Inhibition of Browning Agents in Bisected Protocorm-Derived Callus of Pigeon Orchid (*Dendrobium crumenatum* Swartz)

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ชื่อวิทยานิพนธ์ การยับยั้งการเกิดสารสีน้ำตาลในแคลลัสที่ได้จากการชักนำชิ้นส่วนตัดตามขวางของโพรโทคอร์มกล้วยไม้หวายตะมอย (*Dendrobium crumenatum* Swartz)

ผู้เขียน นางสาวปวีณา แก้วอุบล

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บทคัดย่อ

การชักนำแคลลัสจำเป็นสำหรับการชักนำให้เกิดต้นโดยผ่านแคลลัส นำชิ้นส่วนโพรโทคอร์มตัดตามขวางของกล้วยไม้หวายตะมอย (*Dendrobium crumenatum* Swartz) อายุ 3 เดือน เพาะเลี้ยงบนอาหารแข็งสูตรดัดแปลง Vacin and Went (1949) (VW) ที่เติมน้ำตาลซูโครส 20 กรัมต่อลิตร, ไฟตาเจล 2 กรัมต่อลิตร ร่วมกับ 1-naphthaleneacetic acid (NAA) ที่ความเข้มข้น 0, 0.1, 0.5 มิลลิกรัมต่อลิตร และ 6-benzyladenine (BA) ที่ความเข้มข้น 0, 1, 2 มิลลิกรัมต่อลิตร พบว่าอาหารแข็งสูตรดัดแปลง VW ที่เติม NAA 0.5 มิลลิกรัมต่อลิตร ร่วมกับ BA 1 มิลลิกรัมต่อลิตร สามารถชักนำให้เกิดแคลลัสได้ดีที่สุด โดยแคลลัสเจริญมาจากเซลล์ใต้ชั้นอิพิเดอร์มิสของชิ้นส่วน และเจริญต่อไปเป็นก้อนแคลลัสที่สมบูรณ์ ภายหลังการเพาะเลี้ยงนาน 8 สัปดาห์ พบเซลล์ของแคลลัสมีขนาดเล็ก ไซโทพลาสซึมเข้มข้น นิวเคลียสขนาดใหญ่และมีนิวคลิโอลัส

ขั้นตอนการชักนำแคลลัสมีข้อจำกัด เนื่องจากเซลล์แคลลัสเกิดการเปลี่ยนแปลงเป็นสีน้ำตาล ส่งผลให้ความสามารถในการเจริญเติบโตลดลง จนแคลลัสตาย การทดลองครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของระยะเวลาการเพาะเลี้ยงโดยไม่มีการย้ายเลี้ยงที่มีผลต่อการเกิดการเปลี่ยนแปลงเป็นสีน้ำตาลของแคลลัส ทั้งในระดับเซลล์ ระดับออร์แกเนลล์ และระดับชีวเคมีของเซลล์ โดยทำการตรวจสอบทุกๆ 1 เดือน เป็นเวลา 3 เดือน พบว่าหลังการเพาะเลี้ยงนาน 3 เดือน เซลล์ของแคลลัสมีการจัดเรียงตัวอย่างไม่เป็นระเบียบ และมีการเปลี่ยนแปลงรูปร่างของออร์-แกเนลล์สำคัญ เช่น นิวเคลียส ไมโทคอนเดรีย คลอโรพลาสต์ และพบการสะสมของแทนนินภายในแวคิวโอล ซึ่งแตกต่างจากแคลลัสอายุ 1 เดือน ที่มีสีเขียวและเป็นชุดควบคุม นอกจากนี้การเปลี่ยนแปลงระดับออร์แกเนลล์สามารถบ่งบอกถึงการเกิดการเปลี่ยนแปลงเป็นสีน้ำตาลของแคลลัส โดยเป็นผลมาจากเอนไซม์ที่เกี่ยวข้องกับสภาวะเครียดของพืช จากการศึกษาพบว่าแคลลัสอายุ 3 เดือนที่มีสีน้ำตาล มีกิจกรรมของเอนไซม์พอลิฟีนอลออกซิเดสและเอนไซม์ฟีนิลอลานีน-แอมโมเนียไลเอสค่อนข้างสูง รวมทั้งมีปริมาณลิปิดเปอร์ออกซิเดชันในรูปของปริมาณมาลอน-

ไดอัลดีไฮด์ และปริมาณสารประกอบฟีนอลิกสูงกว่าแคลลัสชุดควบคุม ในขณะที่ปริมาณคลอโรฟิลล์และแคโรทีนอยด์ลดลงเมื่อเปรียบเทียบกับแคลลัสชุดควบคุม การเปลี่ยนแปลงของปัจจัยเหล่านี้ สามารถใช้เป็นตัวชี้วัดความสมบูรณ์ของแคลลัสที่เหมาะสำหรับใช้ในการศึกษาการเจริญต่อไป

การทดลองครั้งนี้จึงจำเป็นต้องศึกษาผลของสารต้านการเกิดสารสีน้ำตาล ได้แก่ กรดแอสคอบิก ที่ความเข้มข้น 0, 0.001 และ 0.01 กรัมต่อลิตร, แอล-ซีสเตอีน ที่ความเข้มข้น 0, 0.01 และ 0.05 กรัมต่อลิตร, พอลิไวนิลไพโรลิโดน ที่ความเข้มข้น 0, 0.5 และ 5 กรัมต่อลิตร รวมทั้งผงถ่านกัมมันต์ ที่ความเข้มข้น 0, 1 และ 2 กรัมต่อลิตร เพื่อยับยั้งการเกิดปัญหาการเปลี่ยนแปลงเป็นสีน้ำตาลของเซลล์แคลลัส พบว่าอาหารแข็งสูตรดัดแปลง VW ที่เติม NAA 0.5 มิลลิกรัมต่อลิตร และ BA 1 มิลลิกรัมต่อลิตร ร่วมกับ กรดแอสคอบิก 0.01 กรัมต่อลิตร สามารถลดการเกิดการเปลี่ยนแปลงเป็นสีน้ำตาลของแคลลัสได้ดีที่สุด โดยยับยั้งกิจกรรมของเอนไซม์พอลิฟีนอลออก-ซิเดสและเอนไซม์ฟีนิลอลานีนแอมโมเนียไลเอส และลดปริมาณการสะสมของผลผลิตของปฏิกิริยาลิปิดเปอร์ออกซิเดชันและปริมาณสารประกอบฟีนอลิก นอกจากนี้ยังพบว่าคลอโรฟิลล์และแคโรทีนอยด์มีปริมาณเพิ่มมากขึ้น ซึ่งผลการทดลองแตกต่างอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับชุดการทดลองที่เติม ผงถ่านกัมมันต์ 1 กรัมต่อลิตรและชุดควบคุม และผลการศึกษาปัจจัยทางชีวเคมีดังกล่าวสอดคล้องกับผลการศึกษาทางเนื้อเยื่อวิทยา โดยพบว่าแคลลัสที่ได้จากการเพาะเลี้ยงบนอาหารแข็งสูตรดัดแปลง VW ที่เติม กรดแอสคอบิก 0.01 กรัมต่อลิตร มีลักษณะเป็นแคลลัสที่หลุดแยกออกจากกันได้ง่าย ภายในเซลล์มีออร์แกเนลล์ที่สมบูรณ์ ได้แก่ ไมโทคอนเดรีย คลอโร-พลาสต์ที่สะสมเม็ดแป้ง และพอลิโซม ซึ่งตรงกันข้ามกับแคลลัสที่เพาะเลี้ยงบนอาหารแข็งสูตรดัดแปลง VW ที่เติมผงถ่านกัมมันต์ 1 กรัมต่อลิตร ซึ่งพบว่าเซลล์ของแคลลัสเหล่านี้มีการจัดเรียงไม่สมบูรณ์และภายในเซลล์มีการเปลี่ยนแปลงของโครงสร้างของเซลล์และออร์แกเนลล์หลายชนิด ได้แก่ ไมโทคอนเดรีย คลอโรพลาสต์ ผนังเซลล์ เยื่อหุ้มเซลล์ และการสะสมของแทนนินภายในแวคิวโอล

การศึกษาครั้งนี้สามารถยืนยันได้ว่า กรดแอสคอบิกเป็นสารต้านการเกิดสารสีน้ำตาลที่มีประสิทธิภาพ โดยสามารถยับยั้งการเกิดการเปลี่ยนแปลงเป็นสีน้ำตาลของเซลล์แคลลัสและรักษาสภาพการแบ่งแยกส่วนระหว่างเอนไซม์และซับสเตรทในแคลลัสที่เพาะเลี้ยงบนอาหารชุดเดิมเป็นระยะเวลานานไว้ได้

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ABSTRACT

Callogenesis is a prerequisite step for the initiation of callus-mediated plant regeneration. Three-month-old protocorms of *Dendrobium crumenatum* Swartz (Pigeon orchid) were bisected transversely and cultured on modified Vacin and Went (1949) (VW) solidified medium containing 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel and various concentrations of 1-naphthaleneacetic acid (NAA) (0, 0.1, 0.5 mg L^{-1}) and 6-benzyladenine (BA) (0, 1, 2 mg L^{-1}). The highest percentage of callus induction was obtained from the explant cultured on a modified VW medium supplemented with 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA. The callus was originated from the subepidermal layer of the bisected protocorm and subsequently formed nodular structure after a culture for 8 weeks. The callus mass contained small isodiametric cells, dense cytoplasm, prominent nuclei and nucleoli.

The browning phenomenon can be a major limitation for 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 on the appearance of tissue browning. Biological alterations at the cellular and subcellular levels, as well as biochemical aspects, were examined. Cultured callus on a modified VW solid medium supplemented with 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA was not subcultured and collected at one-month intervals for 3 months and examined for structural and biochemical alterations associated with browning. Three-month-old unsubcultured callus cells were loosely arranged and major organelles were deformed, exhibiting nuclear envelope breakage, dysfunctional mitochondria, swollen chloroplasts and tannin-filled vesicles, relative to one-month-old green callus, which served as the control. Ultrastructural disorganization involving the nucleus, mitochondria and chloroplasts typified enzymatic oxidative browning. Three-month-old brown callus

had significantly higher polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) activity, lipid peroxidation in terms of malondialdehyde (MDA) content and total phenolic content than those of control callus. Meanwhile, the contents of chlorophyll and carotenoid of three-month-old callus were significantly reduced as compared with control. The level of these factors may serve as useful biochemical markers when selecting suitable callus for subsequent regeneration trials.

Accordingly, the effectiveness of anti-browning agents including L-ascorbic acid (AA; 0, 0.001 and 0.01 g L^{-1}), L-cysteine (Cys; 0, 0.01 and 0.05 g L^{-1}), polyvinylpyrrolidone (PVP; 0, 0.5 and 5 g L^{-1}) and activated charcoal (AC; 0, 1 and 2 g L^{-1}) for prevention of callus browning was then determined. After 3 months of callus culture on the medium added with different concentrations of individual anti-browning agent, the calli were randomly collected and examined for their biological features in structural, ultrastructural and biochemical aspects. The results indicated that 0.01 g L^{-1} AA significantly inhibited the activities of PPO and PAL enzymes, reduced MDA content, decreased total phenolic content, but increased the contents of chlorophyll and carotenoid as compared with 1 g L^{-1} AC and control. Microscopy observation showed that 0.01 g L^{-1} AA-treated friable callus displayed a highly organized system of organelles such as electron-dense mitochondria, chloroplasts containing starch grain and numerous polysomes within dense cytoplasm. Conversely, proliferated callus on 1 g L^{-1} AC showed an unorganized cell arrangement with malformed mitochondrion, distorted chloroplasts, broken cell wall, degraded plasmalemma and numerous osmiophillic material-containing vesicles. This present study confirmed that AA exhibited as an effective anti-browning property for diminishing enzymatic browning and maintaining enzyme-substrate compartmentation in extended callus culture of pigeon orchid.

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LIST OF ABBREVIATIONS

AA	=	Ascorbic acid
AC	=	Activated charcoal
ANOVA	=	Analysis of variance
BA	=	6-Benzyladenine
BAP	=	6-Benzylaminopurine
CDK	=	Cyclin-dependent kinase
Chl^*	=	Excited chlorophyll
CRD	=	Completely randomized design
Cys	=	L-cysteine
DMRT	=	Duncan's multiple range test
ER	=	Endoplasmic reticulum
FAA	=	Formalin-aceto-alcohol
FW	=	Fresh weight
GAE	=	Gallic acid equivalent
H_2O_2	=	Hydrogen peroxide
IBA	=	Indole-3-butyric acid
KC	=	Knudson's C medium
LM	=	Light microscopy
LSD	=	Least significant difference
MDA	=	Malondialdehyde
MS	=	Murashige and Skoog medium
MVW	=	Modified Vacin and Went medium
NAA	=	1-Naphthaleneacetic acid
PAL	=	Phenylalanine ammonia-lyase
PAS	=	Periodic acid-Schiff
PCD	=	Programmed cell death
PGRs	=	Plant growth regulators
PLB	=	Protocorm-like body
POD	=	Peroxidase

LIST OF ABBREVIATIONS (CONTINUED)

PPO	=	Polyphenol oxidase
PVP	=	Polyvinylpyrrolidone
RER	=	Rough ER
ROS	=	Reactive oxygen species
SDS	=	Sodium dodecyl sulfate
SEM	=	Scanning electron microscopy
SER	=	Smooth ER
TBA	=	Thiobarbituric acid
TBARS	=	Thiobarbituric acid reaction
TBO	=	Toluidine blue O
TCA	=	Trichloroacetic acid
TDZ	=	Thidiazuron
TEM	=	Transmission electron microscopy
TTC	=	2,3,5-Triphenyl tetrazolium chloride
VW	=	Vacin and Went medium
$^{1}O_{2}$	=	Singlet oxygen
$^{3}Chl^{*}$	=	Triplet chlorophyll
2,4-D	=	2,4-dichlorophenoxyacetic acid

CHAPTER 1
INTRODUCTION

1.1 Introduction

The pigeon orchid, *Dendrobium crumenatum* Swartz, is a tropical epiphytic orchid widespreaded in South and Southeast Asia. It is valuable not only from the attractive fragrant white flower and gregarious flowering (Meesawat and Kanchanapoom, 2002), but also from its medicinal property (Mangunwardoyo et al., 2012; Umberto, 2012; Sandrasagaran et al., 2014). This orchid is a model plant using for studying in many research areas such as developmental biology, physiology and genetic transformation providing basic knowledge on its growth and consequently enhancing its commercial value.

For this reason, tissue culture technique, especially callus induction, is an appropriate method for rapid in vitro clonal propagation to provide an excellent target material for studying in above areas (Belarmino and Mii, 2000; Musharof Hossain et al., 2013). Moreover, the callus-mediated regeneration can provide a high rate of somatic embryo formation and conversion of somatic embryos into plantlets (Zhao et al., 2008) which is one of the favourite techniques for biotechnological applications widely used in many plants, including orchids (Tokuhara and Mii, 2001; Debnath et al., 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 their slow growth, difficulties in maintaining and their tendency to brown and become necrotic (Begum et al., 1994; Chang and Chang, 1998; Khosravi et al., 2008).

Browning problem is a serious obstacle in the maintenance of plant tissue culture, 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 that many factors are responsible for tissue browning, comprising the presence and action of reactive oxygen species (ROS), phenolic compounds, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and oxidative enzymes including peroxidase (POD, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.10.3.1), all of which are involved in the process of phenolic oxidation. Moreover, the change in photosynthetic pigments, namely chlorophylls and carotenoids, were proved to be the key events observed in lethal browning (Laukkanen et al., 2000; He et al., 2009; Misra et al., 2010).

Although callus can be maintained by frequent subculture onto fresh medium, this technique can give rise to somaclonal variation (Bairu et al., 2011). Meanwhile, culturing callus without subculture can increase the accumulation of free radical, lipid peroxidation and also arrest the morphogenic transition of plant cell. Accordingly, the clearer understanding of the browning phenomenon at biochemical and cellular and/or ultrastructural aspects will provide valuable insight about the browning process and could be used in a practical way to prevent or reduce callus browning during culture. Since the efficient callus culture without signs of browning is a key requirement in orchid tissue culture for enhancing the callus growth.

For preventing browning, the addition of various anti-browning agents, namely ascorbic acid (AA), L-cysteine (Cys), polyvinylpyrrolidone (PVP) and activated charcoal (AC), into the medium have been proposed as an alternative choice for overcoming lethal browning of many plant species such as *Rollinia mucosa* (Jacq.) Baill. (Figueiredo et al., 2001), *Taxus brevifolia* (Khosoushahi et al., 2011), *Psidium guajava* (Liu and Yang, 2011) and *Brachylaena huillensis* (Ndakidemi et al., 2014), but to a very limited extent in orchids.

Therefore, the biological understanding of browning occurrence is the first step to cope with this problem and a well-established callus culture system by overcoming lethal browning is a prerequisite for improving the callus growth of *D. crumenatum*

1.2 Review of Literature

1.2.1 The genus *Dendrobium*

The family Orchidaceae is one of the largest monocotyledonous families in the Angiosperms (flowering plants). This family contains more than 25,000 species in about 870 genera, which are distributed around the world (Chase et al., 2003; Cribb et al., 2003). Most of them are abundant in the tropical regions.

The genus *Dendrobium* is a diverse genus of orchids that comprises many species ranging in size from miniature to giant. There are more than 1,500 species in the world and widely distributed from the Southeast Asia to New Guinea and Australia (Chen and Ji, 1998; Dutta et al., 2011). Most *Dendrobium* species are epiphytic orchids, but some are lithophytic orchids, growing on rock cliff, and terrestrial orchids, growing on grasslands (Puchooa, 2004).

A. Scientific classification of *D. crumenatum*

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Asparagales

Family: Orchidaceae

Subfamily: Epidendroideae

Genus: *Dendrobium*

Species: *D. crumenatum*

B. Characteristics of *D. crumenatum*

D. crumenatum, commonly known as Pigeon orchid, Sparrow Orchid, Dove orchid, Purse-shaped *Dendrobium* or Bag-shaped *Dendrobium*, is a common native epiphytic orchid species of South and Southeast Asia. It is normally found in Sri Lanka, Taiwan, India, Andaman Islands, Myanmar, Thailand, the Philippines, Malaysia, Cambodia, Laos, Vietnam and New Guinea (Leong and Wee, 2013).

Figure 1 Flowers of *D. crumenatum* orchid show full opened white flowers with yellow disc on the labellum.

There are two growth forms in orchid, namely monopodial and sympodial. *D. crumenatum* is a sympodial orchid. It is a robust and branched herb with stems that are slender and elongated up to 60-90 cm in length. The pseudo-bulbs are swollen and conical in shape. The leaves, 5-8 cm long and 1.5-2.5 cm wide, are oblong, thick and very leathery. The inflorescences are comprised of 3 floral buds from which arise white flowers about 4 cm in diameter consisting of three sepals, two petals and one labellum with a yellow disc in the center of the lip to its base (Figure 1, 2). This orchid exhibits synchronous flowering which is triggered by a sudden drop in temperature (about 10 °C), usually after heavy rainfall and then blooms nine days later (Meesawat and Kanchanapoom, 2007). Flowers are strongly fragrant, but short-lived, lasting only 1 or 2 days.

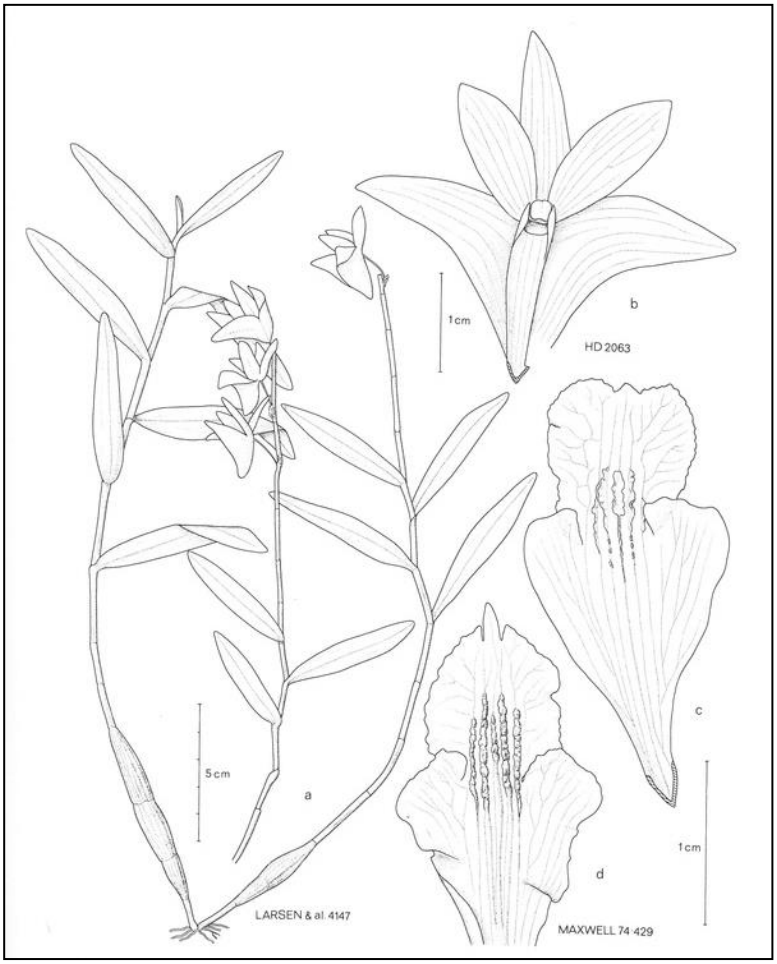


Figure 2 Morphological characteristics of *D. crumenatum*.
(A) growing plant **(B)** flower with lip removed **(C)** lip from different flowers.
(Source: adapted from Seidenfaden, 1985)

1.2.2 Callus induction

In orchid, callus induction is very difficult and few successful cases have been reported due to the explants gradually becoming necrotic after culture and the growth rate of callus being very slow. In previous reports, different concentrations and types of auxin and cytokinin were used to study the induction of callus from various explants. For instance, protocorm-derived callus of *Phalaenopsis* Nebula was obtained on a half-strength Murashige and Skoog (MS) medium added with 0-1 mg L^{-1} thidiazuron (TDZ) and/or 0-10 mg L^{-1} 2,4-dichlorophenoxyacetic

acid (2,4-D) (Chen et al., 2000). Likewise, embryogenic calli of *Oncidium* were induced from root tip and cut end of stem and leaf segment on a modified MS medium supplemented with 0.1-3 mg L^{-1} TDZ and 3-10 mg L^{-1} 2,4-D (Chen and Chang, 2000). Moreover, Lee and Lee (2003) reported that callus of *Cypripedium formosanum* was induced from protocorm segments on a modified MS medium supplemented with 4.54 µM TDZ and 4.52 µM 2,4-D. Similarly, Hong et al. (2008) showed that totipotent callus was induced from seed of *Paphiopedilum* Alma Gavaert when cultured on a modified MS medium supplemented with 4.54 µM TDZ and 22.60 µM 2,4-D. Moreover, callus induction of *P. gigantean* was observed from leaf segment on the medium containing 1 mg L^{-1} 1-naphthaleneacetic acid (NAA) in combination with 0.1 mg L^{-1} TDZ (Niknejad et al., 2011). Tan et al. (2011) also reported that the highest frequency of callus formation from nodal explants of *Vanilla planifolia* was obtained on a MS basal medium containing 2 mg L^{-1} NAA and 1 mg L^{-1} 6-benzyladenine (BA).

