h (Ruzin 1999). Fixed tissues were dehydrated in a tertiary-butyl-alcohol series and embedded in paraffin wax (Histoplast PE; Richard-Allan Scientific, Kalamazoo, MI, USA). Sections 6 µm thick were cut with a rotary microtome (Shandon Southern Product Ltd., Cheshire, UK). Callus sections were stained with Delafield's hematoxylin and safranin (Ruzin 1999) to investigate general structures. To indicate the characteristic meristematic cells based on qualitative observations, histochemical analyses, namely the periodic acid-Schiff (PAS) reaction (Feder and O'Brien 1968) and toluidine blue O (TBO) staining (Ruzin 1999), were performed to examine the presence of carbohydrates (insoluble polysaccharide) and phenolic compounds, respectively. All sections were viewed under an Olympus BX 51 TRF light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and photographs were captured on an Olympus DP 72 digital camera (Olympus Optical Co. Ltd.).

#### Scanning electron microscopy

Scanning electron microscopy (SEM) was performed to investigate alterations in cellular structures during callus culture. Six samples were fixed in SEM fixative [10 %

formaldehyde (Ajax Finechem), 5 % acetic acid (J.T. Baker), 45 % ethanol (Merck) and 1 % Triton X-100 (Panreac, Barcelona, Spain)] at 4 °C for 2 h. Samples were washed in 0.1 M phosphate buffer (pH 7.2) and dehydrated through an ethanol series including 30, 50, 70, 80, 90, 95 and 100 % ethanol (15 min each wash). Samples were dried with a Polaron CPD 7501 critical point dryer (VG. Microtech, East Sussex, UK), covered with gold by a SPI-MODULE sputter coater (SPI Supplies Division of Structure Probe Inc., West Chester, PA, USA), and examined with a Quanta 400 scanning electron microscope (FEI, Brno, Czech Republic) at an accelerating voltage of 10 kV.

#### Ultrastructural analysis

To determine the influence of browning on subcellular structures, six intact callus clumps were fixed overnight in a cold (4 °C) solution of 2.5 % glutaraldehyde (Sigma-Aldrich Co.) in 0.1 M phosphate buffer (pH 7.2) to which 1 % caffeine (Sigma-Aldrich Co.) was added. Caffeine was added to precipitate phenolics, i.e., to localize phenolic compounds within plant cells (Mueller and Greenwood 1978). Pre-fixed samples were washed three times with 0.1 M phosphate buffer (pH 7.2) and post-fixed with 1 % osmium tetroxide (OsO<sub>4</sub>) (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temperature. The post-fixed tissues were washed three times with distilled water, dehydrated through a graded ethanol series (70, 80, 90 and 100 % ethanol) for 15 min each and infiltrated with EMBED 812 (Electron Microscopy Sciences). Ultrathin sections (60 nm thick) were cut using a RMC MTXL ultramicrotome (Boeckeler Instruments, Arizona, TX, USA) and stained in 2 % uranyl acetate (Electron Microscopy Sciences) for 10 min followed by lead citrate (Electron Microscopy Sciences) for 5 min to obtain optimum contrast (Reynolds 1963). Lead citrate was prepared by mixing solution A (1.33 g Pb(Na<sub>3</sub>)<sub>2</sub> in 15 mL of distilled water) with solution B (1.7 g Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>·2H<sub>2</sub>O in 15 mL of distilled water) (at a 1:1 ratio). Sections were viewed with a JEM-2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 160 kV.

#### Preparation of extract

Cultured callus at each collection (1, 2 and 3 months of inoculation) were harvested and prepared as aliquots for the determination of PPO activity and total phenolic content. The extraction was carried out as described by Tang and Newton (2004) with some modifications. Callus (400 mg FW) was homogenized in 400 µL of 0.1 M sodium phosphate buffer (pH 7.2) supplemented with

0.1 % (w/v) sodium dodecyl sulfate (SDS) (EMD Millipore Co., Billerica, MA, USA). The homogenate was centrifuged at  $12,000\times g$  for 10 min at 4 °C in a Mikro 200R refrigerated centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatant was collected, and aliquots (100  $\mu$ L of supernatant) were stored at -20 °C for the determination of PPO activity and total phenolic content.

#### PPO activity assay

To evaluate PPO activity using a modified method of Dai (1993), 10  $\mu$ L of crude extract was mixed with a reaction mixture containing 150  $\mu$ L of 0.5 M catechol (Sigma-Aldrich Co.) and 590  $\mu$ L of 0.1 M sodium phosphate buffer (pH 7.2). The absorbance of samples was measured at 490 nm for 3 min at 15-s intervals with a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific, Somerset, NJ, USA). One unit of PPO activity was defined as the amount of enzyme that caused a change of 0.01 in the absorbance per min (Dai 1993).

#### Determination of total phenolic content

The assay of total phenolic content was performed according to Park et al. (2006) with some modifications to determine the total phenolic content during callus culture without subculture. Briefly, 10  $\mu$ L of the supernatant was mixed with 200  $\mu$ L of Folin-Ciocalteu phenol reagent (Sigma-Aldrich Co.) and 1 mL of 10 % sodium carbonate (Ajax Finechem). After incubating the solution for 20 min at room temperature, the absorbance of the solution was measured at 735 nm with a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific). The total phenolic content was calculated based on a gallic acid calibration curve and expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh weight ( $\text{mg GAE g}^{-1}$  FW).

#### Determination of chlorophyll and carotenoid contents

Callus (100 mg FW) was ground on ice with a chilled mortar and pestle in 2.3 mL of 80 % acetone in the dark. Homogenized tissue was centrifuged at  $10,000\times g$  for 10 min at room temperature with a Sorvall Biofuge PICO centrifuge (Kendro Laboratory Products, Osterode, Germany). The optical density of the supernatant was measured with an HP-8453E UV–visible spectrophotometer (Hewlett Packard, Palo Alto, CA, USA) at 480, 510, 645 and 663 nm. Total chlorophyll (chl), chl *a*, chl *b* and carotenoid contents were calculated in  $\text{mg g}^{-1}$  FW using the following formulae (Misra et al. 2010):

$$\begin{aligned}\text{Total Chl} &= [20.2(A_{645}) + 8.02(A_{663})] \times V/1,000 \times \text{FW}; \\ \text{Chl}a &= [12.7(A_{663}) + 2.63(A_{645})] \times V/1,000 \times \text{FW}; \\ \text{Chl}b &= [22.9(A_{645}) - 4.68(A_{663})] \times V/1,000 \times \text{FW}; \\ \text{Carotenoid} &= [7.6(A_{480}) - 2.63(A_{510})] \times V/1,000 \times \text{FW}.\end{aligned}$$

where  $A_{480}$ ,  $A_{510}$ ,  $A_{645}$  and  $A_{663}$  are the absorbance values (optical density) at 480, 510, 645 nm and 663 nm, respectively; V is the volume of extracted solution (mL); FW is the fresh weight of callus (g).