In *Dendrodium*, Roy and Banerjee (2003) revealed that callus induction from shoot-tip of *D. fimbriatum* Lindl. var *oculatum* Hk. f. was achieved on a modified Knudson's C medium (KC) supplemented with 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA. Moreover, Roy et al. (2007) reported that a modified KC medium supplemented with 2 µM TDZ or 2 µM 6-benzylaminopurine (BAP) was an optimal medium for callus induction from shoot tip of *D. chrysotoxum* Lindl. In *Dendrobium* cv. Serdang Beauty, the highest fresh weight of callus derived from protocorm-like body (PLB) was induced on a MS medium containing 1.5 mg L^{-1} indole-3-butyric acid (IBA) (Khosravi et al., 2008). Besides, callus induction of *D. candidum* Wall ex Lindl. was obtained from longitudinally bisected segments of protocorm on a modified MS medium supplemented with 8.8 µM BA (Zhao et al., 2008). Sunitibala and Kishor (2009) also reported that the highest percentage of callus formation from axillary bud of *D. transparens* L. was observed on a half-strength MS basal medium added with 2 mg L^{-1} BAP. Meanwhile, the highest percentage of callus induction of *D. nanum* was obtained from the rhizome bud after culture on a MS basal medium added with 2.0 µM NAA and 1.2 µM kinetin (Maridass et al., 2010). In addition, Meesawat and Kanchanapoom (2002) also reported that axillary bud-derived callus of *D. crumenatum* was induced on a modified Vacin and Went (VW) medium

supplemented with a combination of 0.1 mg L^{-1} NAA and 1 mg L^{-1} BA. However, such study gave a low frequency of callus induction and did not identify factors affecting callus formation. Accordingly, the effectiveness of callus induction depends on the plant species, type of explants and a balanced combination of plant growth regulators (PGRs).

The joint action of two main classes of PGRs, auxin and cytokinin, plays an important role in the induction of cell division and the control of cell-cycle progression which are key regulators of callus induction (Perrot-Rechenmann, 2010). Auxins are involved in DNA replication since auxin acts as a signal to stimulate cell division by controlling the cell cycle at G1/S transition and the later G2/M transition to complete the mitosis process (Machakova et al., 2008; Perrot-Rechenmann, 2010; Wang and Ruan, 2013). Meanwhile, cytokinin appears to be important for generating an active cyclin-dependent kinase (CDK) which is required for the initiation of cell division by catalyzing the transition from the G2 phase to mitosis (M phase) leading to mitosis, and cytokinin also controls the transition from G1 to S phase (Hopkins and Hüner, 2004).

1.2.3 Browning process

Browning reaction is generally assumed to be a direct consequence of cellular decompartmentation and influenced by oxidative enzymes – PPO and POD – that catalyze the oxidation of phenolic compound. However, PPO played a more important role than POD in browning process even though POD is associated with various physiological process in plant cell (Furumo and Furutani, 2008; He et al., 2009). Accordingly, enzymatic browning is a complex process which can be summarized as follows (Figure 3) (Wang et al., 2010).

(1) Accumulation of free radical

(2) Peroxidation of lipid

(3) Cellular decompartmentation

(4) Release and mixing of substrate and enzymes

(5) Degradation and oxidization of substrate by enzymes

(6) Formation of the brown to dark pigment

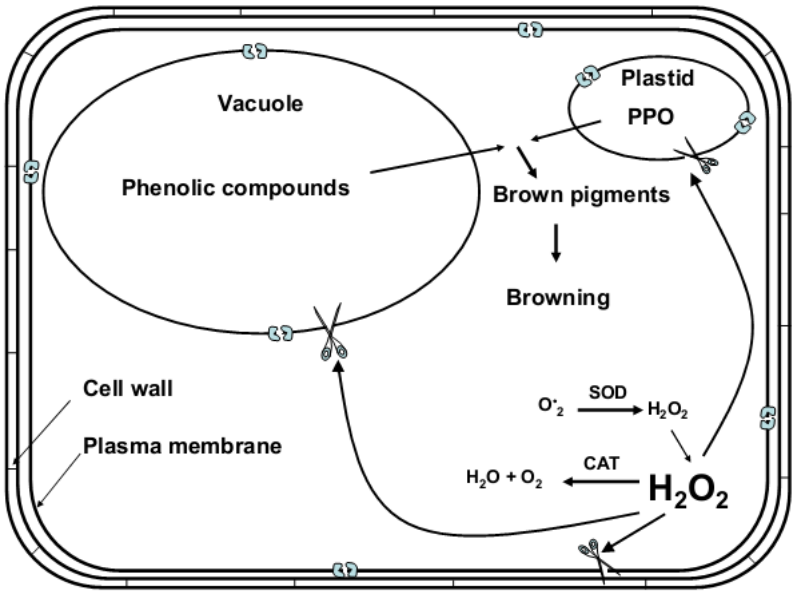


Figure 3 Processes of enzymatic browning.
(Source: adapted from Boonsiri, 2007)

Thus, the occurrence and intensity of browning are affected by various factors as described in 1.2.4.

1.2.4 Biochemical parameters associated with enzymatic browning

A. Phenolic compound

Phenolic compounds occur as secondary metabolites which are generally characterized by an aromatic form that possesses an aromatic benzene ring bearing one or more hydroxyl groups (Kefeli, 2003; Ozyigit, 2008). They are naturally accumulated in plant tissue and their synthesis is associated with the endoplasmic reticulum (ER) (Parham and Kaustinen, 1977; Gleason and Chollet, 2012). Most polyphenolic compounds appear to be formed or deposited in a reduced state within vacuoles or vesicles as fine- and large-granule substance (Beckman, 2000; Vatanpour-Azghandi et al., 2002; Dubravina et al., 2005; Li-Qin et al., 2009), while some phenolic compounds are observed in the chromoplast, cytoplasm and mitochondria (Li-Qin et al., 2009). Reis et al. (2008) also found that membrane-bound vesicles fused with the tonoplast and discharged their contents into the vacuole.

In addition, the phenolic substances are viewed as deleterious because their exudation and oxidation are highly reactive and toxic to the plant cell, causing necrosis and tissue death (Titov et al., 2006; Reis et al., 2008). Particularly, tannin is a phenolic secondary compound 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 high concentration of phenolic compounds can suppress both cell dedifferentiation and proliferation in various plant species such as *Pistacia vera* cv 'Ghazvini' (Vatanpour-Azghandi et al., 2002), *Cypripedium formosanum* (Lee and Lee, 2003) and *Cycas revoluta* (Kiong et al., 2008). Accordingly, phenolic compounds are one of the important factors influencing the browning reaction because they act as potential substrates of oxidative enzymes, in particular PPO.

B. Polyphenol oxidase (PPO)

PPO, also known as tyrosinase, *o*-diphenol oxidase, and catechol oxidase, is a nuclear-encoded copper-containing enzyme (Sapers et al., 2002; Mayer, 2006). PPO is widely distributed in many plant species and exists in a latent form on the thylakoid membrane of chloroplast and in cytoplasm, vesicles and other non-green plastids (Figure 4) (Kim et al., 2001; Toivonen and Brummell, 2008). 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)

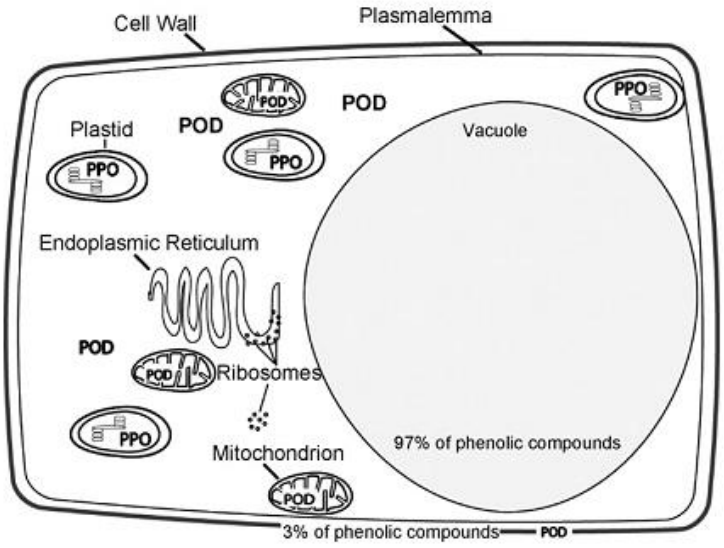


Figure 4 Localization of phenolic compound and oxidative enzymes (PPO and POD) in a typical plant cell.
(Source: Toivonen and Brummell, 2008)

Accordingly, PPO-mediated browning reaction occurs only after a cellular decompartmentalization which allows mixing of vacuolar phenolics and the phenolic oxidizing enzymes (PPO and POD) (Tomás-Barberán et al., 1997; Laukkanen et al., 1999; Laukkanen et al., 2000). Due to the fact that PPO is a copper containing mixed-function oxidase, it is able to catalyze two oxidative reactions involving molecular oxygen: (1) the *o*-hydroxylation of monophenol to *o*-diphenol and (2) the oxidation of colorless *o*-diphenol to *o*-quinone (Figure 5) (Toivonen and Brummell, 2008). Then, these *o*-quinones condense and react non-enzymatically with other compounds, including amino acid, protein, phenolic compounds, to produce dark-colored compounds (Figure 6) (Arnaldos et al., 2001; Sapers et al., 2002; Ni Eidhin et al., 2006). Moreover, Jiang and Miles (1993) previously reported that the production of quinone through PPO-catalyzed reactions can cause the accumulation of hygrogen peroxide (H_2O_2), which is one of the substrates of POD-mediated polyphenol browning.

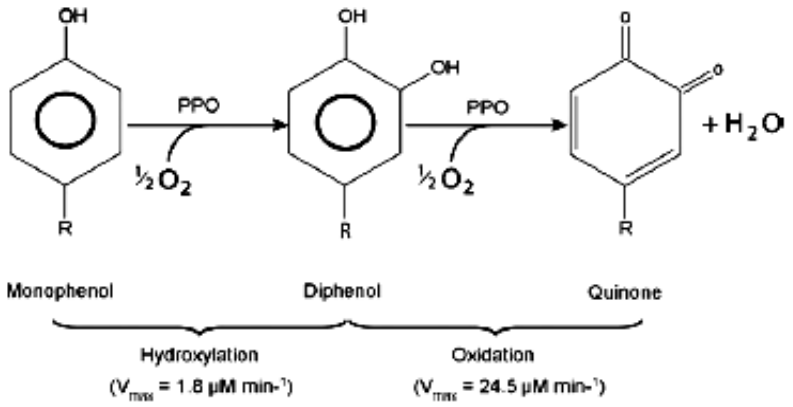


Figure 5 The action mechanism of PPO on monophenol and diphenol oxidations. (Source: Toivonen and Brummell, 2008)

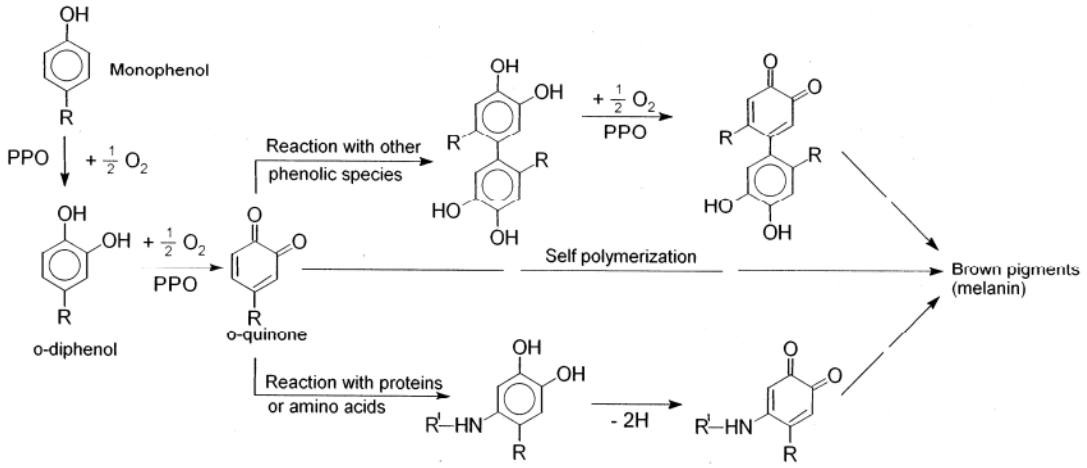


Figure 6 General pathway of PPO-mediated browning reaction. (Source: Gacche et al., 2006)

C. Phenylalanine ammonia-lyase (PAL)

PAL is known to be the key enzyme involved in phenolic biosynthesis through the phenylpropanoid pathway with phenylalanine as a major precursor (Dixon and Paiva, 1995; Fraser and Chapple, 2011). PAL is located in cytoplasm and some organelles consisting of chloroplast, leucoplast, mitochondria, peroxisome and Golgi-derived vesicles (Dixon and Paiva, 1995; Nakashima et al., 1997; Zhou et al., 2009). This enzyme catalyzes the conversion of the amino acid

L-phenylalanine to *trans*-cinnamic acid (Figure 7), the initial step in the biosynthesis of phenolics, which can then be converted to other phenolic compounds such as chlorogenic acid and caffeic acid derivatives (Cheng and Breen, 1991; Hisaminato et al., 2001; Nguyen et al., 2003; Hyun et al., 2011). Moreover, the PAL serves as a biochemical marker for studying various biotic and abiotic stress conditions such as drought, salinity, heavy metals, infection by pathogen, wounding which have been found to increase the activity of PAL through an unknown signal or induced ROS (Saltveit, 1997; Orozco-Cárdenas et al., 2001; Vogt, 2010). Accordingly, PAL activity is involved in plant defense responses (MacDonald and D'Cunha, 2007).

In addition, PAL was found to be closely associated with the occurrence of oxidative browning due to the fact that the PAL-mediated phenolic synthesis via phenylpropanoid pathway can provide natural substrate for oxidative enzymes, PPO (Hisaminato et al., 2001; He and Luo, 2007). Xu and Li (2006) and Yin et al. (2008) reported that the activity of PAL was gradually increased in tissue with browning. Thus, control of PAL activity was considered as an important factor for controlling enzymatic browning by decreasing the phenolic substrate available for PPO.

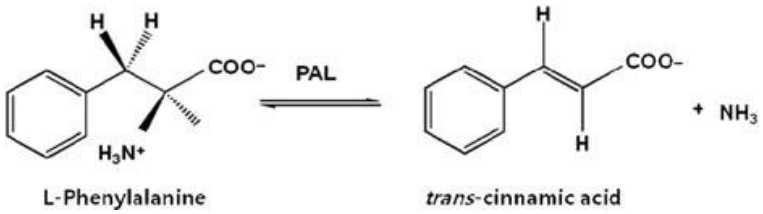


Figure 7 Activity of phenylalanine ammonia-lyase.
(Source: Hyun et al., 2011)

D. Lipid peroxidation

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, also contributes to ROS

production (Wang et al., 2011). Furthermore, culturing callus without subculture can increase the accumulation of free radicals and arrest the morphogenic transition of plant cells (Benson, 2000; George, 2008). Likewise, Liu and Chen (2010) also reported that the production of ROS in *Phalaenopsis* spp., including the hydroxyl radical (•OH), superoxide anion ($O_2•^-$) and hydrogen peroxide (H_2O_2) increased significantly during callus induction. Benson (2000) reported that the increased ROS may be recalcitrant to in vitro culture. Various organelles and cellular structures such as chloroplasts, mitochondria, peroxisomes, plasmalemma, ER and cell wall are the sites of production and scavenging of ROS in response to stress condition (Gill and Tuteja, 2010).

In fact, this excessive generation of ROS is related to lipid peroxidation since ROS can react with polyunsaturated fatty acids in membrane phospholipids, components of cellular and subcellular membrane (Sharma et al., 2012). The overall process of lipid peroxidation consists of three distinct stages: initiation, progression, and termination steps (Figure 8). Accordingly, lipid peroxidation can cause the oxidative damage in several cellular components such as the deformation of chloroplasts, disintegration of the plasmalemma, and breakage of the nuclear membrane (Mittler, 2002; Gill and Tuteja, 2010). These oxidative damages appear to be due to an imbalance between ROS generation and antioxidative enzymes (Tripathy and Oelmüller, 2012). Besides, lipid peroxidation can contribute to the formation of H_2O_2 which is one of the highly toxic ROS in biological systems (Neill et al., 2002; Dolatabadian and Modarressanavy, 2008).

In addition, ROS-mediated lipid peroxidation was found to be involved in tissue browning as shown by an increase in lipid peroxidation during browning (Laukkanen et al., 2000; Tang and Newton, 2004; Tang et al., 2004). Sharma et al. (2012) mentioned that the increment of malondialdehyde (MDA) content can point to the overproduction of free radicals and the existence of membrane lipid damage due to the fact that the amount of end-product of lipid peroxidation, MDA, was released from oxidative damaged membrane. Thus, MDA is considered as a one of the most specific biomarkers of lipid peroxidation and oxidative injury (Janero, 1990; Smirnoff, 1993).

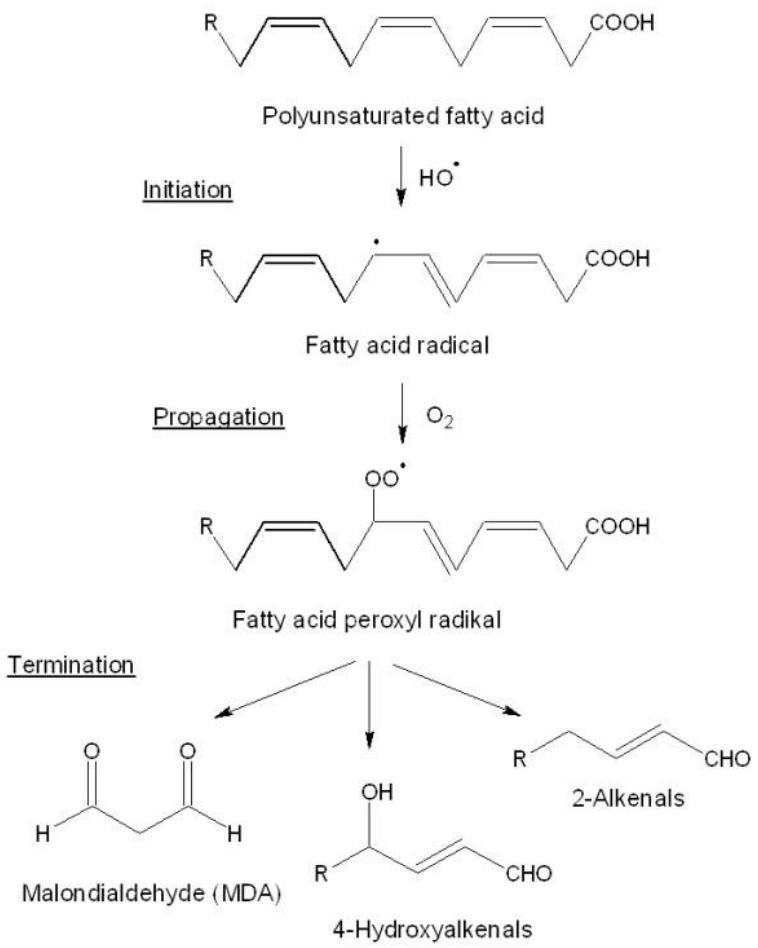


Figure 8 Lipid peroxidation mechanism.
(Source: Mimica-Dukić et al., 2012)

E. Chlorophylls and carotenoids

The change in color is a visible sign of the incidence of tissue browning which was attributed to the gradual loss of chlorophyll and carotenoid contents (Laukkanen et al., 2000; He et al., 2009). Chlorophylls are essential pigments for photosynthesis and their contents have a positive relationship with photosynthetic rate. However, the degradation of chlorophyll can cause the loss of green color leading to the discoloration and the reduction in photosynthetic ability. Yasar et al. (2008) and Khayatnezhad et al. (2011) mentioned that chlorophyll degradation was found to occur as a response to various stress conditions and thus it was considered to be a good indicator of oxidative damage since ROS can cause chlorophyll degradation and lipid peroxidation. In particular, peroxy radical (LOO•)

and phenoxy radical (PhO•) derived from lipid and phenolic oxidation, respectively, were involved in peroxidase-mediated chlorophyll degradation. Moreover, the peroxidase-catalyzed phenol oxidation leads to the production of superoxide anions (O_2 •$^-$) which also directly oxidized the chlorophyll molecule (Figure 9) (Toivonen and Brummell, 2008). Accordingly, the excessive radical production was closely related with chlorophyll breakdown resulting in the loss of chlorophyll and eventually the reduction of plant cell growth.

Carotenoids, lipophilic organic compounds, are also important pigment for photosynthesis by absorbing light and then transferring this light energy to chlorophyll molecule within the photosynthetic apparatus (Polívka and Frank, 2010). Moreover, carotenoids are able to scavenge various forms of ROS due to their antioxidant property leading to protect the photosynthetic system from photo-oxidative damage. By antioxidant property, carotenoids play a role in the inhibition of oxidative damage by reacting with lipid radical to terminate the lipid peroxidation chain reaction, reacting with excited triplet chlorophyll ($^3Chl^*$) or excited chlorophyll (Chl^*) molecules to prevent the singlet oxygen ($^1O_2$) generation and scavenging $^1O_2$ (Figure 10) (Karuppanapandian et al., 2011; Boguszewska and Zagdańska, 2012). Thus, the alteration of chlorophyll and carotenoid contents can cause an arrest of photosynthesis and a reduction in cell growth.

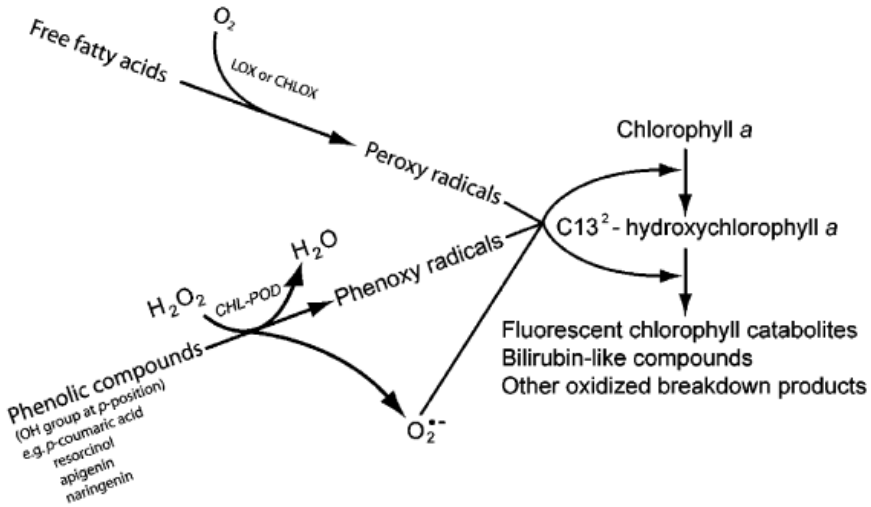
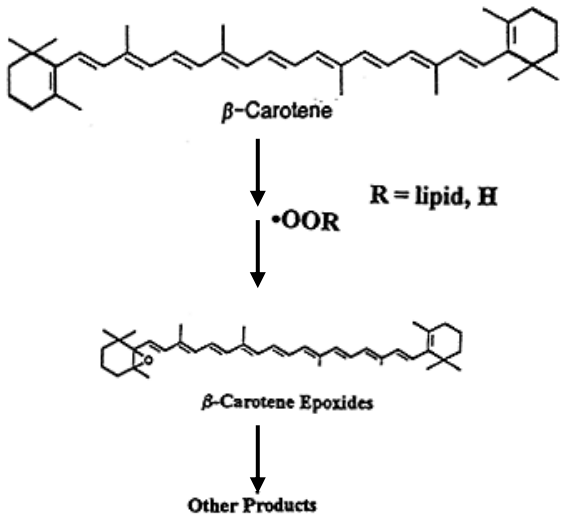


Figure 9 Chlorophyll breakdown pathways in damaged cell of green plant tissue. (Source: Toivonen and Brummell, 2008)

(A)



(B)

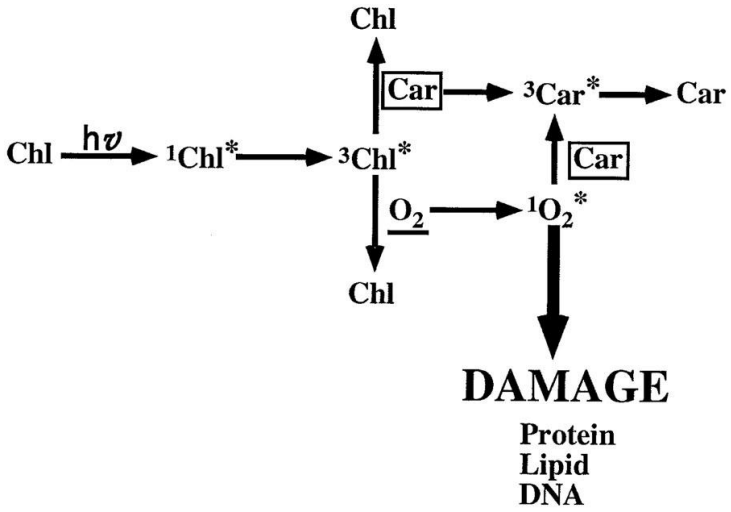


Figure 10 The protective role of carotenoid against oxidative stress.
The reaction between carotenoid and **(A)** lipid radical, and **(B)** triplet chlorophyll and singlet oxygen.
(Source A: adapted from Sturm, 2014; Source B: Ouchane et al., 1997)

1.2.5 Inhibition of enzymatic browning

A. Transferring to fresh medium

Transfer of explants to a fresh medium at short time interval is the way to reduce browning which could be attributed to the reduction of oxidative enzyme and phenolic substrate (Chugh et al., 2009; Abohatem et al., 2011). This technique is simplest and low cost, thus commonly used in many plant species such as *Phalaenopsis* Nebula (Chen et al., 2000), *Capsicum annuum* var. Kulai (Mok and Norzulaani, 2007) and *Phoenix dactylifera* L. (Abohatem et al., 2011). However, frequent subculture can lead to somaclonal variation which is undesirable for plant tissue culture (Sahijram et al., 2003; Bairu et al., 2011).

B. Addition of anti-browning agents

The incorporation of browning inhibitors into culture medium has been used for preventing or delaying the browning reaction. These anti-browning agents can be classified into two groups; antioxidant and adsorbent, and they will be briefly described as follows:

Antioxidants

Antioxidants act as a reducing agent which can neutralize free radicals by donating their electron to these oxidizing agents (Blokhina et al., 2003; Shahidi and Zhong, 2005). Accordingly, antioxidants can be used for preventing the oxidation reaction of oxidizable substrates such as protein, carbohydrate, lipid and DNA.

Moreover, antioxidants can be used as an inhibitor of tissue browning in many plant species, since the addition of appropriate type and concentration of antioxidant could change the oxidation-reduction potential of plant cell leading to prevent the oxidation of polyphenols (Figure 11) (Misra et al., 2010; Zhou et al., 2010). Moreover, antioxidants also reduce the accumulation of peroxidase which is implicated in the oxidation of phenolic compounds (Park et al., 2006). Thus, antioxidants play a role in scavenging ROS and inhibiting ROS generation (Dan, 2008).

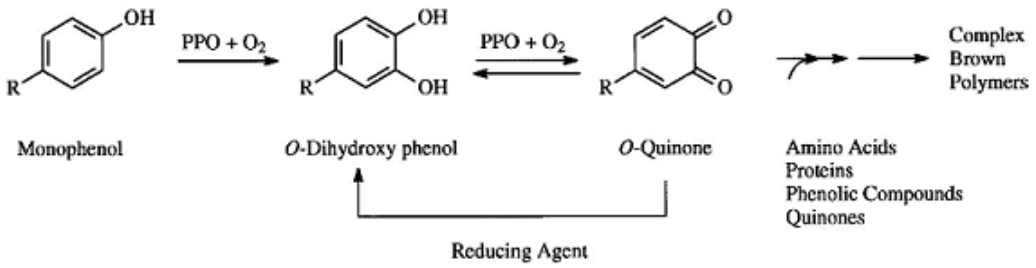


Figure 11 Effect of antioxidant on prevention of the oxidation of phenolic compound.
(Source: Sapers et al., 2002 cited in Sapers, 1993)

Ascorbic acid (AA)

AA ($C_6H_8O_6$) is a water soluble organic compound. It is a hexose derivative and classified as carbohydrate-like substance (Figure 12) (Davey et al., 2000; Oguntibeju, 2008). Moreover, AA contains an enediol group at carbon 2 and 3 which motivates the antioxidant property of AA (Olabisi, 2005; Prakash and Suneetha, 2014). As mentioned earlier, AA act as a powerful antioxidant so that it can directly scavenge or neutralize ROS, leading to an inhibition of lipid peroxidation (Noctor and Foyer, 1998; Horemans et al., 2000; Goveia, 2007; Dolatabadian and Saleh Jouneghani, 2009). In fact, AA in aqueous solution (ascorbate) is the deprotonated form and can donate one electron to free radical and then ascorbate itself becomes the ascorbyl radical (monodehydroascorbate) which is fairly stable and an unreactive free radical. This intermediate is further oxidized to dehydroascorbate which is then reduced to ascorbate by dehydroascorbate reductase via a reaction requiring glutathione, a H_2O_2 detoxifying system (Secenji et al., 2008; Yang et al., 2009). Due to the above mechanism, ascorbate plays an important role in neutralizing free radicals by donating its electron to abundant free radicals (Figure 13). Accordingly, AA has been served as a strong reducing agent in protecting plant cell from oxidative stress (Smirnoff, 1996; Titov et al., 2006; Karuppanapandian et al., 2011). AA was also found to be effective on prevention of lethal browning of many plant species such as Yali,

Aikansui and Abbe Fetel pears (Poudyal et al., 2008) and Cavendish banana (Ko et al., 2009).

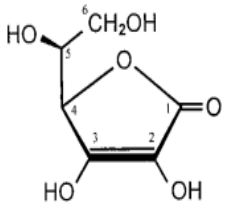
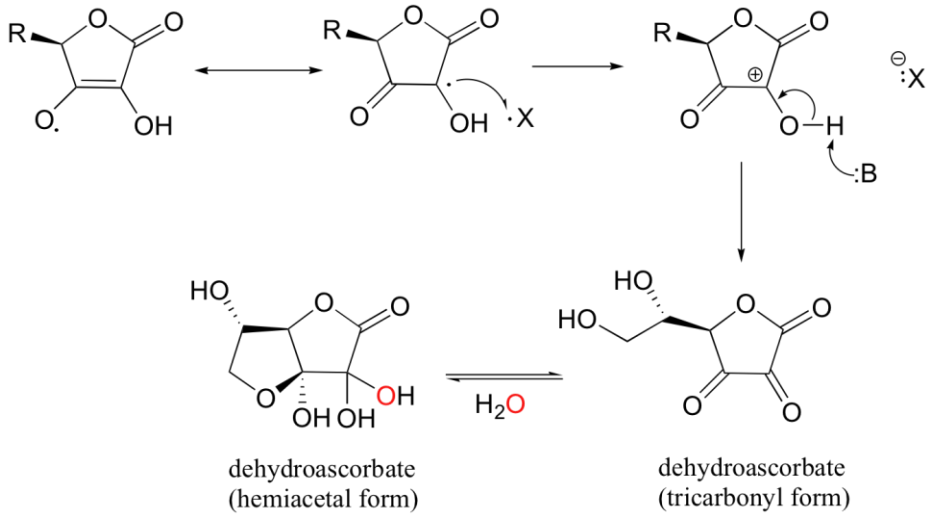


Figure 12 Chemical structure of ascorbic acid.
(Source: Davey et al., 2000)

(A)



(B)

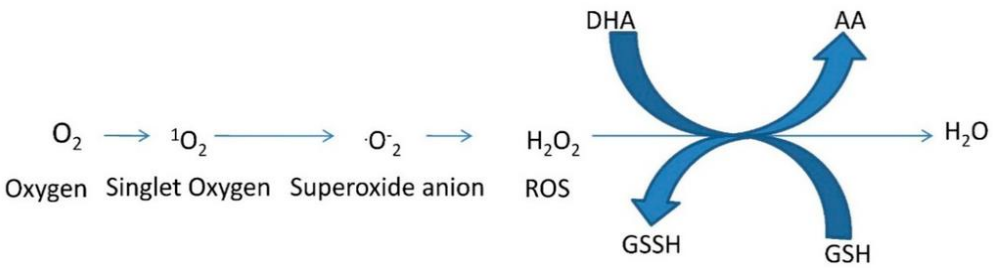


Figure 13 Mechanism of **(A)** ascorbic acid oxidation and **(B)** reversible ascorbate-dehydroascorbate conversion.
(Source A: Soderberg**,** 2013; Source B: Most and Papenbrock, 2015)

L-cysteine (Cys)

Cys ($C_3H_7NO_2S$) is a sulfur-containing amino acid that consists of three functional groups, namely carboxylic acid (-COOH), amino group ($-NH_2$) and thiol groups (-SH) (Figure 14). According to the chemical structure, Cys functions as an antioxidant since the thiol group can undergo redox reactions. Due to its antioxidant property, Cys can scavenge free radical by donating a hydrogen atom or electron to a radical resulting in inhibiting the free radical chain reaction (Figure 15) (Darkwa et al., 1998; Altunkaya and Gökmen, 2008). Moreover, Cys is also reported as an effective PPO inhibitor (Gacche et al., 2006; Manohan and Wai, 2012). Since Cys is able to react with *o*-quinone, produced by PPO-catalyzed oxidation of phenolic substrate, to form stable colorless Cys-quinone adduct which serve as competitive inhibitor of PPO, thus inhibiting PPO activity and consequently stopping pigment formation (Figure 16) (Ding et al., 2002; Altunkaya and Gökmen, 2008; Saeidian, 2013). Accordingly, Cys has been shown to be an effective antioxidant for alleviating enzymatic browning as previously reported in many plant species such as *Rollinia mucosa* (Jacq.) Baill., (Figueiredo et al., 2001); Bamboo (Huang et al., 2002); *Coffea arabica* (Ismail et al., 2003) by using Cys at 50 mg L^{-1}, 1 mM and 10 mg L^{-1}, respectively.

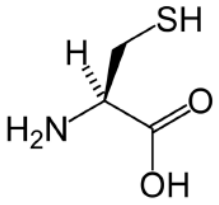
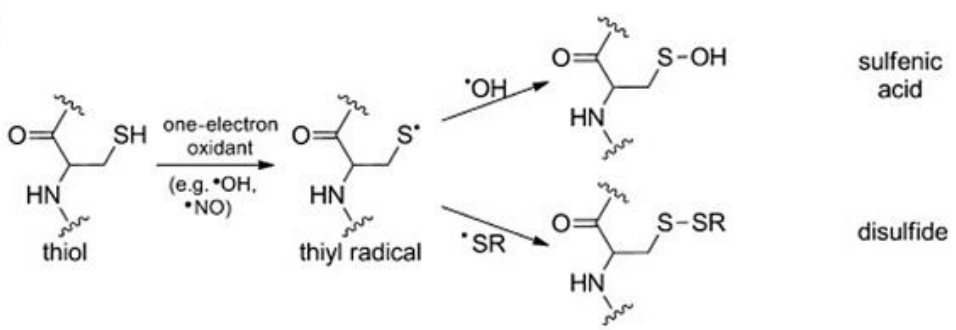


Figure 14 Chemical structure of L-cysteine.
(Source: Birnie-Lefcovitch, 2006)

(A)



(B)

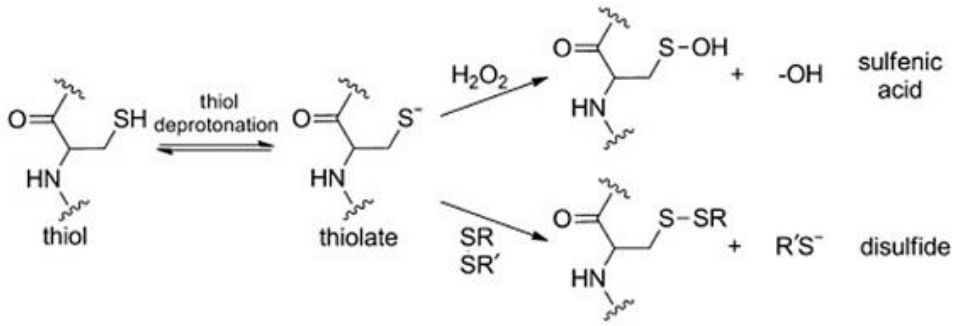


Figure 15 Mechanism of L-cysteine oxidation by **(A)** one-electron and **(B)** two-electron oxidants.
(Source: Hougland et al., 2013)

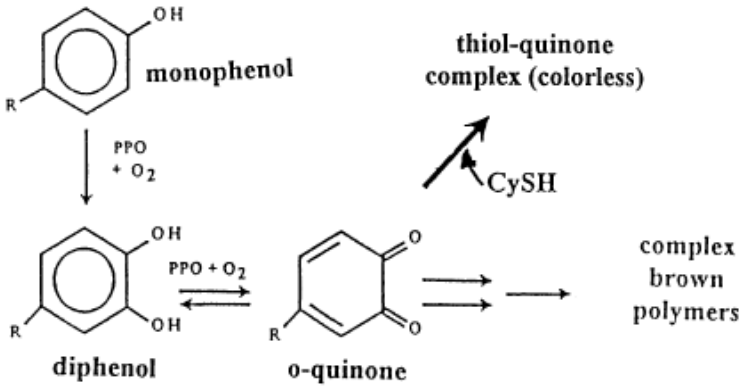


Figure 16 Effect of L-cysteine on inhibition of PPO-mediated reaction.
(Source: Ding et al., 2002)

Adsorbents

Adsorbents are compounds exhibiting adsorption capacity and are able to absorb various substances including toxic substance produced by plant cell. In plant tissue culture technique, there are several types of adsorbent that are widely used to remove toxic substances associated with tissue browning. Particularly, AC and PVP have been commonly used to alleviate tissue browning (Pan and van Staden, 1998; Tang et al., 2004).

Activated charcoal (AC)

AC is a strong adsorptive material that consists of a very fine network of pores with large inner surface area and volume (Figure 17) (Thomas, 2008). According to the porous structure, AC can absorb various organic compounds including excess hormones, vitamins and undesirable substances (Pan and van Staden, 1998; van Winkle et al., 2003; Thomas, 2008). Due to its ability to remove the undesirable compounds, AC was found to effectively reduce the oxidation of phenolic compound by removing phenolic substrate and decrease the accumulation of organic exudate by adsorbing quinone (Tao et al., 2007; North et al., 2012). Accordingly, the addition of AC has been found to be effective for preventing tissue browning of many plant species such as *Piper* species (Madhusudhanan and Rahiman, 2000), *Phalaenopsis* hybrid (Young et al., 2000), *Musa acuminata* L. (Kariyana and Nisyawati, 2013) by added AC at 0.02 %, 0.5 % and 0.2 %, respectively. However, the non-selective adsorption mechanism of AC can cause some adverse side-effects on cultured explants resulting in inhibition of cell growth in vitro or no inhibition of browning (Pan and van Staden, 1998; Zhou et al., 2010; Lajayer et al., 2011; Cui and Wang, 2012).

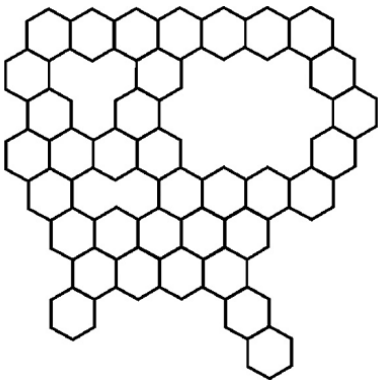
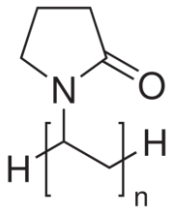


Figure 17 Structure of activated charcoal.
(Source: Wang et al., 2009 cited in Zhang, 1989)

Polyvinylpyrrolidone (PVP)

PVP [$(C_6H_9NO)_n$] is a white synthetic, water-soluble polymer which made from 1-vinyl-2-pyrrolidone monomer and contains carbonyl group (-CO-N) analogizing to peptide bond (-CO-NH-) (Figure 18) (Musbah, 1992; Saeed et al., 2006; Liu et al., 2013). Due to its chemical structure, PVP can form complex with various compounds including polyphenols through the formation strong hydrogen bond between carbonyl group of PVP and hydroxyl group of phenolic compound (Figure 19) (Andersen and Sowers, 1968; Haaf et al., 1985; Musbah, 1992). Moreover, a soluble form of PVP also acts as a competitive inhibitor of PPO by combining with the phenolic substrate moiety of PPO-substrate complex (Lozano-de-Gonzalez, 1991; Musbah, 1992). According to these functions of PVP, the oxidation of phenolic compound can be prevented by reducing the substrate of PPO-mediated oxidation and reducing the activity of PPO (Figueiredo et al., 2001; Sathyanarayana, 2007). There were some previous reports on the application of PVP into the culture medium for prevention of browning, for instance, chestnuts (Abenavoli and Pennisi, 1998), *Dendrocalamus strictus* (Saxena and Dhawan, 1999), *Cycas revoluta* (Kiong et al., 2008) and *Psidium guajava* (Liu and Yang, 2011) by using PVP at 0.25, 1 and 0.25 g L^{-1}, respectively.

(A)



(B)

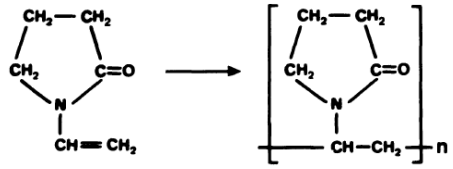


Figure 18 Chemical structure of **(A)** polyvinylpyrrolidone and **(B)** polymerization of *N*-vinylpyrrolidone.
(Source A: Tavlarakis et al., 2011; Source B: Haaf et al., 1985)

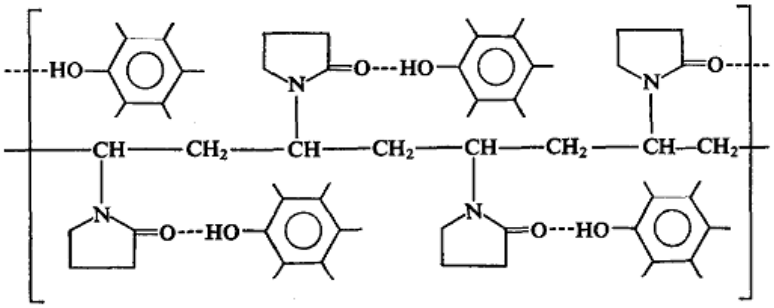


Figure 19 Characterization of hydrogen bond formation between polyvinylpyrrolidone and phenolic compound.
(Source: Andersen and Sowers, 1968)

1.3 Objectives

1. To optimize the concentrations of PGRs, auxin and cytokinin, for callus induction.

2. To elucidate the alterations of biological response at the biochemical and cellular and/or subcellular levels during an extended callus culture

3. To investigate the suitable anti-browning agent for prevention of browning.

CHAPTER 2
RESEARCH METHODOLOGY

2.1 Investigation of PGRs affecting morphogenic responses

To determine the influence of PGRs, NAA and BA, at various concentrations on morphogenic responses, namely callus, PLB and shoot formation.