#### Statistical analyses

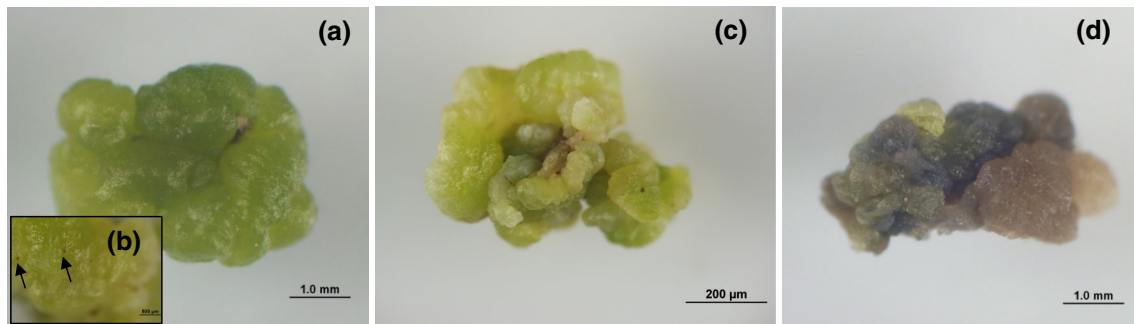
The effects of culture period on PPO activity, total phenolic content and pigment content were determined. Each experiment in the biochemical assays was designed according to a completely randomized design and repeated twice with three replicates per treatment. Data were analyzed statistically using a one-way analysis of variance (ANOVA) and significant differences between means were determined by Duncan's multiple range test at  $P \leq 0.05$  (Bewick et al. 2004) using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) software.

## Results and discussion

### Morphological, histological and histochemical features during callus culture

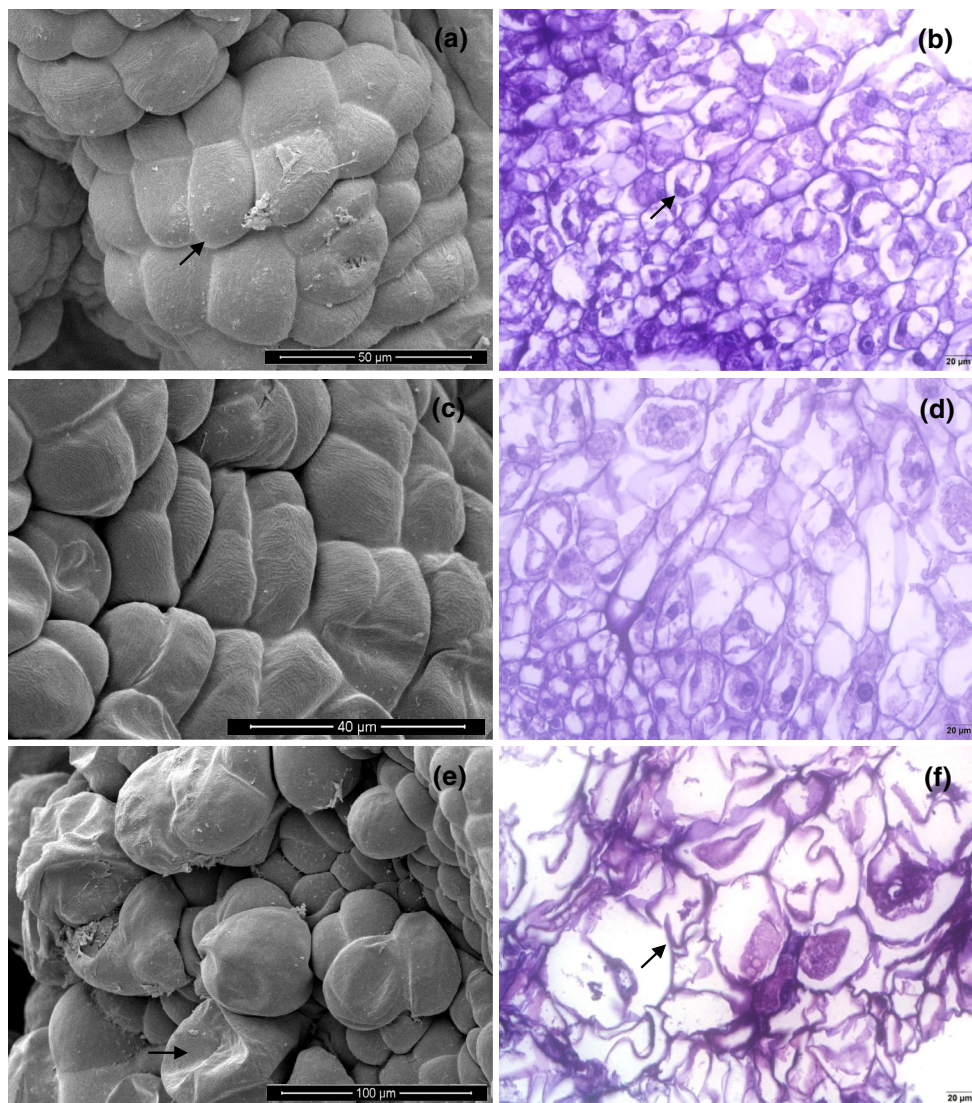
Bisected protocorm-derived callus (Fig. 1a) was cultured on modified VW medium. Callus discoloration was observed after 3–4 weeks as small brown spots on the surface of the callus mass (Fig. 1b). This callus gradually turned pale green after 2 months in culture (Fig. 1c) and finally turned dark brown within 3 months (Fig. 1d). Callus discoloration was attributed to oxidative browning which is a consequence of phenolic oxidation that induces the formation of brown pigments (Sapers et al. 2002).

Changes at the cellular and subcellular levels were also detected and monitored during the 3-month callus culture period. The histological study demonstrated that the 1-month-old callus of *D. crumenatum* consisted of uniform and tightly arranged parenchymatous cells (Fig. 2a, b). However, there were no obvious differences between the 2-month-old callus (Fig. 2c, d) and 1-month-old green callus. In contrast, a loose and disordered cell arrangement was clearly noticed in 3-month-old callus displaying browning (Fig. 2e, f). Laukkanen et al. (2000) reported that yellow-brown callus of Scots pine exhibited a thick cell wall and the absence of cytoplasm compared with green callus. Changes in colour and cell structure of callus were also examined in brown and non-oxidized callus of



**Fig. 1** Morphological features of *Dendrobium crumenatum* callus cultured on modified VW medium. **a** One-month-old callus with green colour; **b** callus with brown spots on its surface after 3–4 weeks

of culture; **c** 2-month-old pale green callus; **d** 3-month-old dark brown callus. (Color figure online)