2.1.1 Plant material preparation

Plants of *D. crumenatum* were cultivated under natural conditions (approximately 28 ± 2 °C) at a photosynthetic photon flux density (PPFD) of 30 µmol m^{-2} s^{-1} in a shaded greenhouse at the Department of Biology, Faculty of Science, Prince of Songkla University. Blooming flowers (at 9 days after a sudden drop in temperature of approximate 10 °C) of more than five-year-old mother plants were hand cross-pollinated to produce orchid pods.

Green capsules were collected at 6 weeks after pollination and surface-sterilized in 1.2 % sodium hypochlorite (NaOCl) solution (1.14 % active chlorine) containing 1-2 drops of Tween 20 for 20 min. They were then thoroughly rinsed with sterile distilled water for 2-3 times and the sterilized capsules were then cut longitudinally into halves. Seeds were scooped out and inoculated into 125 ml Erenmeyer flask containing 30 mL of VW (Vacin and Went, 1949) liquid medium containing 2 % sucrose. The pH of the medium was adjusted to 5.3 with 1 N NaOH or 1 N HCl prior to autoclaving at 121 °C for 20 min. The culture was maintained under 16-h photoperiod provided by cool daylight fluorescent lamps (36 Watts, Philips, Bangkok, Thailand) at 23 µmol m^{-2} s^{-1} on an orbital shaker (120 rpm) at 25 ± 2 °C for 3 months and subcultured at monthly intervals. Moreover, the rest of the seeds were collected for the viability test.

2.1.2 Determination of seed viability

Seeds of *D. crumenatum* from six-week-old capsule were soaked in 1 mL of 1 % solution of 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich Co., St. Louis, MO, USA) for 24 h in the darkness at room temperature. After the incubation period, they were removed from the TTC solution, washed with distilled water 2-3 times and centrifuged for 5 min at 3,000 rpm. The seeds were then examined for the color of the embryo using an Olympus SZH 10 stereomicroscope (Olympus Optical Co. Ltd., Tokyo, Japan) and the images were captured with an Olympus DP 72 digital camera (Olympus Optical Co. Ltd.). The seeds with completely dark red-stained embryos were classified as viable, whereas incompletely stained or unstained embryos were considered as non-viable. The data were collected and calculated as the percentage of viable seeds using the following formula (Vujanovic et al., 2000; Salazar-Mercado, 2012).

$$\text{Percentage of viable seeds} = \frac{\text{Dark red stained embryos} \times 100}{\text{Total number of seeds}}$$

2.1.3 Callus induction

Three-month-old protocorms (approximately 2-3 mm in diameter) at the third stage of developmental growth (Stewart and Zettler, 2002) were transversely bisected without excising the shoot tip. Four segments of bisected protocorms were inoculated cut side down onto 10 mL of modified VW solid medium consisting full-strength macro VW salts, half-strength micro MS salts, 2 % sucrose, 2 % peptone (Becton, Dickinson, and Company, Sparks, MD, USA) and 0.2 % Phytagel (Sigma-Aldrich Co.) in 60 mL glass bottle. Various concentrations of PGRs, NAA (Fluka Chemie GmbH, Buchs, Switzerland) at 0, 0.1, 0.5 mg L^{-1} and BA (Sigma-Aldrich Co.) at 0, 1, 2 mg L^{-1}, were added individually or in combination to the basal medium for examining their inductive effects on morphogenic responses including callus, PLB and shoot. The pH of the media was adjusted to 5.3 with 1 N NaOH or 1 N HCl prior to autoclaving at 121 ºC for 20 min. The explants were incubated in darkness for a week and then transferred to maintain under an illumination at 23 µmol m^{-2} s^{-1}

photosynthetic photon flux density (PPFD) provided by cool daylight fluorescent lamps with a photoperiod of 16-h light and 8-h darkness at 25 ± 2 ºC.

2.1.4 Data collection and statistical analysis

The experiment was performed based on factorial experiments in completely randomized design (CRD). Seven replicates were performed for each treatment. After 8 weeks of culture, the data were recorded and given as the percentage of non-responsive and responsive explants. For morphogenic response, the data were calculated as the percentage of callus, PLB and shoot formation using the following formulae. All data were statistically analyzed separately by analysis of variance (ANOVA) and means were compared by Duncan's multiple range test (DMRT) or least significant difference (LSD) test at a significance level of $P \leq 0.05$ using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) software.

$$\% \text{ Callus formation} = \frac{\text{Number of bisected protocorm producing callus} \times 100}{\text{Number of bisected protocorm inoculated}}$$

$$\% \text{ PLB formation} = \frac{\text{Number of bisected protocorm forming PLB} \times 100}{\text{Number of bisected protocorm inoculated}}$$

$$\% \text{ Shoot formation} = \frac{\text{Number of bisected protocorm forming shoot} \times 100}{\text{Number of bisected protocorm inoculated}}$$

2.1.5 Histological observation

Samples were collected at weekly intervals and prepared by standard paraffin method (Johansen, 1940). Briefly, fresh sample were fixed in FAA II solution (Appendix B) for 48 h at room temperature. The fixed tissue were then dehydrated through a tertiary-butyl-alcohol series and embedded in paraffin wax (Histoplast PE; Richard-Allan Scientific, Michigan, USA). Sections were cut into 6 µm thickness with a rotary microtome (Shandon Southern Product Ltd., Cheshire, England).

The sections were then stained with Delafiled's hematoxylin and safranin (Johansen, 1940; Ruzin, 1999) to observe the origin of callus from the explants and the general structure of various morphological types.

Sections were examined by an Olympus BX 51 TRF light microscope (Olympus Optical Co. Ltd.) and images were captured using an Olympus DP 72 digital camera attached to the microscope.

2.2 Investigation of the alteration of callus during an extended culture period without subculture

To determine the level of browning in response to the inoculation period on callus culture for a better understanding of the biochemical and ultrastructural dynamics underlying callus browning.

2.2.1 Plant material and culture condition

The bisected protocorm-derived calli [100 mg, fresh weight (FW)] were transferred to 60 mL glass bottles containing 10 mL of modified VW supplemented with 2 % sucrose, 2 % peptone and the optimal concentration of PGRs obtained from the callus induction experiment. The medium was solidified with 0.2 % Phytagel. The pH of the medium was adjusted to 5.3 with 1 N NaOH and 1 N HCl before autoclaving at 121 ºC for 20 min. Calli were incubated at 25 ± 2 °C without subculturing and maintained under a 16 h light and 8 h dark cycle with PPFD of 23 µmol m^{-2} s^{-1} provided by cool daylight fluorescent lamps.

2.2.2 Data collection and statistical analysis

The experiment was performed according to CRD with three replicates per treatment. The level of browning of the callus was then calculated using the following formula and statistically analyzed using a one-way ANOVA followed by DMRT.

$$\% \text{ browning callus} = \frac{FW_b \times 100}{FW_f}$$

Where, FW_b : the fresh weight of browning callus (g)

FW_f : the final fresh weight of entire callus mass (g)

One-month-old proliferated callus at the beginning of culture 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 histological, ultrastructural and biochemical analyses.

2.2.3 Histological, structural and ultrastructural observations

A. Light microscopy (LM) observation

Cultured calli were collected at monthly intervals, fixed in FAA II solution and then processed to dehydration, infiltration, embedding and sectioning following the standard paraffin method (Johansen, 1940) as described in 2.1.5.

The sections were then stained with Delafiled's hematoxylin and safranin (Johansen, 1940; Ruzin, 1999) for studying cell arrangement. The sections were also stained with periodic acid-Schiff's (PAS) reaction for insoluble carbohydrate (Feder and O'Brien, 1968) and 1 % (w/v) toluidine blue O (TBO) solution for polyphenolic localization (Yeung, 1998; Ruzin, 1999).

All stained sections were examined by an Olympus BX 51 TRF light microscope and images were taken using an Olympus DP 72 digital camera attached to the microscope.

B. Scanning electron microscopy (SEM) observation

Six samples were collected monthly intervals and fixed in SEM fixative solution (Appendix B) at 4 °C for 2 h. The fixed calli were washed in 0.1 M phosphate buffer (pH 7.2) for 15 min and dehydrated twice through an ethanol series (30 %, 50 %, 70 %, 80 %, 90 %, 95 % and 100 % ethanol) for 15 min each. They were then dried using a Polaron CPD 7501 critical point dryer (VG. Microtech, East

Sussex, UK), mounted on stubs and coated with gold by a SPI214 MODULE sputter coater (SPI Supplies Division of Structure Probe Inc., West Chester, PA, USA).

Prepared samples were examined by a Quanta 400 scanning electron microscope (FEI, Brno, Czech Republic) at an accelerating voltage of 10 kV for investigating the alterations in cellular structure during an extended callus culture.

C. Transmission electron microscopy (TEM) observation

Six intact callus clumps were collected monthly intervals and prepared according to the standard TEM method (Hall and Hawes, 1991; Davies, 1999). Briefly, samples were pre-fixed in a fixative solution containing 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) with the addition of 1% caffeine (Mueller and Greenwood, 1978) at 4 °C for 24 h. Pre-fixed tissues were then washed thrice with 0.1 M phosphate buffer (pH 7.2) and post-fixed with 1 % osmium tetroxide (OsO_4) (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 then rinsed three times with distilled water and dehydrated through a graded ethanol series (70 %, 80 %, 90 % and 100 %) for 15 min each. The fixed samples were infiltrated with propylene oxide and embedded in EMbed 812 (Electron Microscopy Sciences). Semi-thin sections (about 1 µm thick) were cut with glass knife and stained with 1 % TBO in 1 % sodium borate for preliminary light microscope observation.

For TEM viewing, ultrathin sections (60 nm thick) were cut using a RMC MTXL ultramicrotome (Boeckeler Instruments, Arizona, USA) with a diamond knife and then stained with 5 % uranyl acetate (Electron Microscopy Sciences) for 10 min and lead citrate (Electron Microscopy Sciences) for 5 min to obtain optimum contrast.

Sections were viewed with a JEM-2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 160 kV for examining the influence of browning on subcellular structure during an extended callus culture.

2.2.4 Measurements of biochemical parameters

To elucidate the alterations of biochemical features during an extended callus culture for confirmation the occurrence of browning.

A. Preparation of extract

Cultured callus were collected at different periods of inoculation (1, 2 and 3 months of inoculation) for sample extraction as described by Tang and Newton (2004) with some modifications.

Callus about 400 mg in fresh weight was homogenized in 400 µL of extraction buffer containing 0.1 M sodium phosphate buffer (pH 7.2) and 0.1 % (w/v) sodium dodecyl sulfate (SDS) (EMD Millipore Co.). The homogenate was centrifuged at 12,000×*g* for 10 min at 4 °C in a Mikro 200R refrigerated centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatant was collected, aliquoted and stored at -20 °C for the determination of PPO activity, PAL activity, total phenolic content and lipid peroxidation.

B. PPO activity assay

The PPO activity was assayed following the standard method (Dai, 1993). Briefly, the supernatant (10 µL) was mixed with a reaction mixture containing 590 µL of 0.1 M sodium phosphate buffer (pH 7.2) and 150 µL of 0.5 M catechol (Sigma-Aldrich Co.). The change in absorbance of the sample was measured at 490 nm at 15 s intervals for 3 min with a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific, New Jersey, USA) at room temperature.

One unit of PPO activity (U) was defined as the amount of enzyme that caused a change of 0.01 in the absorbance per min under assay condition and expressed as unit per gram of fresh weight (U g^{-1} FW).

C. PAL activity assay

The measurement of PAL activity was carried out according to a modified method of Tan et al. (2014). The supernatant (25 µL) was mixed with 712 µL of reaction mixture consisting of 0.1 M Tris-HCl buffer (pH 8.5), 1 mM β-mercaptoethanol (Sigma-Aldrich Co.) and 0.1 M L-phenylalanine (Sigma-Aldrich

Co.). The mixture was incubated in a water bath at 40 °C for 1 h and the reaction was stopped after incubation period by the addition of 13 µL of 6 N HCl. The denatured protein was then pelleted by centrifugation at room temperature for 10 min at 10,000×*g* on a Sorvall Biofuge Pico centrifuge (Kendro Laboratory Products, Hanau, Germany). The reaction without enzyme extract was used as a blank. The absorbance of sample was measured spectrophotometrically at 290 nm by using an HP-8453E UV-visible spectrophotometer (Hewlett Packard, Palo Alto, CA, USA).

One unit of PAL activity (U) was taken as a change in absorbance of 0.01 per hour under this assay condition and expressed as units per gram of fresh weight (U g^{-1} FW).

D. Determination of phenolic content

The total phenolic content was determined according to Park et al. (2006) with some modifications. Supernatant (10 µL) was mixed with 200 µL of 10 % Folin-Ciocalteu phenol reagent (Sigma-Aldrich Co.) followed by the addition of 1 mL of 10 % sodium carbonate (Ajax Finechem). The mixture was incubated at room temperature for 20 min and the absorbance was measured at 735 nm by using a Genesys 20 visible spectrophotometer. Extraction buffer was used as a blank.

The total phenolic content was calculated based on a gallic acid calibration curve (Appendix C) and expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh weight (mg GAE g^{-1} FW).

E. Lipid peroxidation assay

The level of lipid peroxidation in term of MDA content was measured using the thiobarbituric acid reaction (TBARS) assay (Niknam et al., 2011). A 50 µL of supernatant was mixed with 200 µL of 20 % trichloroacetic acid (TCA) containing 0.5 % thiobarbituric acid (TBA). The mixture was heated at 95 °C for 30 min and quickly cooled in an ice bath. The mixture was then centrifuged at 10,000×*g* for 10 min on a Sorvall Biofuge Pico centrifuge and the absorbance was measured with an HP-8453E UV-visible spectrophotometer at 532 and 600 nm. Extraction buffer was used as a blank.

The MDA content was calculated by the following formula using the extinction coefficient of 155 mmol cm^{-1} and expressed as micromole MDA per gram fresh weight [µmol (MDA) g^{-1} FW] (Niknam et al., 2011) as follows:

$$\text{MDA (µmol g}^{-1}\text{ FW)} = \frac{(A_{532} - A_{600}) \times 1000}{\varepsilon \times FW}$$

Where,

A_{532} : the absorbance value at 532 nm

A_{600} : the absorbance value at 600 nm

ε : the specific extinction coefficient (155 mmol cm^{-1})

FW : fresh weight of callus (g)

F. Determination of chlorophyll and carotenoid contents

Cultured callus (approximately 100 mg FW) was collected at 1, 2 and 3 months of inoculation period and prepared by standard method of Misra et al. (2010). Samples were homogenized in 2.30 mL of 80 % chilled acetone and centrifuged at room temperature for 10 min at 10,000×*g* with a Sorvall Biofuge Pico centrifuge. The optical density of the supernatant was measured at 480, 510, 645 and 663 nm, against an 80% acetone blank in an HP-8453E UV-visible spectrophotometer.

Total chlorophyll, chlorophyll *a*, chlorophyll *b* and carotenoid contents were calculated using the following formulae and expressed in term of milligram per gram fresh weight (mg g^{-1} FW).

Total chlorophyll = $[20.2(A_{645}) + 8.02(A_{663})] \times V /1000 \times FW$

Chlorophyll *a* = $[12.7(A_{663}) + 2.63(A_{645})] \times V /1000 \times FW$

Chlorophyll *b* = $[22.9(A_{645}) - 4.68(A_{663})] \times V /1000 \times FW$

Carotenoid = $[7.6(A_{480}) - 2.63(A_{510})] \times V /1000 \times FW$

Where,

A_{480} : the absorbance value at 480 nm

A_{510} : the absorbance value at 510 nm

A_{645} : the absorbance value at 645 nm

A_{663} : the absorbance value at 663 nm

FW : fresh weight of callus (g)

V : volume of extracted solution (mL)

G. Statistical analysis of biochemical parameters

Independent repetition of each biochemical experiment was performed three replicates per treatment to determine the effects of culture period on biochemical parameters. Each biochemical experiment was designed in CRD. Data were recorded at 1, 2 and 3 months of culture period and statistically analyzed using a one-way ANOVA. Significant differences between means were determined by DMRT at a significance level of $P \leq 0.05$ using SPSS version 19.0 (SPSS Inc.) software.

2.3 Investigation of the effect of anti-browning agents on preventing callus browning

To elucidate the alterations of structural, ultrastructural and biochemical aspects for investigating the potential influence of anti-browning agents on browning prevention.

2.3.1 Callus proliferation

One piece of callus (approximately 100 mg FW) was inoculated onto modified VW medium containing 2 % sucrose, 2 % peptone and PGRs at the best concentration for callus induction as mentioned earlier. To examine the effect of anti-browning agents on callus proliferation, ascorbic acid (Ajax Finechem, Taren Point, Australia) (AA; 0, 0.01 and 0.001 g L^{-1}), L-cysteine (Cys; Sigma-Aldrich Co.) (0, 0.01 and 0.05 g L^{-1}), polyvinylpyrrolidone (Sigma-Aldrich Co.) (PVP; 0, 0.5 and 5

g L^{-1}) and activated charcoal (Sigma-Aldrich Co.) (AC; 0, 1 and 2 g L^{-1}) were added individually into a modified VW medium. The medium was then solidified with 0.2 % Phytagel and the pH was adjusted to 5.3 with 1 N NaOH or 1 N HCl prior to autoclaving at 121 ºC for 20 min. The heat-labile compounds, AA, Cys and PVP, were sterilized by filtration through 0.2 µm sterilizing grade filter (EMD Millipore Co., Billerica, MA, USA) and added to the media after autoclaving. Calli were maintained without any subculture under the same condition as described earlier for callus induction.

2.3.2 Data collection and statistical analysis

The experiment was designed according to CRD. Three replicates were conducted for each treatment and the data recordings were done after 3 months of culture. The increased fresh weight of proliferated callus was calculated using the formula A. The amount of browning callus was separately weighed and given as a percentage of browning callus determining by the formula B as follows.

$$\text{Increased fresh weight of proliferated callus} = \frac{FW_f - FW_i}{FW_i} \quad \text{..... (A)}$$

$$\% \text{ browning callus} = \frac{FW_b \times 100}{FW_f} \quad \text{..... (B)}$$

Where, FW_b : the fresh weight of browning callus (g)

FW_f : the final fresh weight of entire callus mass (g)

FW_i : the initial fresh weight (g)

All data were analyzed independently by a one-way ANOVA and significant differences between treatment means were identified using DMRT at a P value of 0.05 using SPSS version 19.0 (SPSS Inc.) software.

2.3.3 Measurements of biochemical parameters

A. Preparation of extract

Proliferated callus in each treatment was collected after 3 months of culture. About 400 mg FW of collected callus was homogenized and extracted following the standard extraction method as detailed in 2.2.4A. The supernatant was used for the determination of PPO activity, PAL activity, total phenolic content and lipid peroxidation.

B. PPO activity assay

The PPO activity was assayed following the standard method (Dai, 1993) as described in 2.2.4B.

C. PAL activity assay

The measurement of PAL activity was carried out according to a modified method of Tan et al. (2014) as detailed in 2.2.4C.

D. Determination of phenolic content

The total phenolic content was determined by standard protocol (Park et al., 2006) as described in 2.2.4D.

E. Lipid peroxidation assay

The level of lipid peroxidation in term of MDA content was measured using the TBARS assay (Niknam et al., 2011) as described in 2.2.4E.

F. Determination of chlorophyll and carotenoid contents

Proliferated callus was collected after 3 months of culture. About 100 mg FW of callus was extracted and the content of total chlorophyll, chlorophyll *a*, chlorophyll *b* and carotenoid determined by standard method of Misra et al. (2010) as detailed in 2.2.4F.

G. Statistical analysis of biochemical parameters

All experiments were performed twice with three replicates per treatment. Data were recorded after 3 months of culture and statistically analyzed using a one-way ANOVA. Significant differences between means were determined by DMRT at a significance level of $P \leq 0.05$.

2.3.4 Histological and ultrastructural observation

A. LM observation

After 3 months of culture, the proliferated callus were collected, fixed in FAA II solution and then processed to dehydration, infiltration, embedding and sectioning following the standard paraffin method (Johansen, 1940) as described in 2.1.5.

The sections were then stained with Delafiled's hematoxylin and safranin (Ruzin, 1999). Stained sections were examined by an Olympus BX 51 TRF light microscope and images were captured using an Olympus DP 72 digital camera attached to the microscope for investigating the effect of anti-browning agent on histological features.

B. TEM observation

After callus proliferation for 3 months, the proliferated callus were collected and fixed in TEM fixative solution, followed by dehydration, infiltration, embedding, sectioning and staining according to the standard TEM method (Hall and Hawes, 1991; Davies, 1999) as detailed in 2.2.3C.

Sections were viewed with a JEM-2010 transmission electron microscope at an accelerating voltage of 160 kV for investigating the effect of anti-browning agent on subcellular structure.

CHAPTER 3
RESULTS

3.1 Influence of PGRs on morphogenic responses

3.1.1 Determination of seed viability

Seed viability of six-week-old *D. crumenatum* capsule (Figure 20A) was determined by using standard protocol of TTC staining. The results showed that average seed viability was about 97.15 ± 0.88 %. After culturing, viable seeds (Figure 20B, 20C) gradually swelled and developed into protocorms. Approximately three-month-old protocorms at the third stage of developmental growth with the appearance of the protomeristem (Stewart and Zettler, 2002) were obtained after culture seed on the VW liquid medium containing 2 % sucrose. These protocorms (Figure 20D) were used as the explants for further experiment.

3.1.2 Callus, PLB and shoot formations

Transversely bisected protocorms of *D. crumenatum* were cultured on a modified VW solidified medium supplemented with various types and concentrations of PGRs, NAA (0, 0.1, 0.5 mg L^{-1}) and BA (0, 1, 2 mg L^{-1}). It was found that some explants became necrotic within 8 weeks before any morphogenic response took place, whereas more than 50 % of explants in all treatments showed various types of developmental morphology. Such results clearly showed that the explants could be categorized as responsive and non-responsive explants, but there were no significant difference between the PGR treatment and control group (Table 1). After 8 weeks of culture, the responsive explants showed different three morphogenic responses, namely callus, PLB and shoot as shown in Figure 21 and Figure 22. However, this study only focused on the formation of callus.

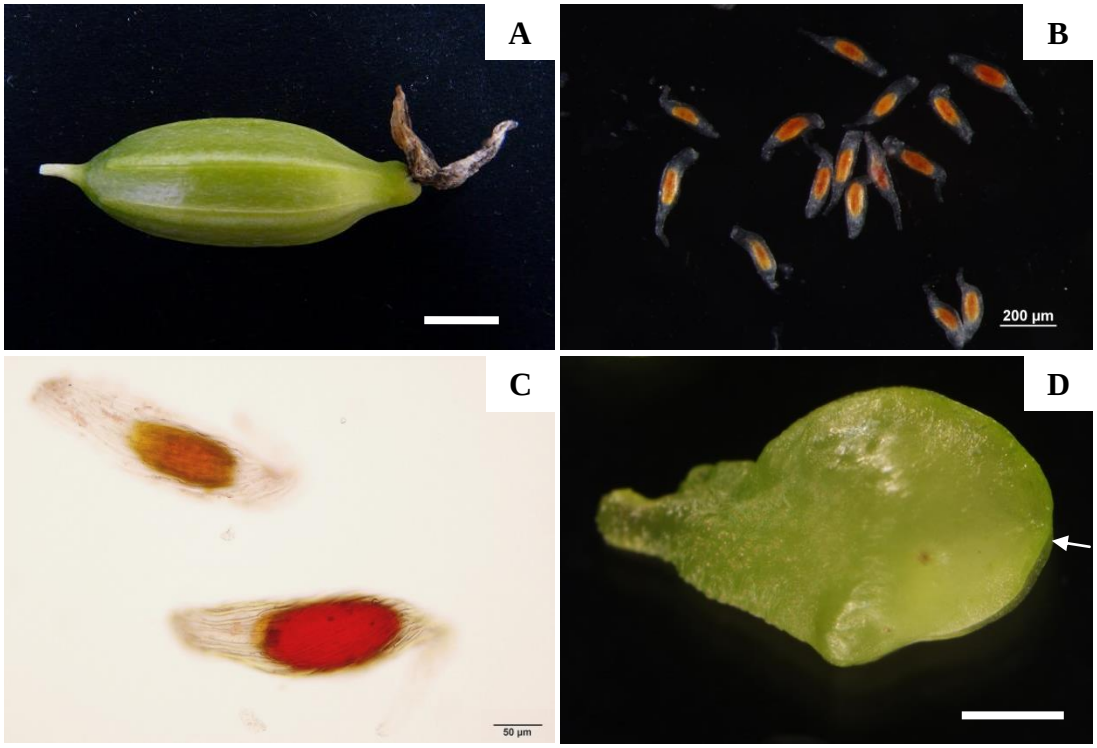


Figure 20 Morphological characteristic of capsule and seed of *D. crumenatum*. **(A)** Six-week-old capsule (*bar* = 0.7 cm); **(B-C)** viable seeds showing red-stained embryos examined by TTC test; **(D)** three-month-old protocorm exhibiting the appearance of shoot region (*arrow*) (*bar* = 1 mm).

Callus formation: callus was observed on all treatments at different frequency (Figure 21). The control treatment gave the lowest frequency of callus formation (7.14 ± 4.61 %). Likewise, the percentage of callus formation on treatments with NAA alone at 0.1 mg L^{-1} (14.29 ± 5.05 %) and 0.5 mg L^{-1} (10.71 ± 5.05 %) and BA alone at 1 mg L^{-1} (25.00 ± 5.46 %) and 2 mg L^{-1} (25.00 ± 10.91 %) were very low and not significantly different from control. Meanwhile, PGRs combination treatments in particular 0.5 mg L^{-1} NAA plus 1 mg L^{-1} BA was found to be a potent treatment for callus formation which gave the highest percentage of explants forming callus (42.86 ± 8.99 %) as compared with control. This induced callus remained healthy and vigorous morphological appearance. Moreover, 0.5 mg L^{-1} NAA combined with 2 mg L^{-1} BA also provided the high percentage of callus

formation (32.14 ± 7.14 %), but this callus was retarded and eventually underwent browning. Accordingly, the medium containing NAA in combination with BA at lower concentration appeared to significantly enhance the formation of callus (Table 2).

PLB formation: PLB was also found in all PGR treatment including control (Figure 21). However, NAA alone at 0.1 mg L^{-1} produced the highest percentage of PLB formation (50.00 ± 10.91%) followed by 0.5 mg L^{-1} NAA (42.86 ± 15.15 %), 2 mg L^{-1} BA (39.29 ± 10.71 %) and 1 mg L^{-1} BA (35.71 ± 9.22 %), respectively. In addition, the PGR combination treatments also gave PLB formation, but lower than individual PGR treatments and there were not significantly different from control (21.43 ± 11.48%).

Shoot formation: shoot was also observed in all treatment at different frequency after bisected protocorm culture on the medium supplemented with various PGR types and concentrations (Figure 21). Particularly, the presence of 1 mg L^{-1} BA gave the highest frequency of shoot formation (25.00 ± 7.72 %), but not statistically difference from the other treatments.

Table 1 Effect of NAA and BA on the responses of protocorm segments of *D. crumenatum* after culture for 8 weeks on a modified VW solid medium.

Plant growth regulators			
NAA (mg L^{-1})	BA (mg L^{-1})	Non-responsive explants (% ± S.E.) ^{ns}	Responsive explants (% ± S.E.) ^{ns}
0	0	46.43 ± 15.84	53.57 ± 15.84
0	1	28.57 ± 10.10	71.43 ± 10.10
0	2	39.29 ± 12.02	60.71 ± 12.02
0.1	0	35.71 ± 13.20	64.29 ± 13.20
0.1	1	50.00 ± 12.20	50.00 ± 12.20
0.1	2	39.29 ± 9.22	60.71 ± 9.22
0.5	0	35.71 ± 12.02	64.29 ± 12.02
0.5	1	28.57 ± 10.10	71.43 ± 10.10
0.5	2	39.29 ± 13.20	60.71 ± 13.20

Data shown the mean of seven replicates ± standard error (S.E.). Comparison of the mean values was analyzed using the DMRT at $P \leq 0.05$.

ns : no significant difference

Table 2 Analysis of variance for effect of PGRs on callus, PLB and shoot formation from transversely bisected protocorm of *D. crumenatum*.

Source of variation	Degrees of freedom	Mean square		
		Callus formation	PLB formation	Shoot formation
NAA	2	833.33 ^{ns}	158.73 ^{ns}	9.92 ^{ns}
BA	2	2172.62*	307.54 ^{ns}	128.97 ^{ns}
NAA$^{\times}$BA	4	416.67 ^{ns}	1289.68 ^{ns}	337.30 ^{ns}
Error	54	340.61	770.50	343.92

* significant at $P \leq 0.05$

ns : non significant difference

Figure 21 Effect of NAA and BA on callus (silver colour), PLB (gray colour) and shoot formation (dark gray colour) from bisected segment of protocorm.

Comparison of the mean value was analyzed using the DMRT or LSD at $P \leq 0.05$. The S.E. bars marked with different letters within morphogenic response indicate significant differences among treatments.

ns : no significant difference between treatment for shoot

* Detailed of various PGRs in a modified Vacin and Went (MVW) medium

1 : MVW	4 : MVW + 0.1 mg L^{-1} NAA	7 : MVW + 0.5 mg L^{-1} NAA
2 : MVW + 1 mg L^{-1} BA	5 : MVW + 0.1 mg L^{-1} NAA + 1 mg L^{-1} BA	8 : MVW + 0.5 mg L^{-1} NAA + 1 mg L^{-1} BA
3 : MVW + 2 mg L^{-1} BA	6 : MVW + 0.1 mg L^{-1} NAA + 2 mg L^{-1} BA	9 : MVW + 0.5 mg L^{-1} NAA + 2 mg L^{-1} BA

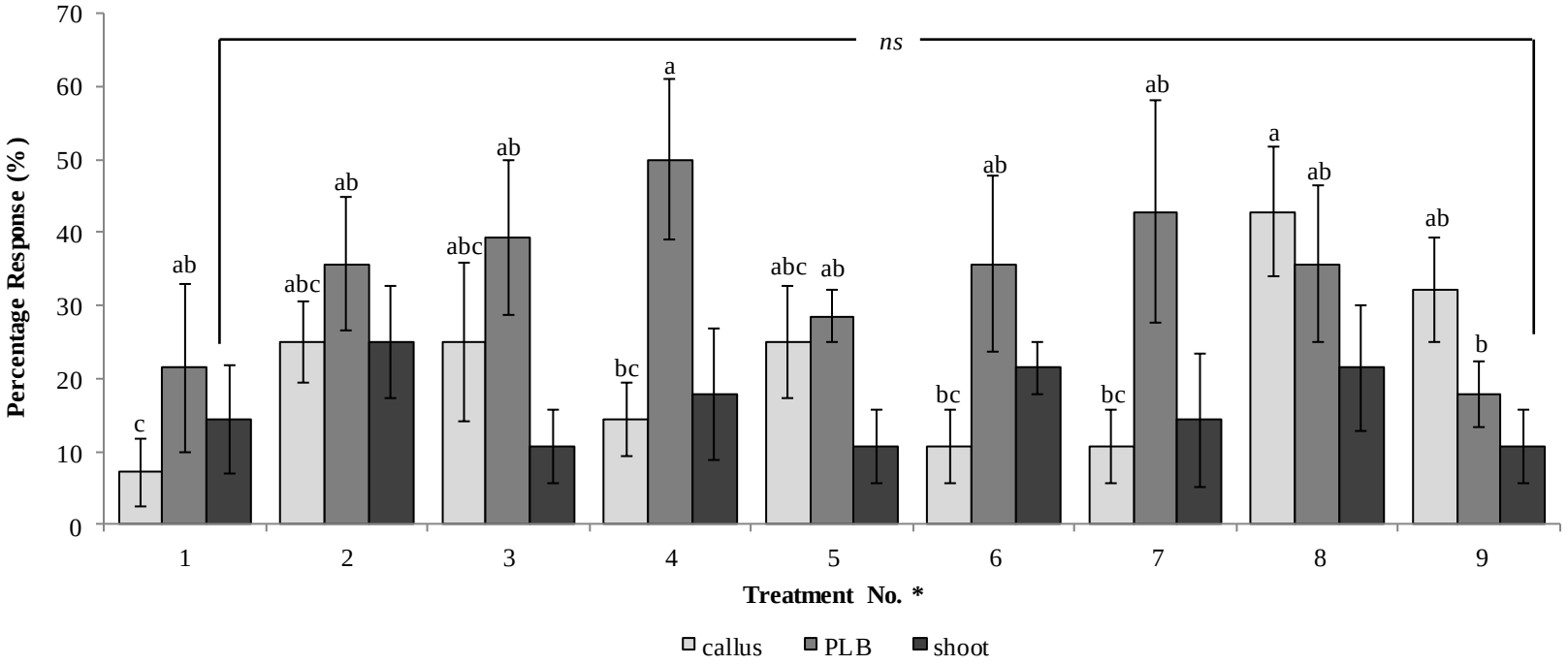


Figure 21 (see facing page for legend)

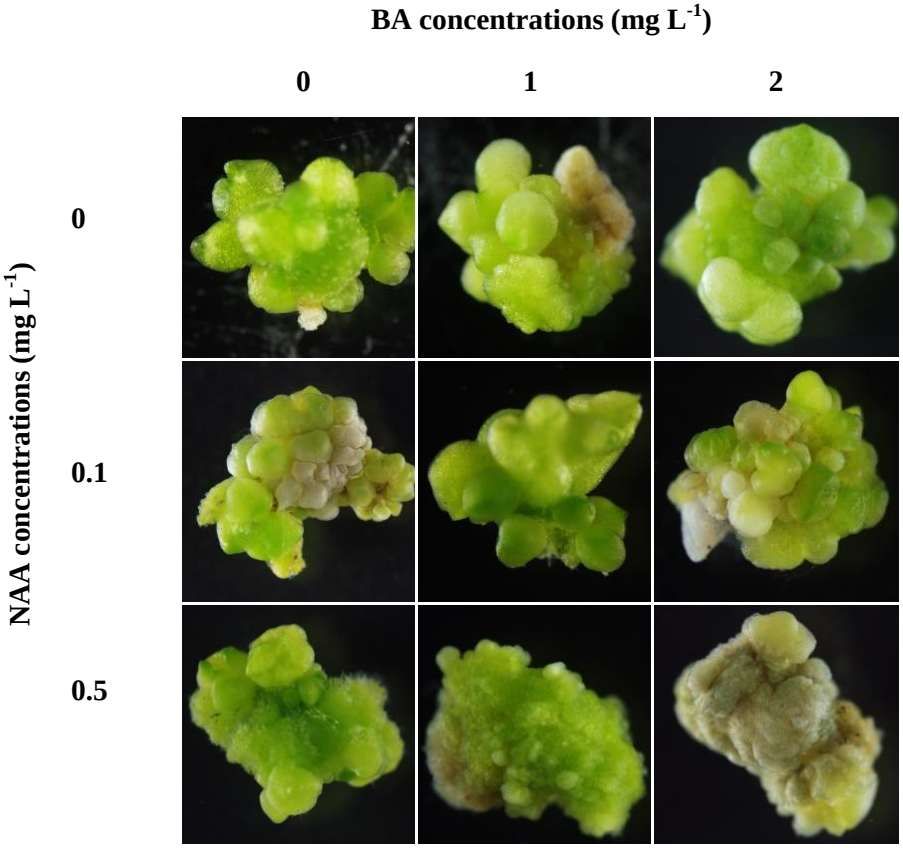


Figure 22 Effect of different ratios of NAA to BA on the developmental pattern of protocorm segment after culture for 8 weeks on a modified VW medium.

3.1.3 Histological observation of callogenesis

Histological examination of callogenesis in bisected protocorm segment was carried out to determine which cells of the explants undergo dedifferentiation giving rise to callus. The origin of callus and its ontogeny were investigated during bisected protocorm culture on a modified VW medium added with 0.5 mg L^{-1} NAA incorporate with 1 mg L^{-1} BA. After culture for a week, the explants began to swell (Figure 23A) and showed the onset of cell division near the wound site which was possible to observe from a single subepidermal cell having a dense cytoplasm and large nucleus (Figure 24A, 24B). Then, the single originated callus cells continued to divide in anticlinal orientation for producing a greater amount of cells leading to increase the size of callus mass (Figure 23B, 24C, 24D). Four weeks after culture, the callus mass became more obvious (Figure 23C, 24E) and the number of cells increased by periclinal and anticlinal divisions (Figure 24F). The obtained

callus cells were clearly distinguished from the original cell of explants. The callus mass continued to grow and eventually formed compact green callus within 8 weeks of culture (Figure 23D). This compact callus also showed nodular structure comprising small and isodiametric parenchyma cells with a prominent nucleus and a conspicuous nucleolus within densely staining cytoplasm (Figure 24G, 24H).

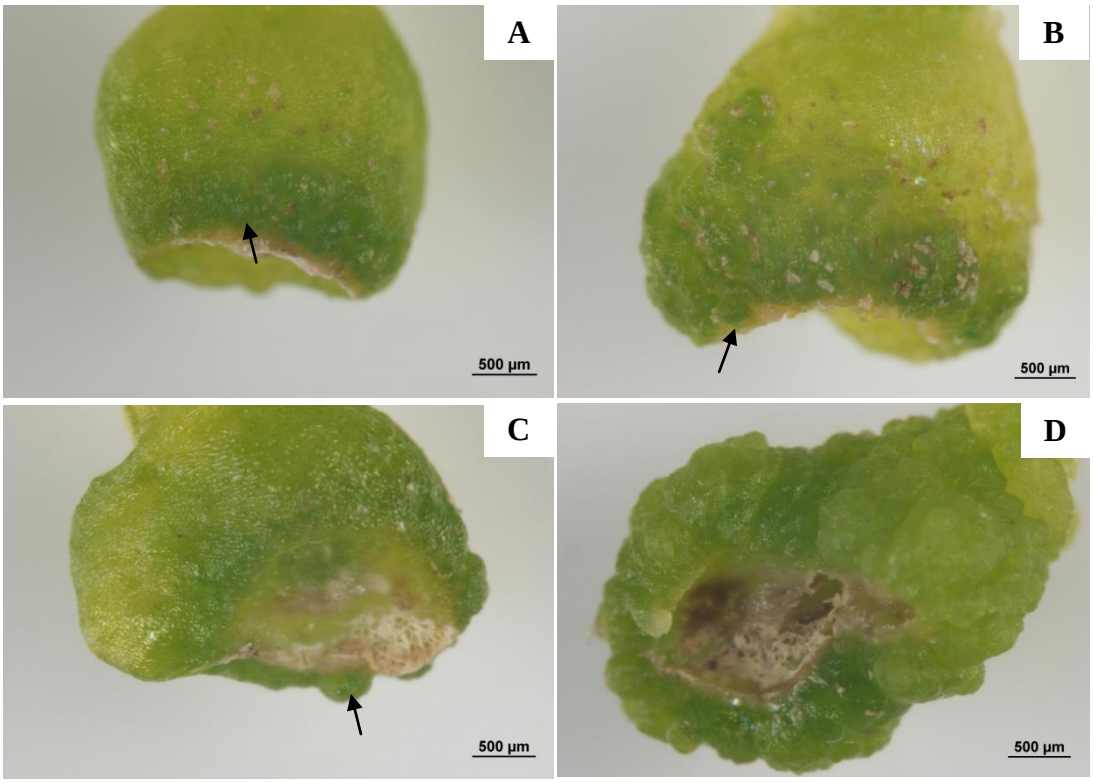


Figure 23 Callogenesis of *D. crumenatum* on a modified VW medium supplemented with 0.5 mg L^{-1} NAA plus 1 mg L^{-1} BA.
Three-month-old protocorms were bisected transversely showing **(A)** swollen protocorm and early callus formation (*arrow*), **(B)** small clusters of callus (*arrow*), **(C)** callus mass (*arrow*) and **(D)** large amount of compact greenish callus mass after culture for 1, 2, 4 and 8 weeks, respectively.

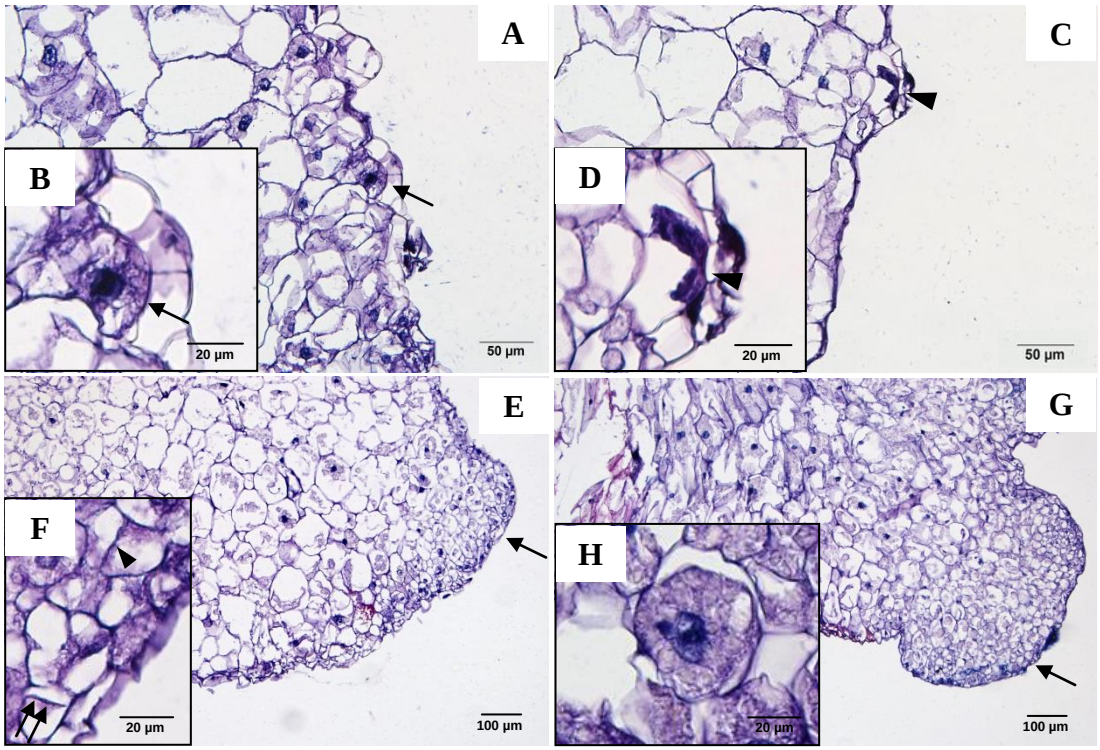


Figure 24 Histological analysis of callogenesis of *D. crumenatum*.
(A-B) Callus was originated from the subepidermal cell (*arrows*) of protocorm segment after 1 week of culture; **(C-D)** Subepidermal cell divided in an anticlinal plane (*arrow head*s) after 2 weeks of culture; **(E-F)** Obvious callus mass (*arrow*) containing meristematic cells undergo periclinal (*arrow head*) and anticlinal divisions (*double arrows*) after 4 weeks of culture; **(G-H)** Nodular compact callus (*arrow*) comprising small isodiametric cells with a conspicuous nucleus and nucleolus within densely stained cytoplasm after 8 weeks of culture.