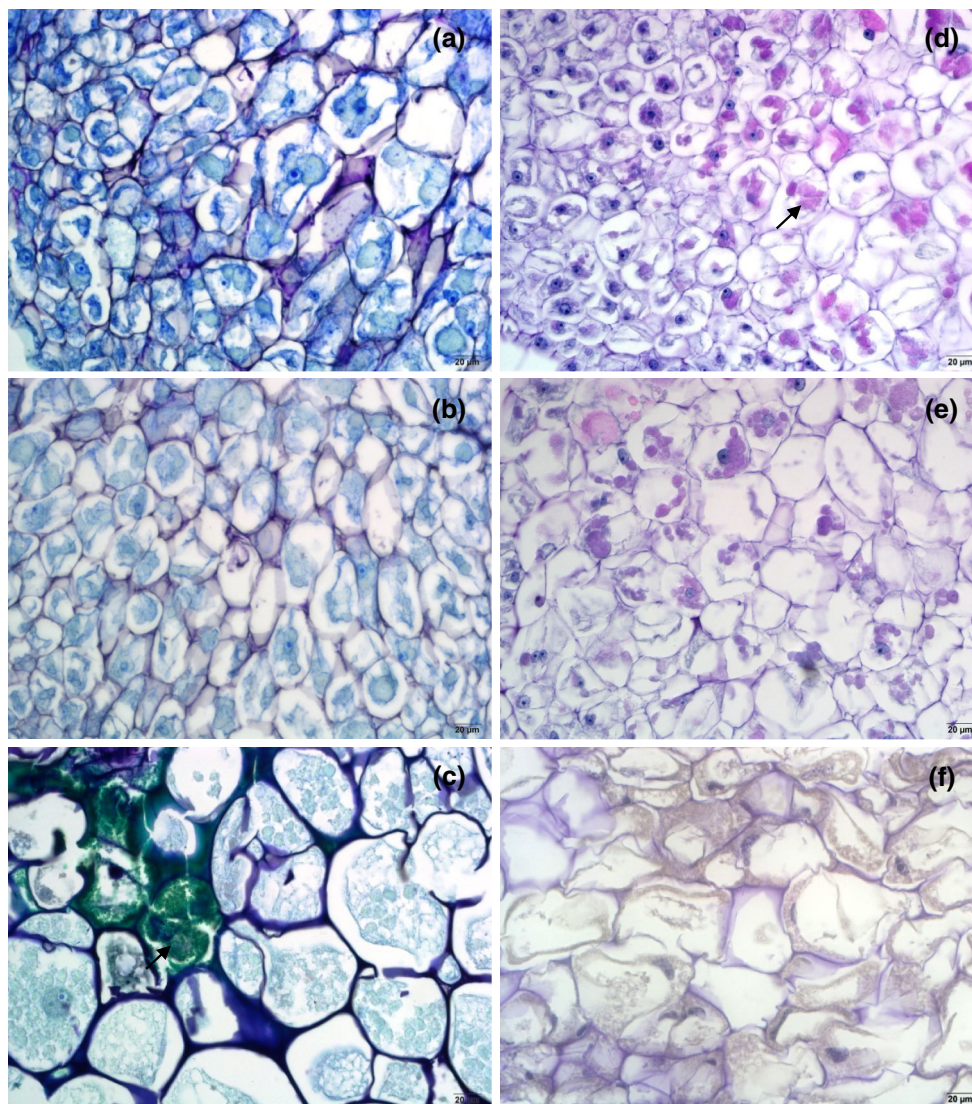
**Fig. 2** Cellular structure of bisected protocorm-derived callus of *Dendrobium crumenatum*. **a, b** 1- and **c, d** 2-month-old callus exhibiting uniform and tightly arranged parenchyma cells (arrows)

compared to **e, f** 3-month-old browning callus showing variable and disordered cells with partially collapsed cells (arrows)

*Jatropha curcas* (He et al. 2009). In the latter study, it was revealed that cells of non-oxidized callus were spheroid and compactly arranged while irregular and loosely arranged cells were observed in brown callus.

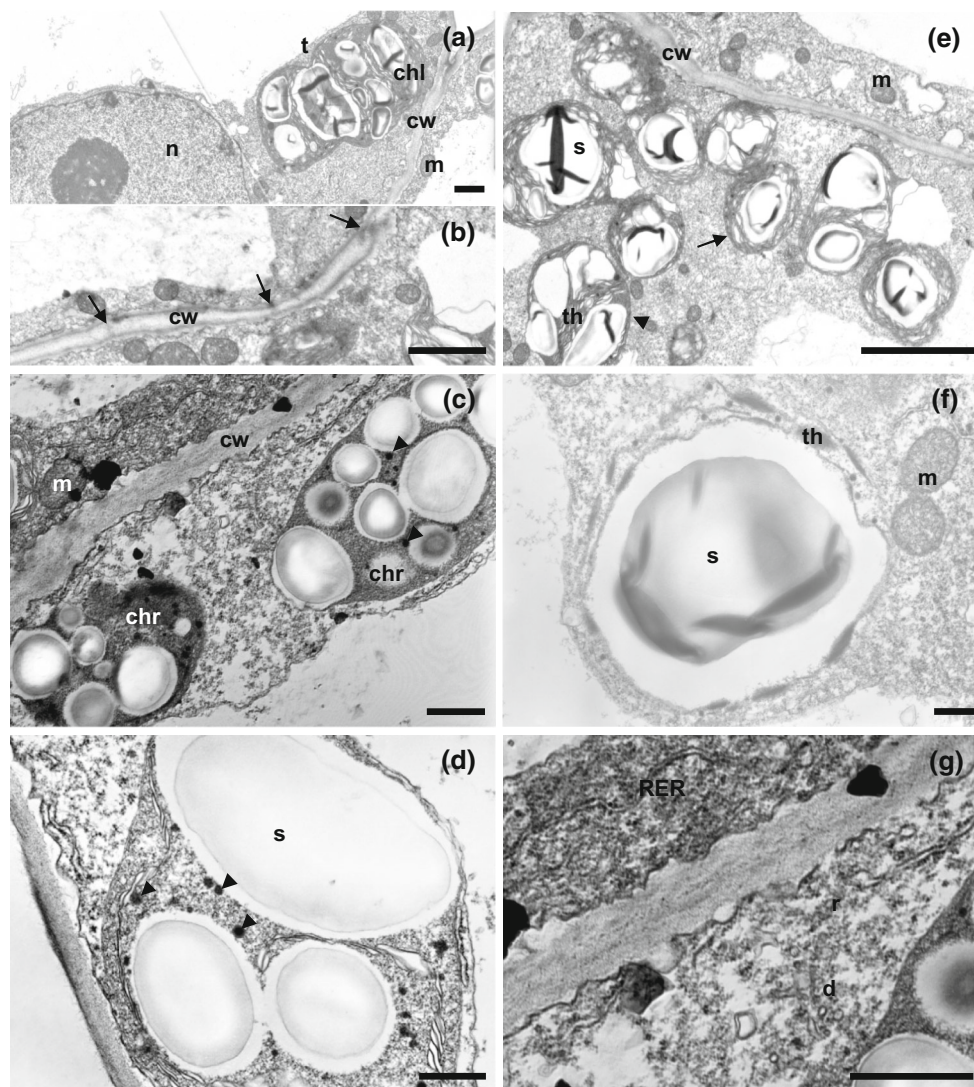
Histochemical observations showed the accumulation of ergastic substances such as tannin (TBO staining)—a phenolic compound—and insoluble carbohydrate (PAS reaction) during callus culture. One- and 2-month-old callus exhibited a blue to dark blue colour which indicated clear nuclei from which tannin was absent (Fig. 3a, b). In contrast, 3-month-old callus with a greenish-blue colour (Fig. 3c) after TBO staining revealed an increase in tannin accumulation more than that of 1-month-old callus. Tannin