Moreover, histological data also showed the two major morphogenic responses including PLB (Figure 25A) and shoot (Figure 25B). Firstly, PLB formation (Figure 25C) occurred directly from transversely bisected protocorm segment. These PLBs showed the elongated shape with densely packed cells and a depression at the apical region (Figure 25C, arrow head). In the other morphogenic response, shoot (Figure 25D) was directly induced from the explants. These shoots had a shoot apical meristem, leaf primordia and vascular strand (Figure 25D, 25E).

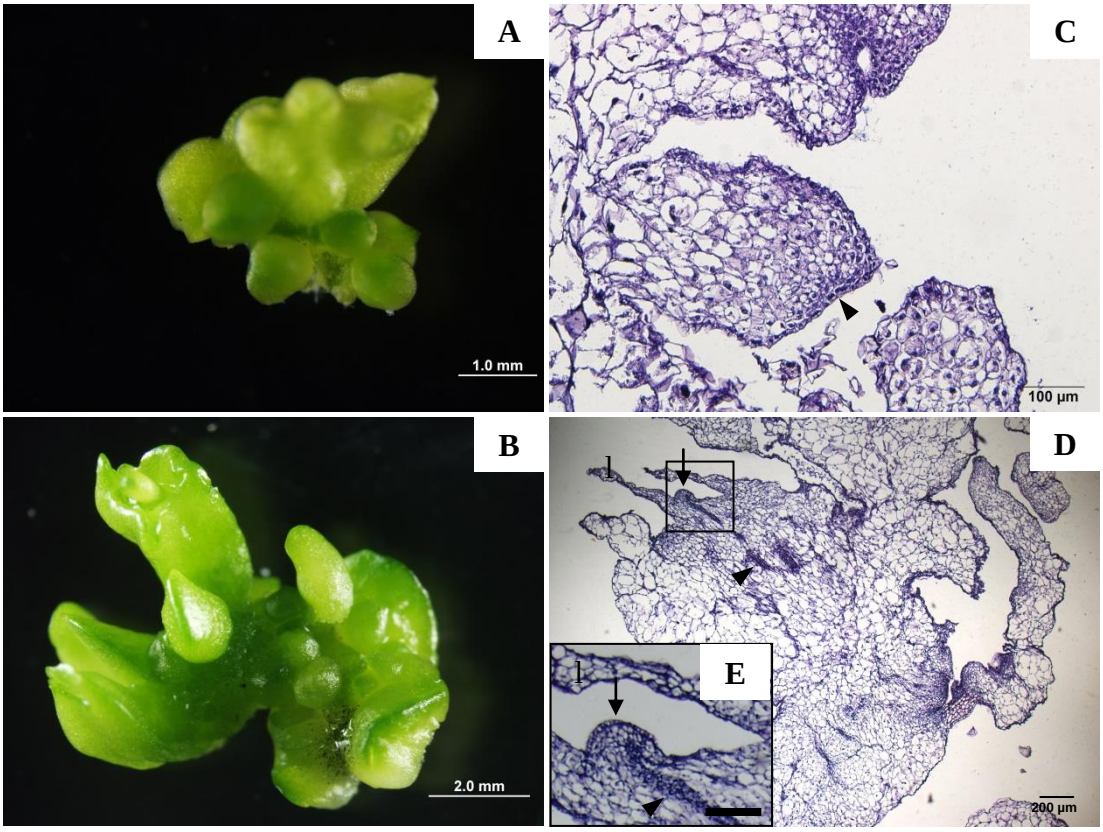


Figure 25 In vitro morphogenesis of *D. crumenatum* after culture bisected protocorm for 8 weeks on a modified VW medium containing NAA and BA.

The explants were cultured on medium supplemented with 0.1 mg L^{-1} NAA alone and 1 mg L^{-1} BA alone showing **(A)** PLB and **(B)** shoot formation. Longitudinal section of explants exhibiting **(C)** PLB with a small depression at shoot apex (*arrow head*); **(D)** shoot with shoot apical meristem (*arrow*), leaf primordium (l) and vascular strand (*arrow head*); **(E)** magnified view of boxed region of (D) (*bar* = 100 µm).

3.2 Influence of an extended culture period on the alteration of callus

3.2.1 Effect of incubation period on browning problem

After the extended period of callus culture on the same medium, the incidence of callus browning markedly increased (Figure 26). There was a significant difference in the percentage of browning callus among the different culture period. The present results showed that the lowest frequency of browning callus (9.49 ± 1.18 %) was obtained at the initial incubation. However, the rate of browning callus gradually increased and then exhibited the highest rate of browning callus (43.85 ± 3.71 %) after 3 months of incubation on a modified VW medium without any subculture.

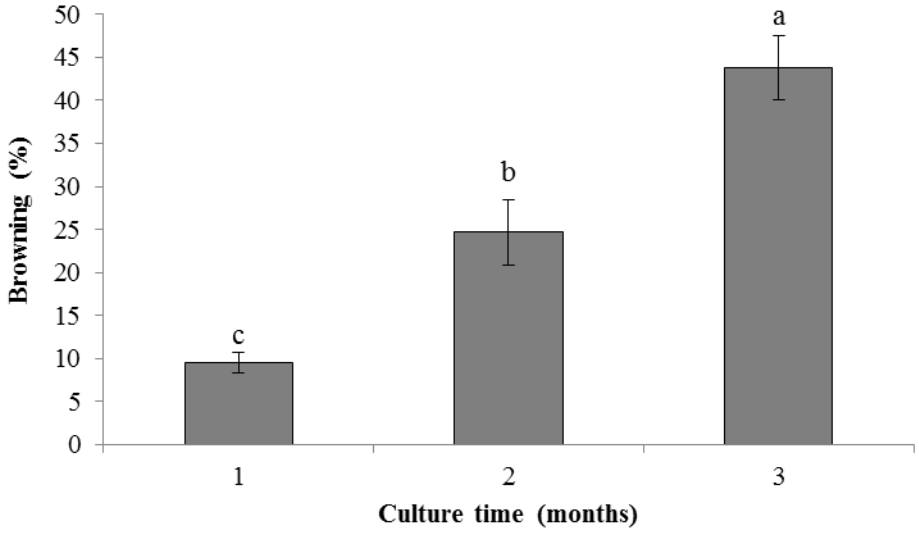


Figure 26 Browning percentage of *D. crumenatum* callus cultured on modified VW medium at 1, 2 and 3 months of culture incubation.
Different letters above S.E. bars indicate significant differences at $P \leq 0.05$ according to DMRT.

3.2.2 Morphological, histological and histochemical features during callus culture

After callus culture, the color appearance of callus in the difference age was observed (Table 3). Strong green callus (Figure 27A) derived from bisected protocorm were obtained on a modified VW medium supplemented with 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA. During callus culture, the discoloration of the callus was observed and first appeared as a small brown spot on the surface of callus mass after culture for 3-4 weeks (Figure 27B). Then, this callus gradually became pale green after 2 months of culture (Figure 27C) and finally turned into a darker brown color within 3 months (Figure 27D).

Table 3 The color appearance of *D. crumenatum* callus cultured on a modified VW medium supplemented with 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA without any subcultures.

Culture incubation (months)	Callus color (exceeding 80 %)
1	Green
2	Pale green
3	Brown

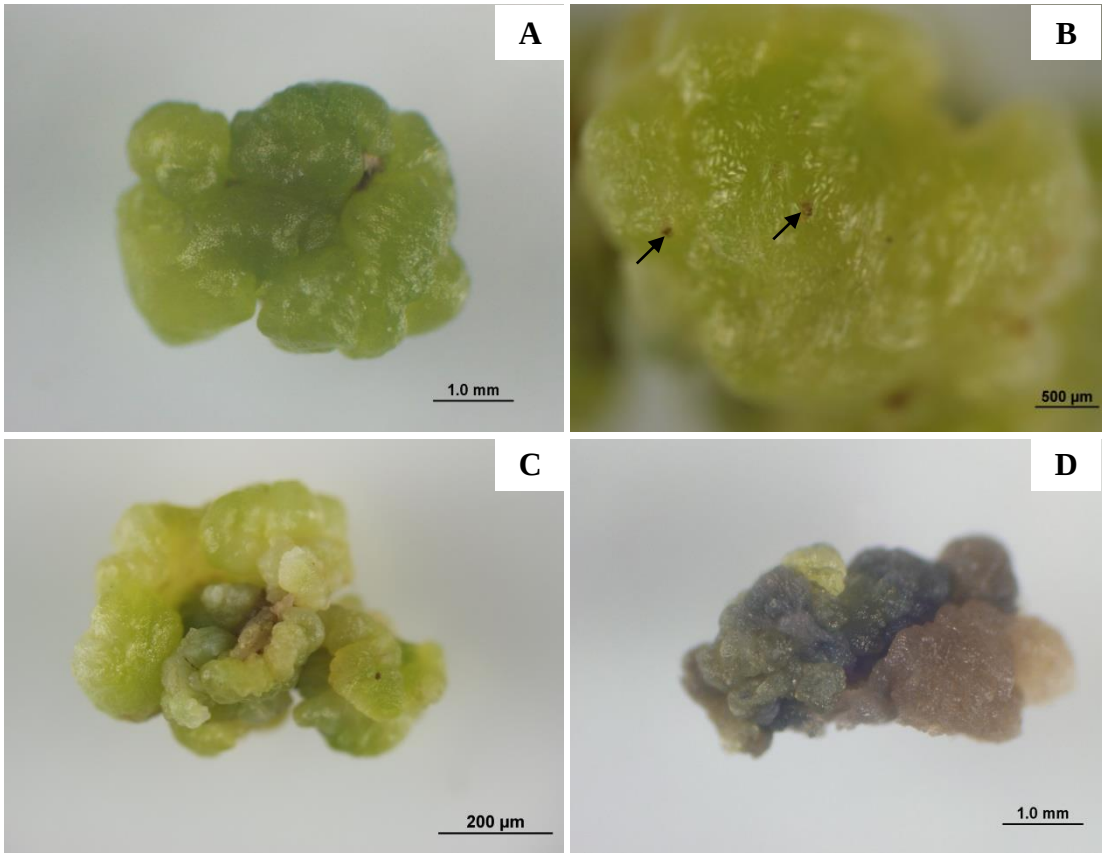


Figure 27 Morphological features of *D. crumenatum* callus cultured on modified VW medium.
(A) One-month-old callus with green colour; **(B)** callus with brown spots (*arrows*) on its surface after 3-4 weeks of culture; **(C)** two-month-old pale green callus; **(D)** three-month-old dark brown callus.

Alteration at the cellular level during an extended callus culture without subculture was detected by microscopic observation. The results demonstrated that the one-month-old callus (Figure 28A, 28B) and two-month-old callus (Figure 28C, 28D) had no obvious differences in their cellular features. These cells were comprised of uniform and compactly arranged cells which were seen to contain dense-staining cytoplasm and nucleus. Three months later, the cellular structure of the vigorous callus clearly showed desirable changes which included a loose and disordered cell arrangement and a lack of both cytoplasm and nucleus (Figure 28E, 28F).

In addition, the accumulation of ergastic substances, for instance phenolic compound and insoluble carbohydrate, during a prolonged culture of callus on the same medium was investigated by histochemical observations. The results showed that a one- (Figure 29A) and two-month-old (Figure 29B) callus exhibited a blue to dark blue color after TBO staining which indicated the absence of phenolic compound. On the other hand, a three-month-old callus gave a greenish blue color showing the abundance of phenolic compound within this cell (Figure 29C). Moreover, the one- (Figure 29D) and two-month-old (Figure 29E) callus showed a deep magenta color after PAS staining which indicated a greater accumulation of insoluble carbohydrate than in a three-month-old callus (Figure 29F).

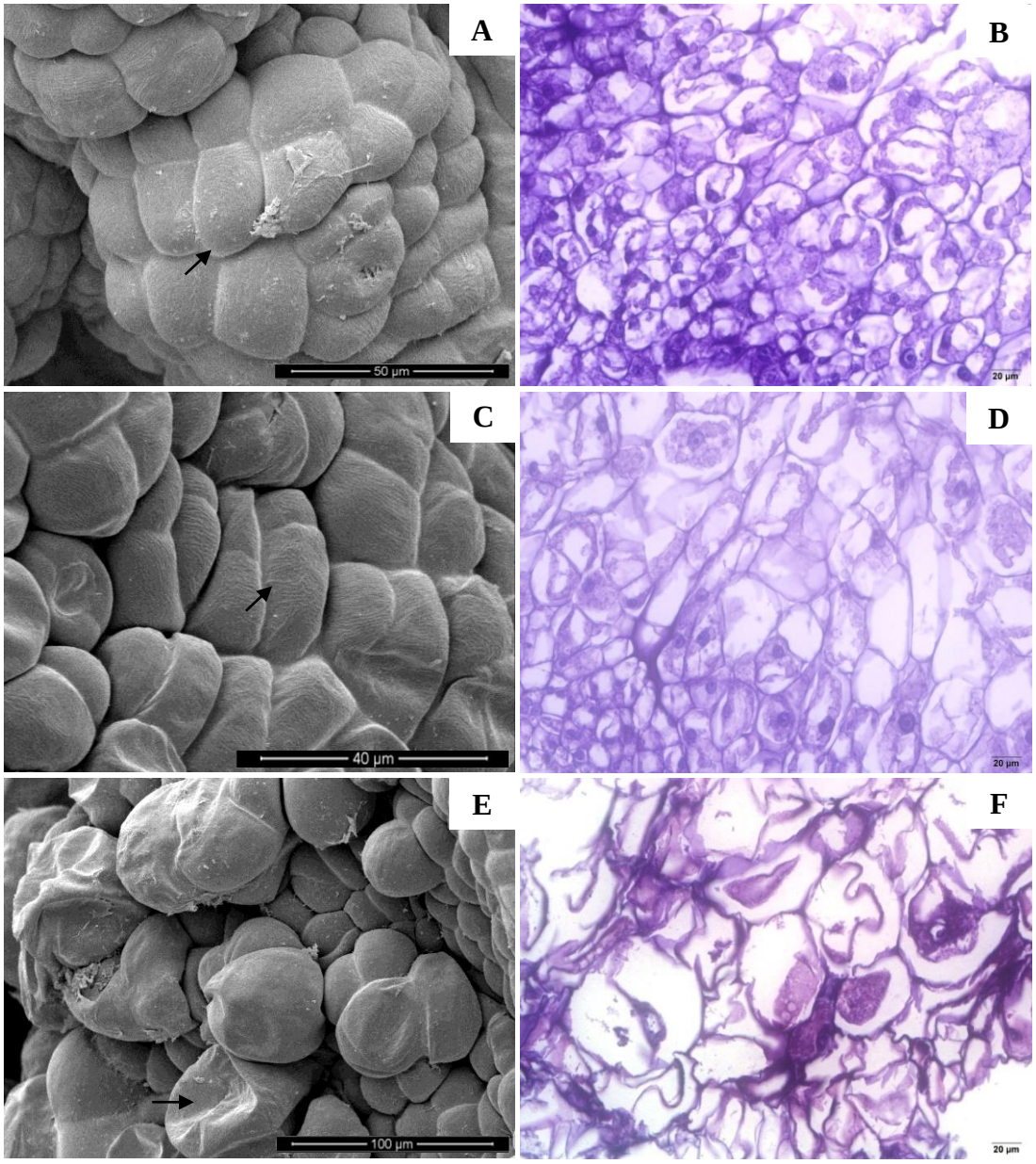


Figure 28 Cellular structure of bisected protocorm-derived callus of *D. crumenatum*. **(A-B)** One- and **(C-D)** two-month-old callus exhibiting uniform and tightly arranged parenchyma cells (*arrows*) compared to **(E-F)** three-month-old browning callus showing variable and disordered cells with partially collapsed cells (*arrow*).

Figure 29 Histochemical observations in different ages of callus.

(A, D) one-month-old callus, **(B, E)** two-month-old callus and **(C, F)** three-month-old callus obtained from bisected *D. crumenatum* protocorms. **(A-C)** after TBO staining, **(A)** one- and **(B)** two-month-old callus show a blue colour indicating the absence/or a low amount of tannin-like material, whereas **(C)** three-month-old browning callus exhibits a green colour showing the accumulation of tannin (*arrow*). **(D-F)** PAS reaction detects the presence of insoluble polysaccharides with a magenta colour (*arrow*) in the **(D)** one- and **(E)** two-month-old callus compared to **(F)** negative PAS staining observed in the browning callus.

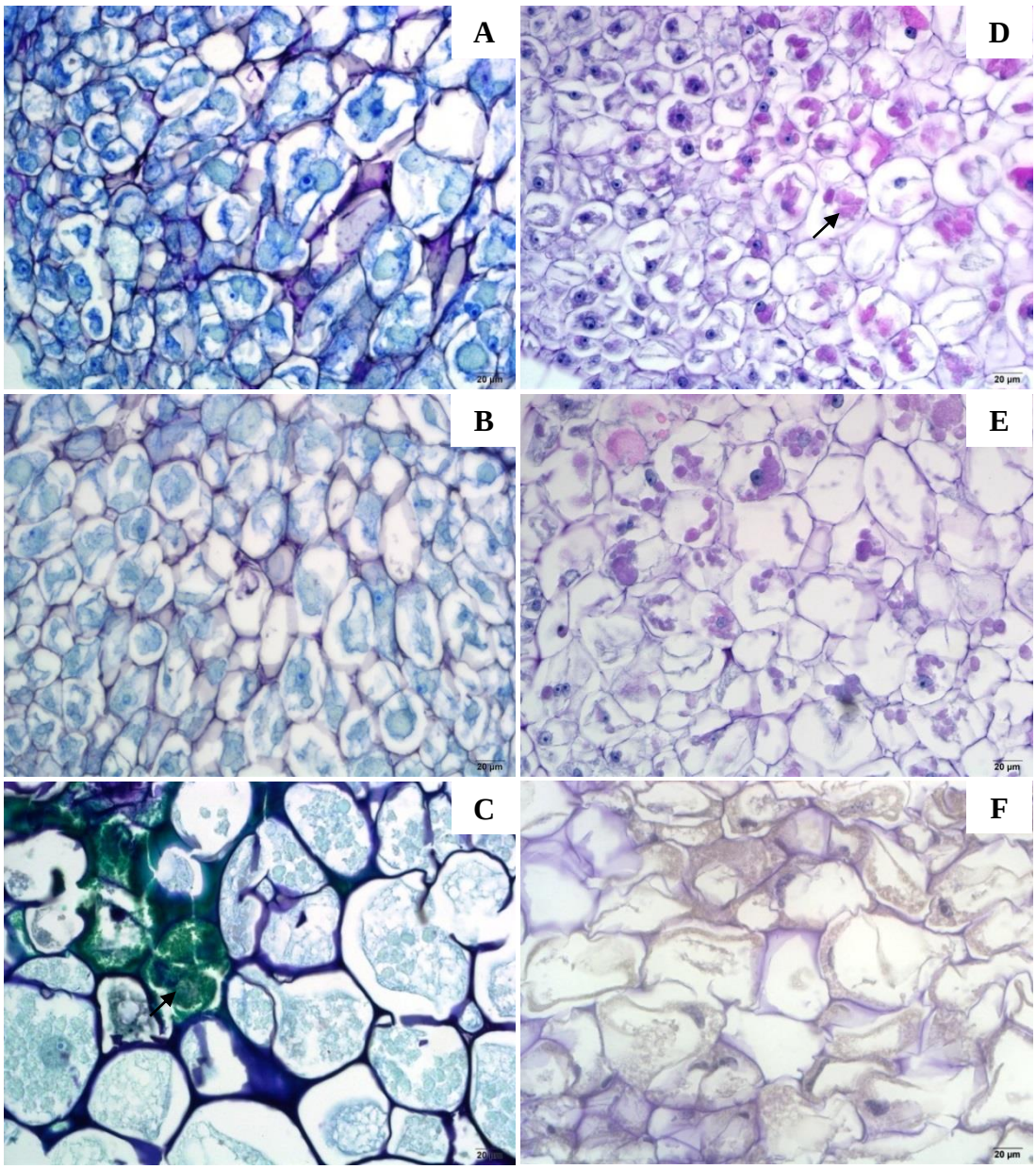


Figure 29 (see facing page for legend)

3.2.3 Ultrastructural alterations during callus culture

The change of subcellular features during an extended culture of callus was investigated and described as follows, based on the age of callus:

One-month-old callus

The ultrastructure of one-month-old callus showed distinct appearance of a thin cell wall, tonoplast and plasmodesmata (Figure 30A). The well-organized organelles, including nucleus, mitochondria and chloroplasts, were very apparent in the cytoplasm (Figure 30B). Globular chromoplasts (Figure 30C) and other plastids, including chloro-chromoplasts (Figure 30D), were noticed. These plastids contained small plastoglobuli (Figure 30C, 30D, arrows). This study also exhibited two types of starch-containing plastids namely chloroplasts (Figure 30E) and amyloplast (Figure 30F). Amyloplast contained a starch grain and still retained some thylakoid stacks. Furthermore, a dense cytoplasm that was rich in free ribosomes, cisternae of granular or rough ER (RER) and dictyosomes was also present (Figure 30G).

The presence of crucial organelles, particularly nucleus, mitochondria and chloroplast are subsequently described in the following context (3.2.4).

Two-month-old callus

Ultrastructural observation of two-month-old callus revealed that these callus cells contained unusual structures including empty simple and concentric whorl double-membranous vesicles (Figure 31A, 31B). Besides, the secretion of cytoplasmic material deposited at the cell walls was obviously present (Figure 31C). Moreover, clear plasmalemma, obvious organellar membranes and distinct vesicles containing less osmiophilic materials were also observed (Figure 31C, 31D). The alteration and initial degradation of crucial organelles including nucleus, mitochondria and chloroplast – relative to the one-month-old callus – were also noticed as described below in 3.2.4.

Figure 30 TEM micrographs of one-month-old callus cultured on modified VW medium.

(A) Panoramic view of one-month-old cell showing distinct tonoplast, thin cell wall, regular nucleus and numerous mitochondria (*bar* = 2 µm). **(B-G)** Magnified views of **(B)** plasmodesmata (*arrows*) (*bar* = 1.25 µm), **(C)** chromoplast with plastoglobuli (*arrow heads*) (*bar* = 1 µm), **(D)** plastoglobuli (*arrow heads*) in chloro-chromoplast (*bar* = 500 nm), **(E)** chloroplasts containing starch grain (*bar* = 5 µm), **(F)** amyloplast (*bar* = 500 nm) and **(G)** dictyosome, free ribosomes and RER within the cytoplasm (*bar* = 500 nm).

(chloroplasts: chl; chromoplast: chr; cell wall: cw; dictyosome: d; mitochondria: m; nucleus: n; ribosomes: r; rough endoplasmic reticulum: RER; starch grain: s; tonoplast: t; thylakoid membrane: th)

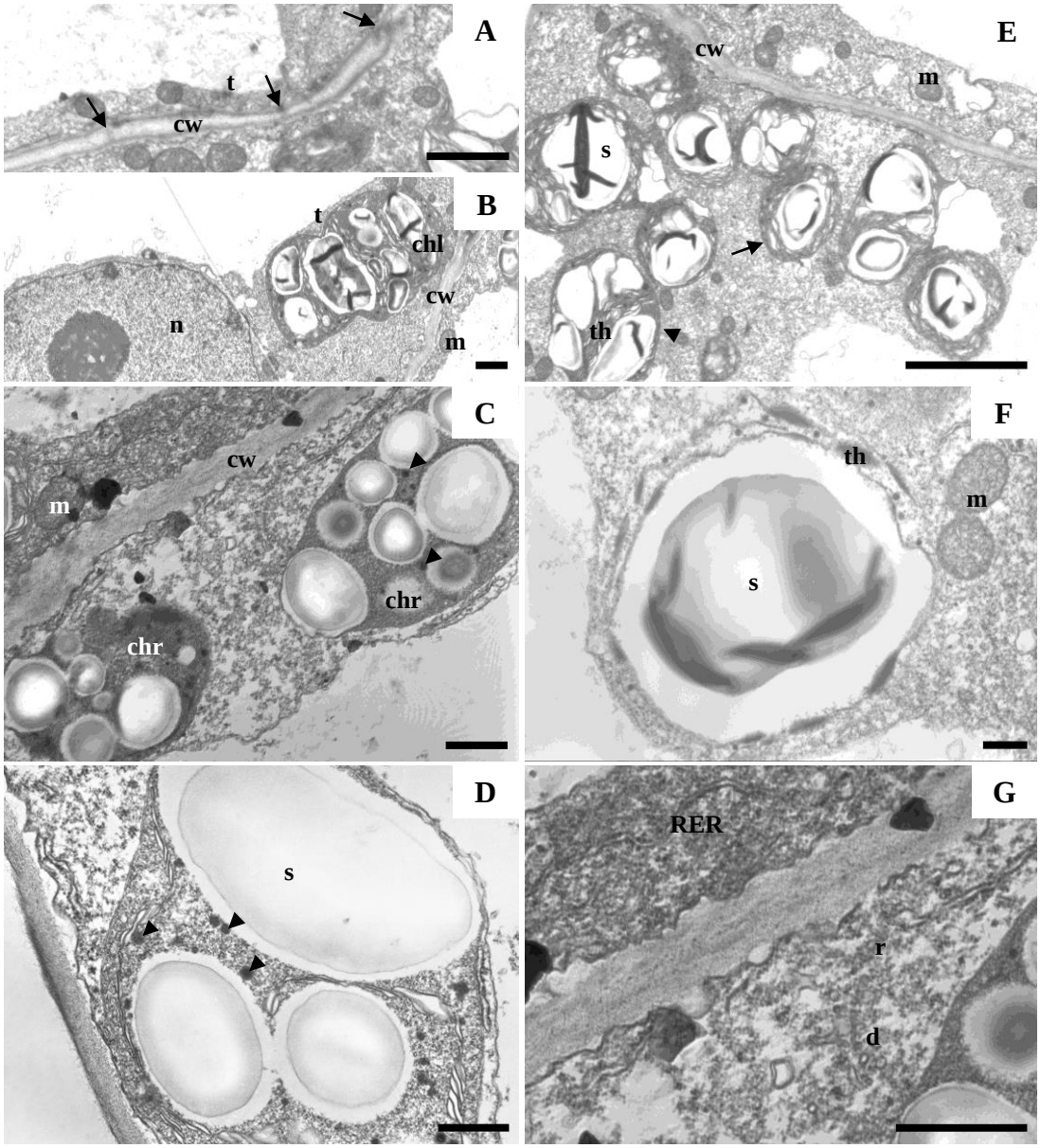


Figure 30 (see facing page for legend)

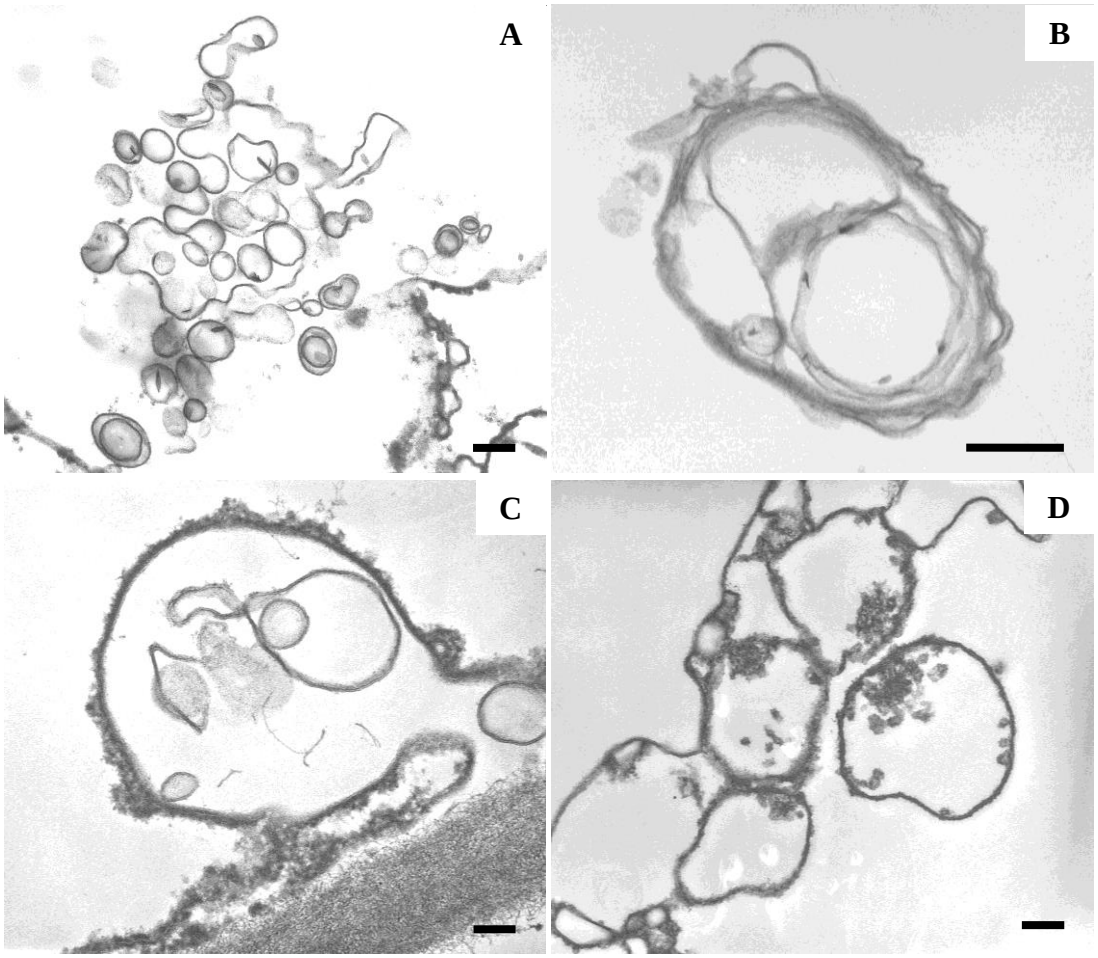


Figure 31 TEM micrographs of browning callus after a two-month culture period on modified VW medium.

(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 cell wall (*bar* = 100 nm); **(D)** distinct vesicles containing less osmiophilic materials

Three-month-old callus

In three-month-old callus, various organelles displayed an obvious alteration including a reduction in cytoplasm, a decreased number of plastids, a detachment of the plasmalemma from the cell wall as seen by the shrunken membrane and a distortion in thickened cell walls (Figure 32A). Moreover, this callus showed a greater abundance of microvesicles as compared with one-month-old callus, in which such vesicles were absent (Figure 32B). These vesicles contained a dark pigmentation of osmiophilic materials which was attached to the inner surface of the tonoplast (Figure 32C). Besides, dark osmiophilic materials were also found to be deposited in the cytoplasm (Figure 32C, arrow). In addition, a dense fibrillar structure was existed between cell wall of neighboring cells in three-month-old callus (Figure 32D). Numerous smooth ER (SER) (Figure 32E, 32F), many peroxisomes with great crystal-like structure and lipid bodies (Figure 32G) were also present, but dictyosomes were not clearly observed. Moreover, there was a complete disorganization of important organelles, mainly nucleus, mitochondria and chloroplasts, which was detailed in below context (3.2.4).

Figure 32 TEM micrographs of browning callus after a three-month culture period on modified VW medium.

(A) Brown cell showing disorganized organelles and twisted cell wall with shrunken membrane (*arrow*) (*bar* = 5 µm). Magnified images of **(B)**, an area of the cytoplasm showing numerous small vesicles (*arrows*) and osmiophilic material (*arrow head*) (*bar* = 1 µm). **(C)** Magnified view of osmiophilic deposition (*arrow*) that appears to be discharged into the vacuole 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 µm), **(E, F)** numerous SER (*bar* = 500, 100 nm), **(G)** peroxisomes and lipid bodies (*bar* = 500 nm).

(cell wall: cw; lipid body: lb; nucleus: n; peroxisome: p; smooth endoplasmic reticulum: SER; tonoplast: t; vacuole: v)

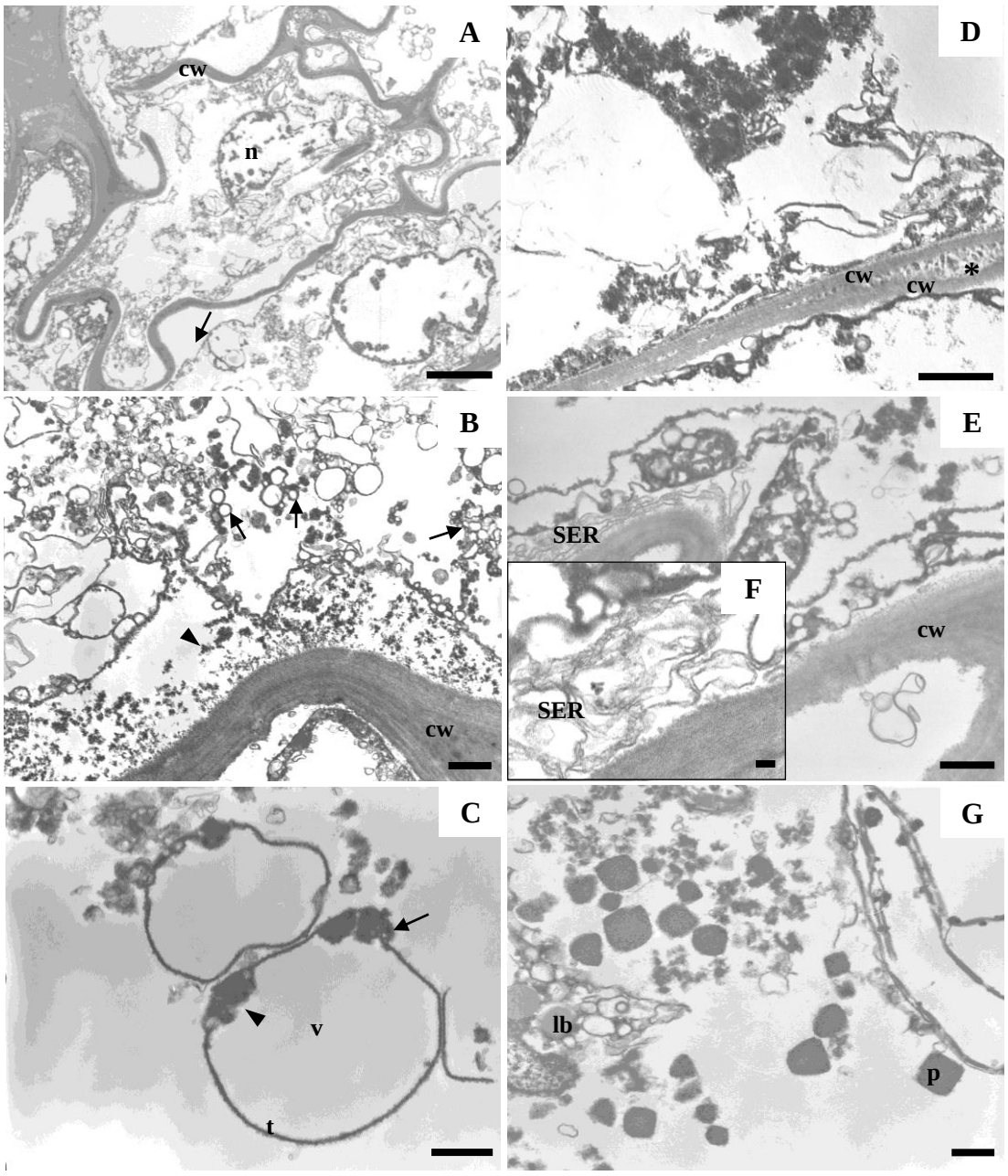


Figure 32 (see facing page for legend)

3.2.4 Ultrastructural alteration in crucial organelles (nucleus, mitochondria and chloroplasts)

Many organelles underwent progressive alterations during an extended callus culture. However, the crucial organelles, particularly the nucleus, mitochondria and chloroplasts, showed a distinct deformation.

Ultrastructural observation revealed that the one-month-old callus contained a regular nucleus which had a dense nucleolus, a distinct nuclear envelope and two types of chromatin, namely heterochromatin and euchromatin (Figure 33A). This callus cell also exhibited plentiful round mitochondria with well-defined cristae, clear matrix material and distinctive double-membrane envelope (Figure 33B). Moreover, chloroplasts loaded with either a large starch grain or several starch grains were clearly present (Figure 33C, 33D).

After 2 months of culture, cells in callus showed a slight alteration in the appearance of organelle. This callus had a nucleus with a moderately dense nucleolus, an obvious nuclear pore and two distinct types of chromatins, namely peripheral and nucleoplasmic heterochromatins, which were detected in greater amount than the euochromatin state (Figure 34A). Moreover, the nuclear envelope showed minimal change with invagination of double-layered nuclear envelope into the nucleus causing an unusual arrangement of nuclear pores (Figure 34B). Likewise, spherical mitochondria showing enlarged cristae and less matrix material were also detected (Figure 34C). Alterations in ultrastructure of chloroplasts were observed in two-month-old callus. These altered chloroplasts with swollen thylakoid membrane had no starch grains, but contained lipid droplets (Figure 34D).

Three-month-old callus showed full ultrastructural alteration in the main organelles. The results exhibited chromatin condensation at the periphery of the nucleus causing breakage in some areas of the nuclear envelope (Figure 35A). Moreover, swollen mitochondria with deformed inner and outer membranes were seen (Figure 35B). Likewise, abnormal chloroplasts with a swelling thylakoid membrane were evident in three-month-old callus (Figure 35C).

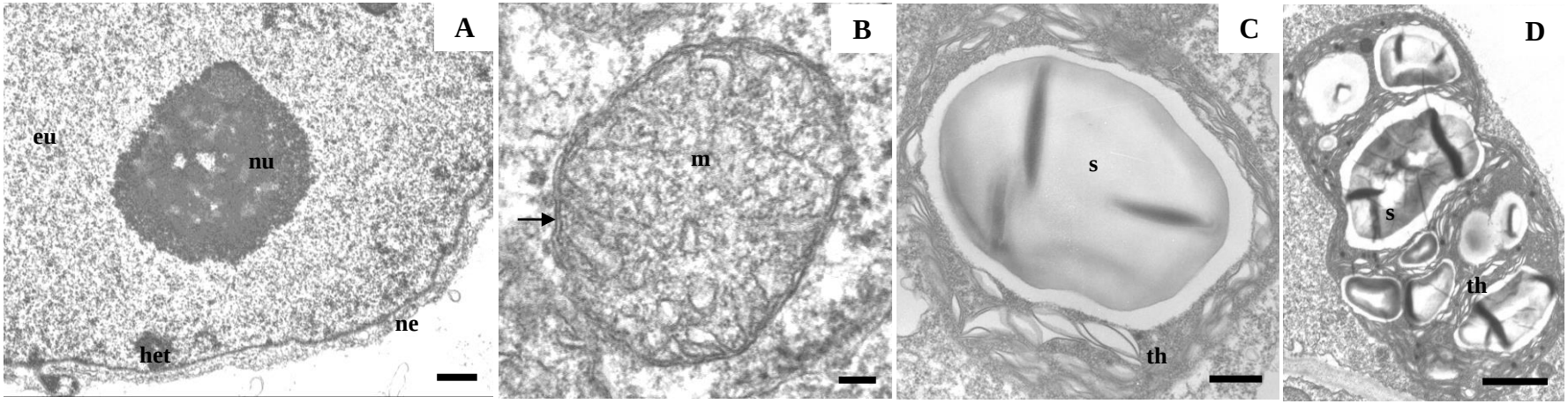


Figure 33 TEM micrographs of ultrastructural alteration of important organelles of one-month-old callus.

(A) a nucleus with a clear nuclear envelope, nucleolus, euchromatin and heterochromatin (*bar* = 1 µm); **(B)** well-organized mitochondrion with a mitochondrial double-membrane system (*arrow*) and numerous cristae (*bar* = 100 nm); **(C-D)** chloroplast with one or several starch grains and a well-developed thylakoid membrane system (*bar* = 500 nm, 2 µm).

(euchromatin: eu; heterochromatin: het; mitochondria: m; nuclear envelope: ne; nucleolus: nu; starch grain: s; thylakoid membrane: th)

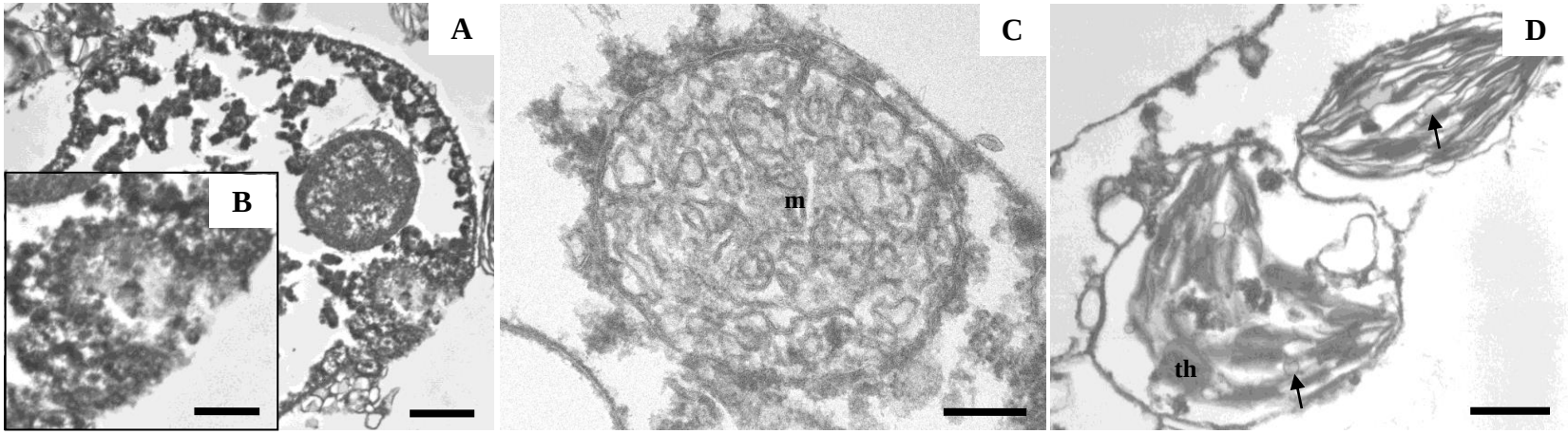


Figure 34 TEM micrographs of ultrastructural alteration of important organelles of two-month-old callus.

(A) peripheral and nucleoplasmic heterochromatin (*bar* = 1 µm); **(B)** magnified images of nuclear envelope exhibiting invagination of double-layered nuclear membrane (*bar* = 500 nm); **(C)** spherical mitochondria with swollen cristae and less dense matrix material (*bar* = 100 nm); **(D)** chloroplast containing lipid bodies (*arrows*) and no starch grains (*bar* = 500 nm). (mitochondria: m; thylakoid membrane: th)

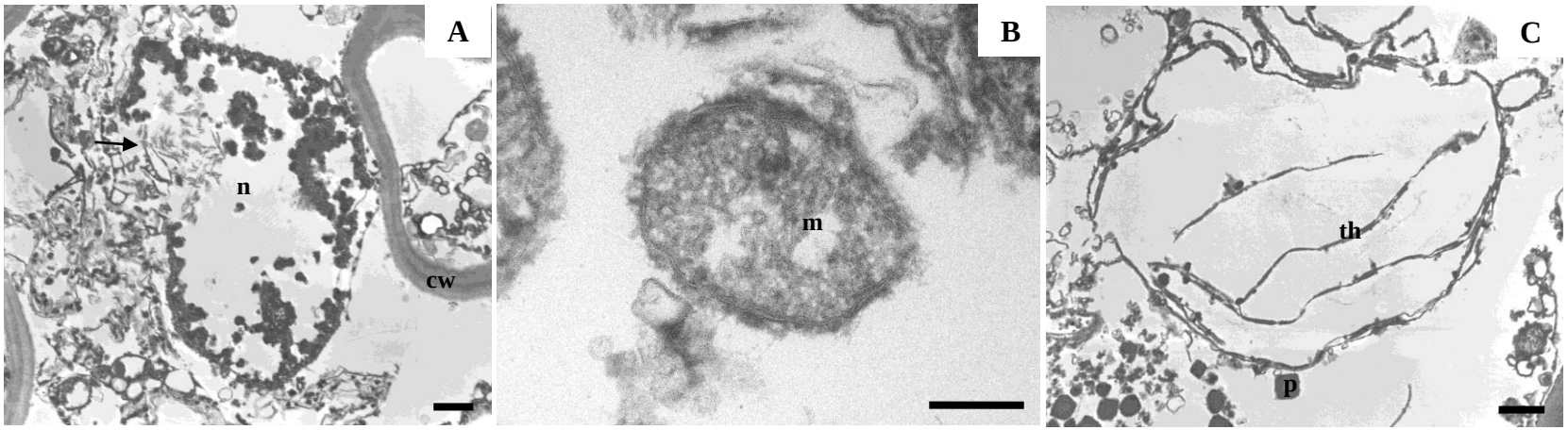


Figure 35 TEM micrographs of ultrastructural alteration of important organelles of three-month-old callus.