is a phenolic by-product and is considered to be a waste product that has adverse physiological effects on plant cells, contributing to cell growth inhibition and eventually cell death (Santiago et al. 2000). Moreover, the 1- and 2-month-old callus showed a greater accumulation of insoluble carbohydrate (Fig. 3d, e) than in 3-month-old callus (Fig. 3f). This corresponds to the histological detection of carbohydrates in the embryogenic callus of *Vanilla planifolia* (Palama et al. 2010). Thus, the present results indicate that browning callus had accumulated little insoluble carbohydrates leading to a lack of an energy sources required for further development which could result in the loss of totipotency in morphogenically competent cells (George 2008).



**Fig. 3** Histochemical observations in **a, d** 1-month-old callus, **b, e** 2-month-old callus and **c, f** 3-month-old callus obtained from bisected *Dendrobium crumenatum* protocorms. After TBO staining, **a** 1- and **b** 2-month-old callus show a blue colour indicating the absence/or a low amount of tannin-like material whereas **c** 3-month-old browning

callus exhibits a green colour showing the accumulation of tannin (arrow). PAS reaction detects the presence of insoluble polysaccharides with a magenta colour (arrow) in the **d** 1- and **e** 2-month-old callus compared to **f** negative PAS staining observed in the browning callus. (Color figure online)



**Fig. 4** TEM micrographs of 1-month-old callus cultured on modified VW medium. **a** Panoramic view of 1-month-old cell showing distinct tonoplast (*t*), thinning cell wall (*cw*), regular nucleus (*n*) and numerous mitochondria (*m*) (*bar* 2  $\mu\text{m}$ ). Magnified views of **b** plasmodesmata (*arrows*) (*bar* 1.25  $\mu\text{m}$ ), **c** chromoplast (*chr*) with

plastoglobuli (*arrow heads*) (*bar* 1  $\mu\text{m}$ ), **d** plastoglobuli (*arrow heads*) in chloro-chromoplast (*bar* 500 nm), **e** chloroplasts (*chl*) containing starch grain (*s*) (*bar* 5  $\mu\text{m}$ ), **f** amyloplast (*bar* 500 nm), **g** dictyosome (*d*), free ribosomes (*r*) and rough ER (RER) within the cytoplasm (*bar* 500 nm)

### Ultrastructural analysis

Alterations to subcellular characteristics during callus culture are described next based on the age of the callus. Throughout this paper, the term callus has been used to represent the singular and plural forms of callus (Teixeira da Silva 2012).

#### One-month-old callus

In the 1-month-old callus showing a strong green colour, a thin cell wall, tonoplast and plasmodesmata were clearly observed (Fig. 4a, b). The well-organized organelles,

including a nucleus, mitochondria and chloroplasts, were very apparent in the cytoplasm (Fig. 4c–g). Globular chromoplasts (Fig. 4c) and other plastids, including chloro-chromoplasts (Fig. 4d), were noticed. These plastids contained small plastoglobuli, which are lipoprotein particles in plastids that contain lipid isoprenoid-derived metabolites (Br  h  lin et al. 2007). Plastoglobuli have been shown to play a key role in the chloroplast to chromoplast transition and carotenoid metabolism (Bian et al. 2011).

This study exhibited two types of starch-containing plastids: chloroplasts (Fig. 4e) and amyloplast (Fig. 4f). This peculiarity was consistent with the ability of plastids to differentiate or redifferentiate between these and several



other forms, depending on the function in the cell, as shown in duckweed (*Spirodela polyrhiza*), a monocotyledonous plant (Wang and Messing 2012). Moreover, this starch-containing amyloplast could function as a source and sink organelle for starch biosynthesis as it still retained a simple thylakoid membrane (Wang and Messing 2012). The appearance of this organelle at the subcellular level coincided with the presence of carbohydrates after PAS staining, indicating that large numbers of amyloplasts in plant cells exist, representing active metabolism of cells, and might play an important role in a cell's ability to regenerate from non-browning callus of pigeon orchid (Zenkteler and Kwaśna 2007).

Furthermore, a dense cytoplasm that was rich in free ribosomes, cisternae of rough or granular ER and dictyosomes was also present (Fig. 4g). Appezzato-da-Glória and Machado (2004) revealed that a ribosome-rich cytoplasm, a conspicuous dictyosome and rough ER (RER) were observed in the meristemoid cells of *Bauhinia forficata* Link and *Glycine max* (L.) Merrill. The presence of these organelles indicated that these cells had high metabolic activity during plant cell growth as they were able to synthesize protein and cell wall components (Pihakaski-Maunsbach et al. 1993; Appezzato-da-Glória and Machado 2004).

These well-organized organelles, including a clearly defined nucleus, rounded mitochondria, plastids (chloroplasts, chromoplasts and amyloplasts), numerous free ribosomes, RER and an active dictyosome suspended in dense cytoplasm reported in our study (Fig. 4) are consistent with observations made by others for embryogenic and organogenic callus of banana and peach palm which could develop further to form plantlets (Steinmacher et al. 2011; de Oliveira Ribeiro et al. 2012).

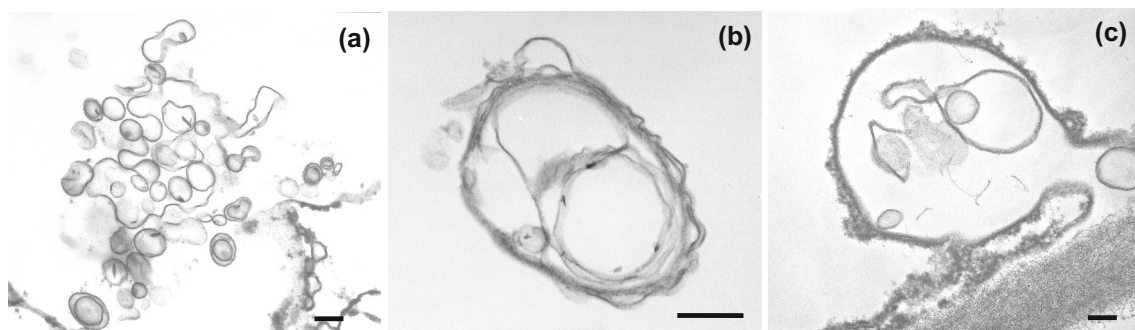
#### Two-month-old callus

Two-month-old callus was mostly pale green. The plasmalemma and organellar membranes were evident. In addition, an empty double-membranous vesicle which was

very similar to an autophagosome-like vesicle detected in *Arabidopsis* cells undergoing programmed cell death (PCD) (Hofius et al. 2009) was obviously present (Fig. 5a, b). The secretion of cytoplasmic material to be deposited at the cell wall was observed (Fig. 5c). The appearance of these membranous vesicles was also prevalent in the cortical cells of cucumber (*Cucumis sativus* L.) after exposure to chilling stress (Lee et al. 2002). The alteration and initial degeneration of crucial organelles, particularly the nucleus, mitochondria and chloroplasts—relative to 1-month-old callus—were also noticed in 3-month-old callus (Fig. 7).

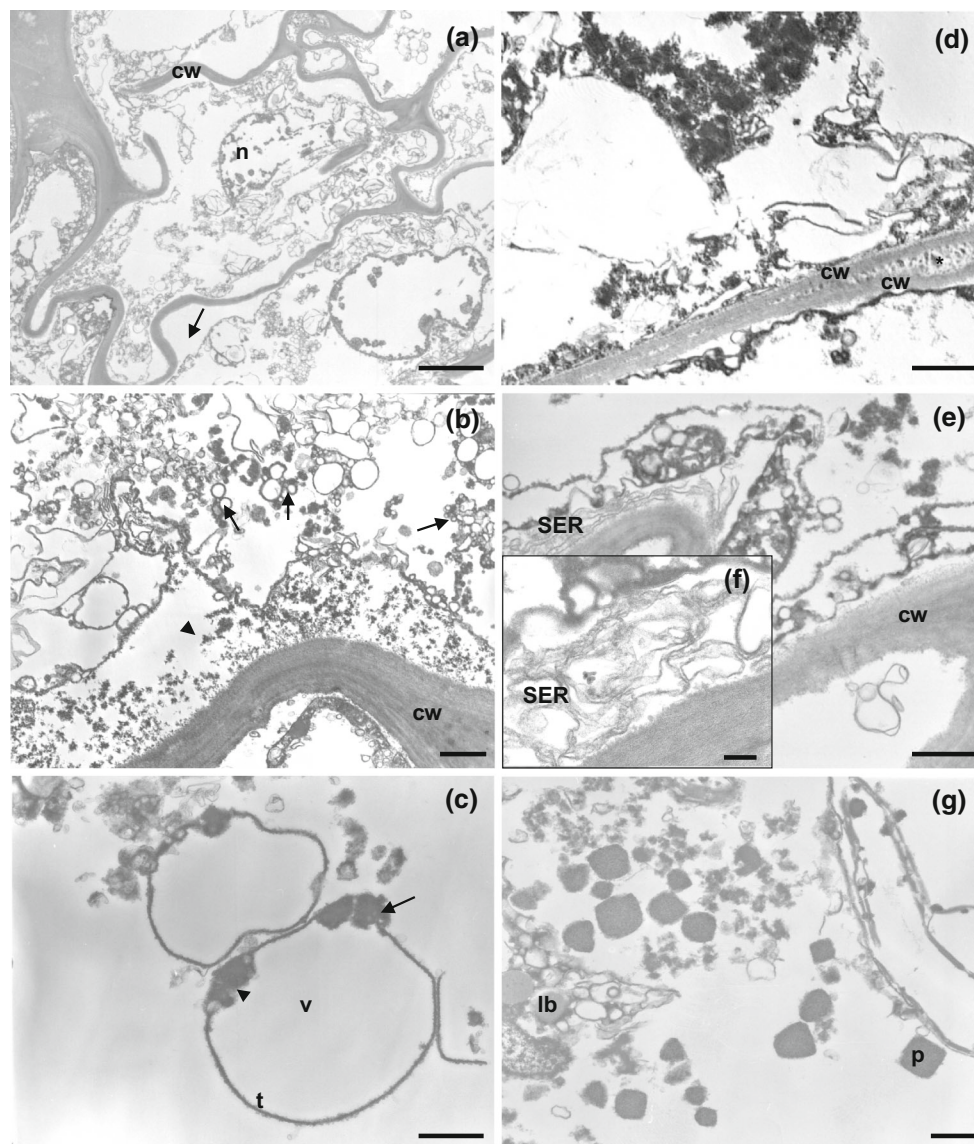
#### Three-month-old callus

Three-month-old callus was light to dark brown in colour. Various organelles in 3-month-old callus cells were completely disorganized. Brown cells had minimal cytoplasm, numerous degraded thylakoid-containing plastids and shrunken plasmalemma resulting in detachment of the plasmalemma from the cell wall (Fig. 6a). A cell with a shrunken membrane indicates membrane dysfunction, an increase in cell membrane permeability and the leakage of ions which are distinguishing features of the response to oxidative stress (Sunkar 2010), similar to the alteration of organelles in (oxidative) stress-induced cells of other plants and in the browning callus of *D. crumenatum* in this study. Oxidative stress caused by the over-production of ROS can occur in *in vitro* culture (Cassells and Curry 2001). Thus, explant wounding and various environmental stresses such as hormone, mineral composition, water and light can lead to enhanced ROS production (Cassells and Curry 2001). When *D. crumenatum* callus is not subcultured onto fresh medium, factors leading to oxidative stress *in vitro* might have been caused by wounding of the callus mass since the beginning of callus culture on CIM, prolonged culture period with nutrient deficiency, environmental stress, long-term oxidative stress and negatively impacted metabolic processes of plant cells, including photosynthesis and



**Fig. 5** TEM micrographs of browning callus after a 2-month culture period on modified VW medium. Callus cells exhibiting **a** a simple double-membranous structure (*bar* 200 nm) and **b** concentric

membranous whorls (*bar* 250 nm). **c** Membranous vesicle showing the secretion of cytoplasmic material to be deposited at the walls (*bar* 100 nm)



**Fig. 6** TEM micrographs of browning callus after a 3-month culture period on modified VW medium. Necrotic cells exhibiting signs of cell damage and eventually cell death. **a** Brown cell showing disorganized organelles and twisted cell wall with shrunken membrane (*arrow*) (*bar* 5  $\mu\text{m}$ ). Magnified images of (**b**), an area of the cytoplasm showing numerous small vesicles (*arrows*) and

osmiophilic material (*arrow head*) (*bar* 1  $\mu\text{m}$ ). **c** Magnified view of osmiophilic deposition (*arrow*) that appears to be discharged into the vacuole (*v*) and attached to the inner surface of the tonoplast (*arrow head*) (*bar* 500 nm). **d** Dense fibrillar structures (*asterisk*) between the cell wall (*bar* 1  $\mu\text{m}$ ), **e**, **f** numerous smooth ER (*SER*) (*bar* 500, 100 nm), **g** peroxisomes (*p*) and lipid bodies (*lb*) (*bar* 500 nm)

respiration. Factors such as these contribute to ROS production resulting in an imbalance between ROS and ROS scavengers (Adelberg et al. 1997; Apel and Hirt 2004; Liu and Chen 2010). Three-month-old callus also had distortions in thickened cell walls (Fig. 6a), similar to malformed cell walls observed by Gunawardena et al. (2007) in lace plant where cell wall degradation was caused by the action of ROS that lead to rapid cell death. Cell wall thickening also causes the arrest of cell growth and is attributed to reduced extensibility of the cell wall (Singh and Prasad 2009).

Three-month-old callus exhibited numerous microvesicles compared with 1-month-old callus, in which such vesicles were absent (Fig. 6b). These vesicles contained dense pigmentation consisting of a dark osmiophilic material, tannin, which was attached to the inner surface of the vacuolar membrane (Fig. 6c). Tannin was also deposited in the cytoplasm (Fig. 6c, *arrow*). Laukkanen et al. (2000) reported that tannin accumulated to a high level in the cytoplasm and intercellular spaces and could lead to tissue browning in *Pinus sylvestris* L. callus cells. In particular, the accumulation of condensed tannin in the

cytoplasm was one factor causing browning and death of plant cells due to the oxidation of tannin and other polyphenolic compounds (Laukkanen et al. 2000; Ahmad et al. 2013). In addition, condensed tannin can be deposited in the browning cells of *Phalaenopsis* leaves when cultured in vitro (Xu et al. 2005).

Laukkanen et al. (2000) also reported the presence of dense fibrillar structures between neighboring cell walls in *P. sylvestris* browning callus, indicating high oxidative stress in these cells. Similar fibrillar components could be found in 3-month-old callus cells of *D. crumenatum* (Fig. 6d). Three-month-old callus displayed more smooth ER (SER) (Fig. 6e, f), peroxisomes with a large crystal-like structure and lipid bodies (Fig. 6g) compared to 1-month-old callus.

Peroxisomes are the site of  $H_2O_2$  scavenging in response to  $H_2O_2$  that is formed by photorespiration and  $\beta$ -oxidation of fatty acids (Sandalio et al. 2013). These peroxisomes, which contain an antioxidant enzyme catalase, form by budding off from a specific segment of the SER (Hu et al. 2012). The SER also plays a role in the formation of lipid bodies both in the cytoplasm and plastids, and are produced in response to oxidative stress (Chapman et al. 2012). Moreover, increased dilation and vesiculation of SER are evidence of the response to oxidative stress (Lee et al. 2002). However, dictyosomes were not clearly observed in 2-month-old callus or in 3-month-old callus in this study. The disappearance of dictyosomes in browning callus may imply that the formation of vesicles may be mainly involved with ER although previous reports revealed that vesicles could originate from both the ER and dictyosomes (Matile and Moor 1968; Stefanowska et al. 2002; Mauseth 2014).

#### Ultrastructural alteration in important organelles

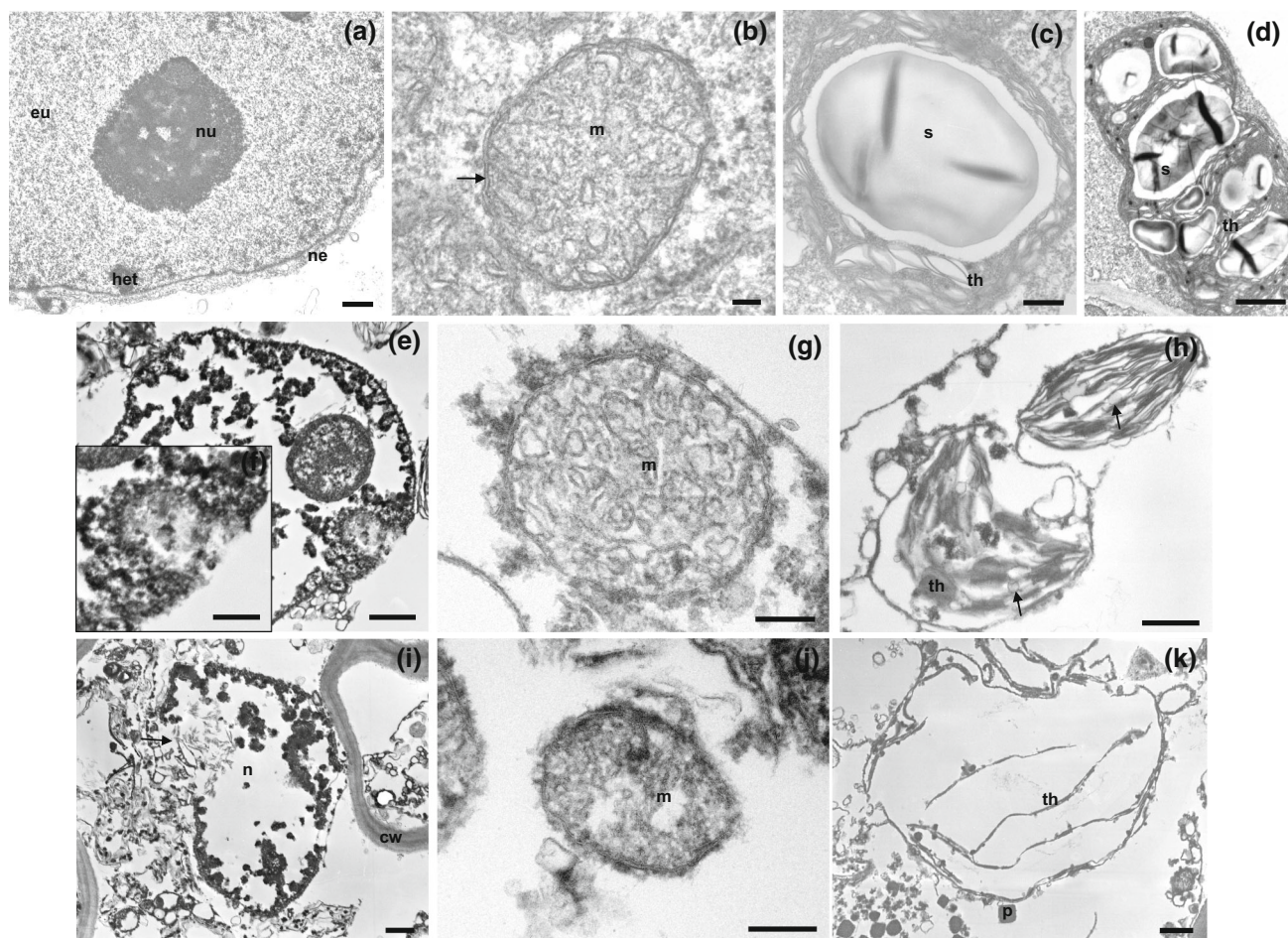
Various organelles underwent changes during callus culture. However, the important organelles, particularly the nucleus, mitochondria and chloroplasts showed distinct alterations.

One-month-old callus exhibited a nucleus with a nucleolus, a distinct nuclear envelope and two types of chromatin (heterochromatin and euchromatin) (Fig. 7a). These structures are related to the characteristics of totipotent embryogenic cells that have a nucleus containing less perinuclear heterochromatin but more euchromatin (Verdeil et al. 2007). The 1-month-old callus still exhibited numerous round mitochondria, each with a distinct double-membrane envelope (Fig. 7b). The isodiametric shape of the mitochondria is an important site of both high metabolic activity and respiration rate, which could be observed in the embryogenic cells of *Inga vera* Willd. subsp. *Affinis* (DC.) T.D. Penn. (Stein et al. 2010). The mitochondrion is

an important organelle involved in a wide range of biosynthetic reactions that maintain eukaryotic life and that is involved with the response of a plant to oxidative stress (Bi et al. 2009). Chloroplasts containing either a large starch grain or several starch grains were clearly observed (Fig. 7c, d). Forters and Pais (2000) reported that starch accumulated in callus cells and prenodular structures of *Humulus lupulus* var. Nugget which could provide large amounts of energy required for organ initiation and further development. One or more starch grain-containing chloroplasts were clearly observed during organogenesis of *Glycine max* (L.) Merrill and *Bauhinia forficata* Link (Appezato-da-Glória and Machado 2004) and *Zea mays* (Marín-Méndez et al. 2009), indicating the relationship between these plastids and the organogenic potential of plant cells. Moreover, the presence of starch grains was also related to the acquisition of embryogenic potential in the plant species *Drosera spatulata* Labill. (Bobák et al. 2004), *Picea abies* and *Picea omorika* (Hazubska-Przybył et al. 2008) and embryogenic cells of *Inga vera* (Stein et al. 2010). Thus, starch accumulation might be an important factor for supporting a morphogenic pathway, both organogenesis and embryogenesis, which are energy-requiring processes (George 2008).

Two-month-old callus showed a nucleus with a nucleolus, a nuclear pore and two distinct types of chromatins, peripheral and nucleoplasmic heterochromatins, which were detected more than in the euchromatin state (Fig. 7e). The nuclear envelope showed minimal changes with invagination into the nucleus causing an unusual arrangement of nuclear pores (Fig. 7f). Spherical mitochondria showing enlarged cristae and less matrix material were also detected (Fig. 7g). Moreover, alterations in chloroplasts, a major site of PPO (Yoruk and Marshall 2003; Mayer 2006), were observed. Chloroplasts appeared to lack starch grains. A swollen thylakoid membrane and lipid droplets were also observed in some chloroplasts (Fig. 7h). These facets were consistent with the characteristics of plant cells after being exposed to chilling stress (Kratsch and Wise 2000). These injured cells, caused by chilling stress, exhibited swelling chloroplasts with dilated thylakoids, reduced size and number of starch grains as well as the appearance of lipid droplet-containing chloroplasts which were similar to 3-month-old *D. crumenatum* callus. It is possible that browning callus could be a consequence of oxidative stress due to organelle disorganization and increased lipid peroxidation during callus culture without subculture. An increase in lipid peroxidation as a result of oxidative stress has been attributed to an imbalance between ROS generation and antioxidative enzymes (Tripathy and Oelmüller 2012).

Three-month-old callus showed the condensation of chromatin at the periphery of the nucleus resulting from



**Fig. 7** TEM micrographs of ultrastructural alteration of important organelles during callus culture. One-month-old callus exhibits **a** a nucleus with a clear nuclear envelope (*ne*), nucleolus (*nu*), euchromatin (*eu*) and heterochromatin (*het*) (*bar* 1  $\mu$ m). **b** Well-organized mitochondrion (*m*) with a mitochondrial double-membrane system (*arrow*) and numerous cristae (*bar* 100 nm). High magnification of chloroplast with **c** one or **d** several starch grains (*s*) and a well-developed thylakoid membrane system (*th*) (*bar* 500 nm, 2  $\mu$ m). Two-month-old callus showing **e** peripheral and nucleoplasmic

heterochromatin (*bar* 1  $\mu$ m). **f** Magnified images of nuclear envelope exhibiting invagination of double-layered nuclear membrane (*bar* 500 nm). **g** Spherical mitochondria with swollen cristae and less dense matrix material (*bar* 200 nm). **h** Chloroplast containing lipid bodies (*arrows*) and no starch grains; the thylakoid membrane starts to swell (*bar* 500 nm). Three-month-old callus showing **i** disintegrated nucleus (*arrow*) (*bar* 1  $\mu$ m). **j** Irregular mitochondrion with unclear cristae (*bar* 200 nm). **k** Chloroplast with strongly dilated thylakoid membranes (*bar* 1  $\mu$ m)

breakage in some areas of the nuclear envelope (Fig. 7i). Peripheral nuclear chromatin was also noted in plant cells both undergoing PCD (Gunawardena et al. 2005) and plants induced by oxidative stress (Speranza et al. 2007). Swollen mitochondria with deformed inner and outer membranes were noticed (Fig. 7j). These changes were consistent with the typical structure of dysfunctional mitochondria in necrotic cells as a consequence of lipid peroxidation and over-generate ROS (Lee et al. 2002; Yoshinaga et al. 2005). Swollen chloroplasts with swollen thylakoid membranes were still evident (Fig. 7k). These malformed chloroplasts might be attributed to an excess production of ROS during long-term callus culture under stress conditions which was indicated by an increased amount of lipid peroxidation (data not shown). Dilatation of

thylakoid membranes and the loss of granal stacking in chloroplasts were also observed in *Arabidopsis* seedlings (Wi et al. 2005) maintained under growth cabinet conditions and in the callus of *Nicotiana bigelovii* var. *bigelovii* (Bennici and Tani 2009) after gamma irradiation and salinity stress. These alterations to chloroplasts could reduce the photosynthetic capacity of that organ or tissue (Wu et al. 2009). In contrast, callus that was subcultured at a suitable interval showed chloroplasts with a normal shape since ROS-induced damage could be reduced by subculturing (Peng and Zhang 2009). Unfortunately, subcultures can still induce somaclonal variation (Bairu et al. 2011). Moreover, Brillouet et al. (2013) revealed that swollen chloroplasts containing unstacked thylakoids are the origin of tannosome, an organelle involved in the formation of

tannin storage, and the production of condensed tannin. As mentioned above, tannin is a polyphenolic compound that participates in enzymatic browning caused by its oxidation, and resulting in the formation of toxic *o*-quinone (Ahmad et al. 2013).

These ultrastructural features indicate that browning-related changes involve a wide range of organelles. In particular, chloroplasts, the nucleus and mitochondria are the primary organelles affected by oxidative stress and alterations to these organelles subsequently indicate signs of necrosis. These findings are consistent with ultrastructural changes caused by many oxidative stresses, as described above. Thus, a browning-induced incubation period is a (new) form of abiotic stress.

### PPO activity

PPO is a crucial enzyme for defense against oxidative stress in plant cells and is located in the thylakoid membrane of the chloroplast (Mayer 2006). PPO is involved in the enzymatic oxidation of phenolic compounds that result in discoloration and eventual death of explants (Mayer 2006). In *D. crumenatum* callus cultures, PPO activity increased gradually and continuously throughout the 3-month incubation period. However, maximum enzyme activity was observed in 3-month-old callus and was significantly higher than 1-month-old callus (Table 1). This is in agreement with an increase in PPO activity observed in the browning of bamboo shoots (Huang et al. 2002), *Pinus virginiana* Mill callus (Tang and Newton 2004), and tree peony roots (Fu et al. 2011). Moreover, changes in PPO activity might be noticed after wounding (Mayer 2006) and several other oxidative stress-inducing situations such as salinity (Weisany et al. 2012) and drought (Terzi et al. 2013).

**Table 1** The colour appearance of callus, PPO activity and total phenolic contents in callus culture on modified VW medium supplemented with 0.5 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA without any subcultures

Culture incubation (months)	Callus colour (exceeding 80 %)	PPO activity (U <sup>a</sup> µg <sup>-1</sup> protein)	Total phenolic contents (mg GAE g <sup>-1</sup> FW)
1	Green	656.71 ± 29.56b	0.467 ± 0.004b
2	Pale green	669.60 ± 31.24b	0.452 ± 0.001c
3	Brown	799.19 ± 37.32a	0.500 ± 0.003a

Mean ± SE of six replicates. Values with different letters are significantly different at  $P \leq 0.05$  (Duncan's multiple range test)

<sup>a</sup> One unit of enzyme activity is defined as the absorbance change of 0.01 min<sup>-1</sup>

### Total phenolic content

Phenolics are one of the important factors in the browning reaction since they act as substrates of oxidative enzymes. The total phenolic content of 3-month-old *D. crumenatum* callus was significantly higher than that of the 1- and 2-month-old callus (Table 1). The over-production of phenolics adversely influenced callus growth and a high amount of phenolics resulted in tissue browning as reported in European and Canadian yew (*Taxus baccata* L. and *T. Canadensis* Marsh.) (Dubravina et al. 2005) and *Cicer arietinum* (Naz et al. 2008). Leng et al. (2009) also reported that the total phenolic content and percentage browning in *Pistacia vera* L. callus gradually increased and was approximately 2- and 2.3-fold higher than the controls, respectively. Thus, the long-term culture of *D. crumenatum* callus showed a positive relation between the quantity of total phenolic content and the degree of browning. This is consistent with previous reports on browning in *Phalaenopsis* tissue culture (Xu and Li 2006; Xu et al. 2010; Yin et al. 2006).

### Chl and carotenoid contents

A decrease in Chl and carotenoid contents is associated with the deterioration of callus caused by tissue browning (Laukkanen et al. 2000; He et al. 2009). The degradation of Chl, one of the major causes of changes in colour, is a consequence of Chl oxidation which is directly oxidized by the phenoxy radical derived from phenolic oxidation (Yamauchi et al. 2004; Toivonen and Brummell 2008). Moreover, carotenoids are important pigments that act as antioxidants by scavenging singlet oxygen, the triplet state of Chl and other harmful free radicals, thus preventing the destruction of Chl (Zhang et al. 2005; Boguszewska and Zagdanska 2012). Chlorophyll is an essential pigment for photosynthesis, which is one of the most central biological processes. The reduction of Chl is attributed to the degradation of Chl and the inhibition of Chl biosynthesis results in the arrest of photosynthesis and cell growth (Najafi and Jamei 2014). The contents of total Chl, Chl *a*, Chl *b* and carotenoids decreased significantly when callus was maintained on CIM for 3 months without subculture (Table 2). A gradual decrease in Chl and carotenoid concentrations were also observed during *Jatropha curcas* callus culture (He et al. 2009) and shoot proliferation (Misra et al. 2010). In addition, the loss of Chl content is normally considered to be the consequence of Chl degradation resulting from oxidative stress and this incidence also increased ROS production (Misra et al. 2010). The reduction of Chl content induced by abiotic stress, which then stimulates the overproduction of ROS and increased activity of Chl-degrading enzymes (Sevengor et al. 2011),

**Table 2** Changes in the content of chlorophylls and carotenoids during callus culture on modified VW medium supplemented with 0.5 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA without any subcultures

Culture incubation (months)	Total chlorophyll (mg g <sup>-1</sup> FW ± SE)	Chlorophyll <i>a</i> (mg g <sup>-1</sup> FW ± SE)	Chlorophyll <i>b</i> (mg g <sup>-1</sup> FW ± SE)	Carotenoid (mg g <sup>-1</sup> FW ± SE)
1	0.081 ± 0.003a	0.054 ± 0.001a	0.040 ± 0.002a	0.027 ± 0.001a
2	0.064 ± 0.007b	0.044 ± 0.006a	0.031 ± 0.003b	0.026 ± 0.001a
3	0.021 ± 0.003c	0.014 ± 0.002b	0.011 ± 0.002c	0.011 ± 0.002b

Values with different letters within each column are significantly different at  $P \leq 0.05$  (Duncan's multiple range test)

would support a possible mechanism at work in *D. crumenatum* callus cultures in which the lack of subcultures would be equivalent to an abiotic stress. Water stress also caused a reduction in Chl content in wheat (*Triticum durum*) by inhibiting Chl synthesis (Ghozène et al. 2013).

Carotenoids play an important role in overcoming oxidative stress and preventing lipid peroxidation in membranes by decreasing carotenoid content (Amirjani 2010; Boguszewska and Zagdanska 2012) and are effective scavengers that eliminate singlet oxygen and lipid radicals (Ramel et al. 2012). This concept is fortified by studies that observed a significant decrease in carotenoid content with increasing level of salt stress in barley (*Hordeum vulgare*) grains (El-Tayeb 2005). Yang et al. (2013) revealed that the carotenoid content of wheat (*Triticum aestivum* L.) seedlings decreased after exposure to UV-B radiation.

## Conclusion

Bisected *D. crumenatum* protocorm-derived callus gradually turns brown as culture period is prolonged, which is a common phenomenon for many orchid callus cultures. The occurrence of browning was confirmed by biochemical assays and by cellular and subcellular observations. These findings demonstrate that tissue browning is related to the period of in vitro tissue culture which can serve as oxidative abiotic stress if no sub-cultures are performed, resulting in alterations to the shape and appearance of many organelles and cellular structures. Early tissue browning can also serve as a sign for the need to sub-culture, before callus dies. Understanding the causes of observed changes during callus browning can provide important clues for predicting the onset of browning. In the future, investigations of enzymatic browning at the molecular level should be done to establish ways to prevent it.

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