(A) disintegrated nucleus (*arrow*) (*bar* = 1 µm); **(B)** irregular mitochondrion with unclear cristae (*bar* = 200 nm); **(C)** chloroplast with strongly dilated thylakoid membranes (*bar* = 1 µm).

(cell wall: cw; mitochondria: m; nucleus: n; peroxisome: p; thylakoid membrane: th)

3.2.5 Changes in biochemical factors during callus culture

A. Enzymatic activity

The activities of enzymes were studied at 1, 2 and 3 month intervals of extended incubation without any subculture**.**

PPO activity

The results revealed low PPO activity (335.54 ± 7.88 U g^{-1} FW) after an initial incubation on a culture medium. However, changes in PPO activity after 2 months of incubation (337.29 ± 8.92 U g^{-1} FW) were not significantly different from those at one month of incubation. Meanwhile, this present result showed that PPO activity increased gradually and continuously during prolonged incubation period for 3 month. The maximum enzymatic activity of PPO (382.89 ± 17.36 U g^{-1} FW) was observed in three-month-old callus and was significantly higher than that of one- and two-month-old callus (Figure 36A).

PAL activity

The results demonstrated that PAL exhibited maximum activity at the initial incubation (78.81 ± 4.63 U g^{-1} FW) and statistically decreased after 2 months of culture (62.71 ± 2.74 U g^{-1} FW). However, the PAL activity of cultured callus became increase when the period of culture was extended to 3 months (70.26 ± 2.36 U g^{-1} FW) (Figure 36B).

B. Total phenolic content

The content of total phenolics was determined during the whole culture period. The results showed that the total phenolic content was significantly increased and reached to the highest level after 3 months of culture (0.500 ± 0.003 mg GAE g^{-1} FW), even though the content of total phenolic was significantly decreased after 2 months of culture (0.455 ± 0.001 mg GAE g^{-1} FW) in comparison with 1 month of culture (0.478 ± 0.004 mg GAE g^{-1} FW) (Figure 36C).

C. Lipid peroxidation in terms of MDA contents

After an extended period of culture, the level of lipid peroxidation was determined by quantifying the content of MDA. The result showed that the MDA content was significantly increased (116.42 ± 3.49 µmol g^{-1} FW) after 2 months of culture as compared with one month of culture (105.95 ± 1.33 µmol g^{-1} FW). Especially, after extended incubation for 3 months, the content of MDA was significantly increased (129.14 ± 0.53 µmol g^{-1} FW) to a level approximately 1.2-fold compared to the content of MDA at one month of incubation (Figure 36D).

D. Chlorophyll and carotenoid contents

The contents of chlorophyll and carotenoids were determined after extended culture on the same medium. The result revealed that the highest content of total chlorophyll (0.092 ± 0.002 mg g^{-1} FW) and chlorophyll *a* (0.062 ± 0.001 mg g^{-1} FW) were obtained after culture for one month and showed a significant higher in their contents as compared with 2 and 3 months of culture. Similarly, the highest content of carotenoids (0.033 ± 0.001 mg g^{-1} FW) was also obtained after one month of culture but this was not significantly different from a two-month culture period. Meanwhile, chlorophyll *b* content showed the highest level (0.045 ± 0.001 mg g^{-1} FW) after culture for 2 months. However, the contents of total chlorophyll, chlorophyll *a*, chlorophyll *b* and carotenoids decreased significantly to their lowest levels (0.070 ± 0.001, 0.050 ± 0.001, 0.031 ± 0.001, 0.029 ± 0.000 mg g^{-1} FW, respectively) when callus was maintained on callus induction medium for 3 months without any subculture (Figure 37).

Figure 36 Measurement of biochemical data in *D. crumenatum* callus after 1, 2 and 3 months of culture incubation on modified VW medium.

(A) PPO activity, **(B)** PAL activity, **(C)** Total phenolic content and **(D)** MDA content. Different letters above S.E. bars indicate significant differences at $P \leq 0.05$ according to DMRT.

* One unit of enzyme activity is defined as the absorbance change of 0.01 min^{-1}.

** One unit of enzyme activity is defined as the absorbance change of 0.01 h^{-1}.

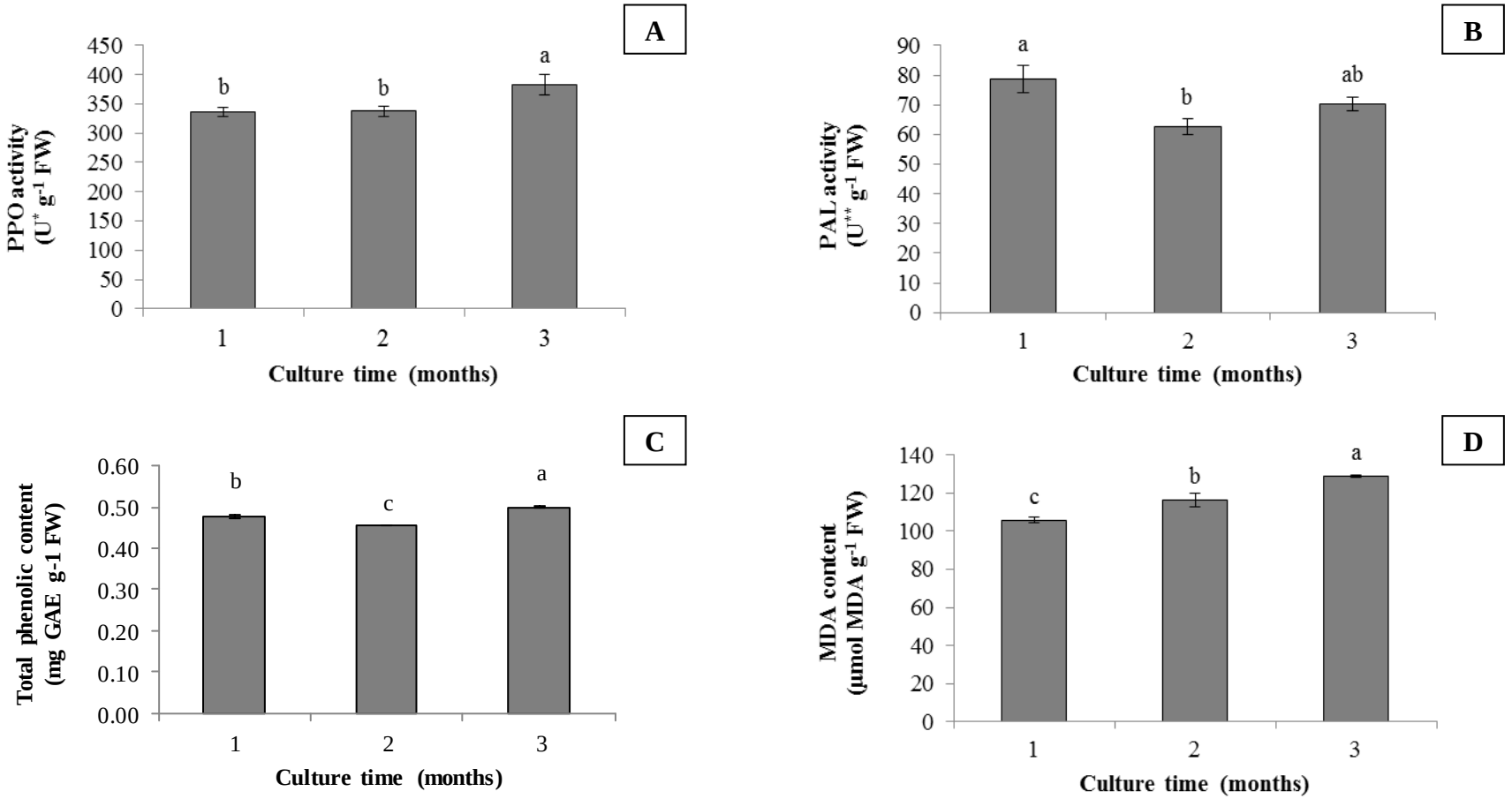


Figure 36 (see facing page for legend)

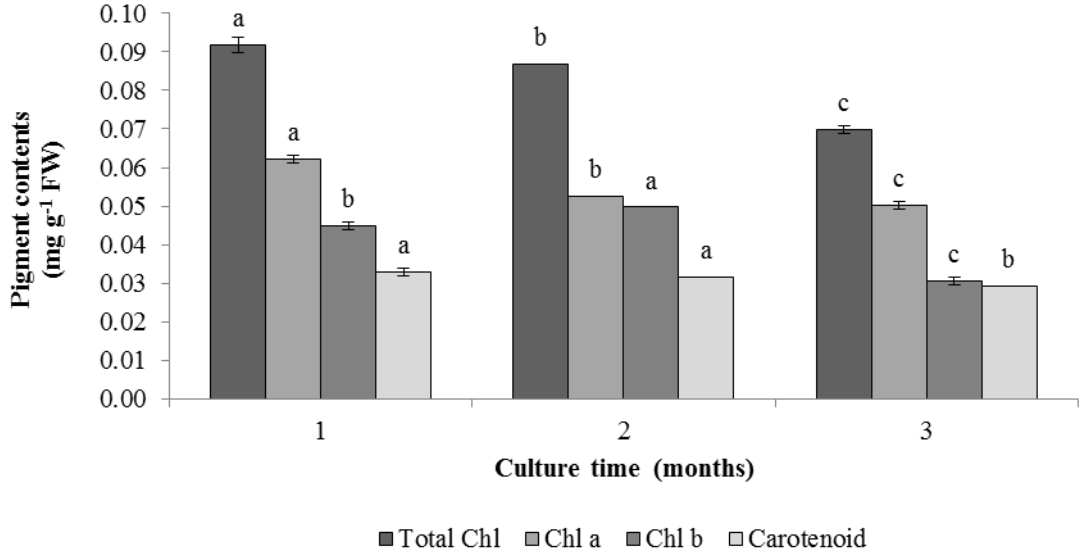


Figure 37 Estimation of pigment contents in *D. crumenatum* callus after 1, 2 and 3 months of culture incubation on modified VW medium.

Different letters above S.E. bars within substance indicate significant differences at $P \leq 0.05$ according to DMRT.

3.3 Influence of anti-browning agents on preventing the browning

3.3.1 Effect of anti-browning agents on callus growth

After extended period of callus culture for 3 months, the effects of two groups of anti-browning agents namely antioxidants (AA and Cys) and adsorbents (PVP and AC) on increased fresh weight of proliferated callus were investigated.

The results showed that AA (0.001 and 0.01 g L^{-1}), Cys (0.01 and 0.05 g L^{-1}) and PVP (0.5 and 5 g L^{-1}) gave a greater increased fresh weight of callus, but each treatment was not significant difference from the control. Especially, AA at 0.01 g L^{-1} gave the highest increased callus fresh weight (3.78 ± 0.41 g g^{-1} FW). It showed a significant difference in comparison with the medium supplemented with 1 g L^{-1} AC which provided the lowest increased fresh weight of callus (0.86 ± 0.29 g g^{-1} FW) (Figure 38).

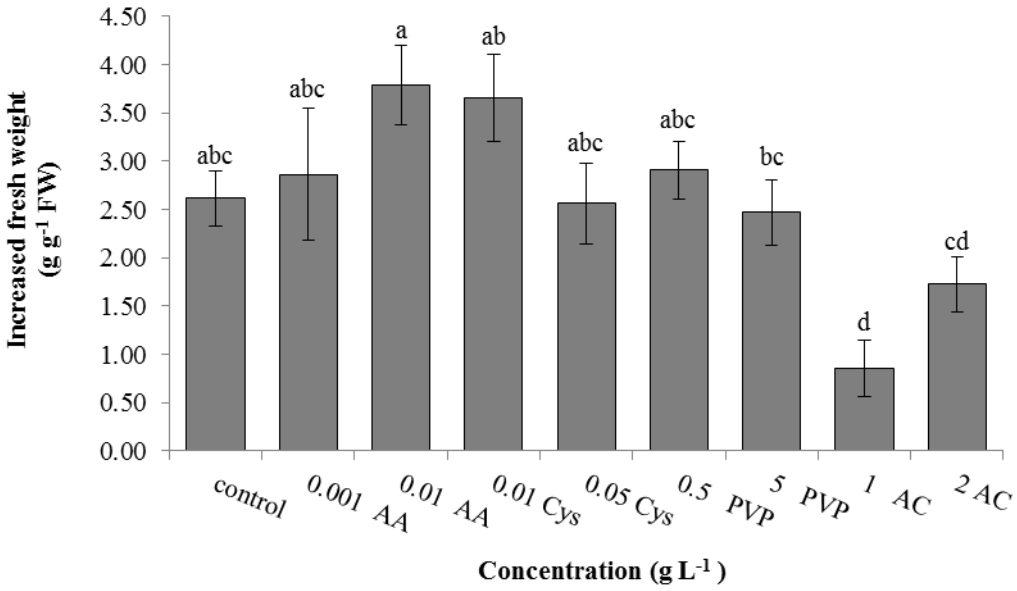


Figure 38 Effect of anti-browning agents on callus fresh weight after extended period of culture for 3 months.

Different letters above S.E. bars indicate the significant differences at $P \leq 0.05$ according to DMRT.

AA = ascorbic acid; Cys = L-cysteine;

PVP = polyvinylpyrrolidone; AC = activated charcoal

3.3.2 Effect of anti-browning agents on callus browning

The browning occurrence in callus was investigated after extended callus culture for 3 months on modified VW medium supplemented with various anti-browning agents. The results showed that AA (0.001 and 0.01 g L^{-1}), Cys (0.01 and 0.05 g L^{-1}) and PVP (0.5 and 5 g L^{-1}) could significantly reduce the frequency of browning callus when compared to control. The lowest frequency of browning callus was obtained on a medium containing 0.01 g L^{-1} AA (8.36 ± 4.35 %) followed by 0.05 g L^{-1} Cys (10.94 ± 2.60 %) and 0.5 g L^{-1} PVP (10.96 ± 4.81 %), respectively (Table 4). Even though the culture period was extended and without any subculture to the fresh medium, the treated callus with AA was still fresh and green (Figure 39C).

In contrast, a high frequency of browning callus could be observed on medium containing AC. The AC-treated media at 1 and 2 g L^{-1} exhibited the browning percentages of 58.54 ± 13.87 % and 46.44 ± 16.19 %, respectively, which were similar to the control group (67.93 ± 11.17 %). The proliferated callus on 1 g L^{-1} AC treatment exhibited discoloration and stunted growth (Figure 39H).

Table 4 Changes in percentage of browning callus after an extended callus culture for 3 months on modified VW medium supplemented with various anti-browning agents.

Anti-browning agents	Concentrations ($g\ L^{-1}$)	Browning callus (% ± S.E.)
Control	0	67.93 ± 11.17 [a]
Ascorbic acid	0.001	24.73 ± 12.53 [cd]
	0.01	8.36 ± 4.35 [d]
L-cysteine	0.01	16.36 ± 9.47 [cd]
	0.05	10.94 ± 2.60 [d]
Polyvinylpyrrolidone	0.5	10.96 ± 4.81 [d]
	5	30.03 ± 13.66 [bcd]
Activated charcoal	1	58.54 ± 13.87 [ab]
	2	46.44 ± 16.19 [abc]

The values represent the mean ± S.E. of six replicates of each treatment. Mean values within a column with different superscripts are significantly different at $P \leq 0.05$ by DMRT.

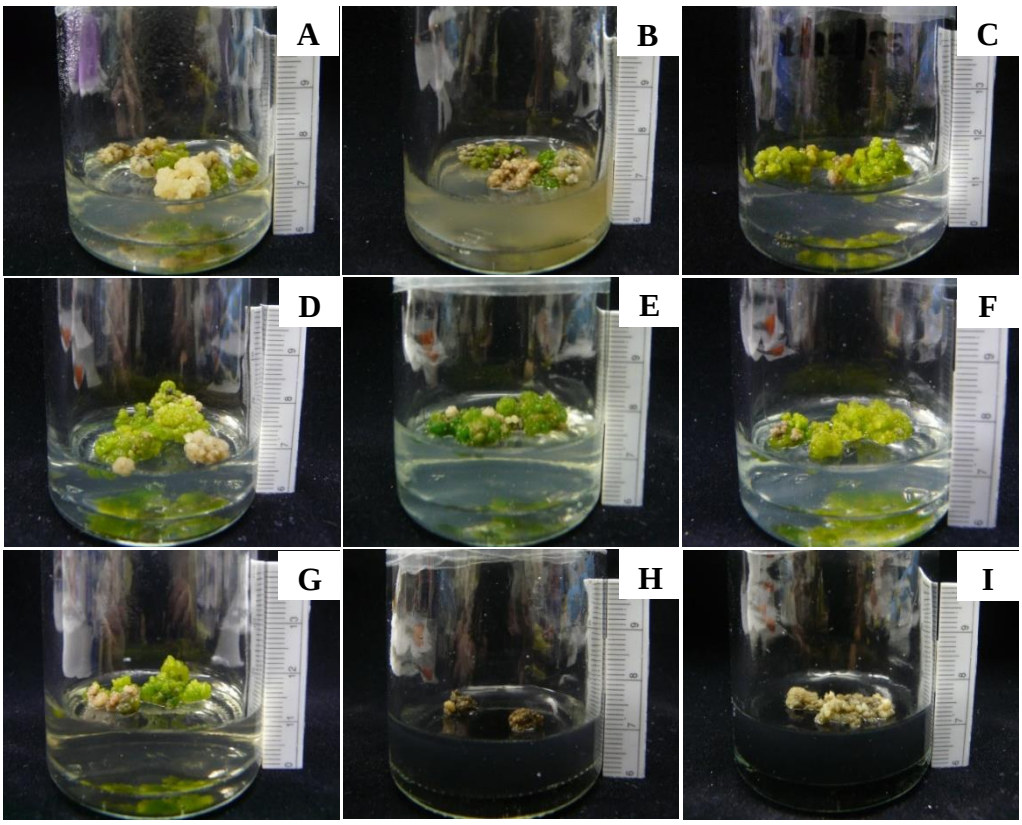


Figure 39 Effect of anti-browning agents on callus browning of *D. crumenatum* after 3 months of culture.

(A) Control group **(B)** 0.001 g L^{-1} AA **(C)** 0.01 g L^{-1} AA **(D)** 0.01 g L^{-1} Cys **(E)** 0.05 g L^{-1} Cys **(F)** 0.5 g L^{-1} PVP **(G)** 5 g L^{-1} PVP **(H)** 1 g L^{-1} AC **(I)** 2 g L^{-1} AC

3.3.3 Effect of anti-browning agents on biochemical changes

A. PPO activity

The effect of anti-browning agents on PPO activity was evaluated after extended culture for 3 months. The results showed that the presence of AA at 0.01 g L^{-1} gave the lowest level of PPO activity at 276.89 ± 14.95 followed by 0.001 g L^{-1} AA at 280.28 ± 9.32 U g^{-1} FW, statistically significant difference compared with control (365.70 ± 10.93 U g^{-1} FW). Conversely, the highest activity of PPO (393.86 ± 16.36 U g^{-1} FW) was found in the presence of 1 g L^{-1} AC followed by 2 g L^{-1} AC (347.67 ± 5.95 U g^{-1} FW), 0.5 g L^{-1} PVP (335.71 ± 8.50 U g^{-1} FW), 0.05 g L^{-1} Cys (334.85 ± 12.55 U g^{-1} FW) and 0.01 g L^{-1} Cys (327.20 ± 17.77 U g^{-1} FW), but there was significantly lower than that of control (Table 5).

B. PAL activity

The effect on PAL activity was evaluated after extended culture for 3 months. Of the four anti-browning agents tested, the PAL activity was significantly decreased when AA (0.001 and 0.01 g L^{-1}) and Cys (0.01 and 0.05 g L^{-1}) were added into the culture medium. Particularly, the callus cultured on medium containing 0.01 g L^{-1} AA gave the significant lowest activity of PAL (19.79 ± 3.76 U g^{-1} FW) as compared to control (99.33 ± 20.32 U g^{-1} FW). In contrast, the presence of 1 g L^{-1} AC exhibited a strong increase in PAL activity (210.60 ± 34.71 U g^{-1} FW) followed by 2 g L^{-1} AC treatment (165.76 ± 24.20 U g^{-1} FW), which was significantly different from control (Table 5).

Table 5 Changes in the PPO and PAL activities after an extended callus culture for 3 months on modified VW medium supplemented with various anti-browning agents.

Anti-browning agents	Concentrations ($g\ L^{-1}$)	PPO activity ($U^*\ g^{-1}$ FW ± S.E.)	PAL activity ($U^*\ g^{-1}$ FW ± S.E.)
Control	0	365.70 ± 10.93 ^{ab}	99.33 ± 20.32 ^{b}
Ascorbic acid	0.001	280.28 ± 9.32 ^{d}	33.62 ± 3.50 ^{c}
	0.01	276.89 ± 14.95 ^{d}	19.79 ± 3.76 ^{c}
L-cysteine	0.01	327.20 ± 17.77 ^{bc}	32.32 ± 9.34 ^{c}
	0.05	334.85 ± 12.55 ^{bc}	43.42 ± 15.30 ^{c}
Polyvinylpyrrolidone	0.5	335.71 ± 8.50 ^{bc}	68.00 ± 5.37 ^{bc}
	5	299.36 ± 10.97 ^{cd}	70.06 ± 7.05 ^{bc}
Activated charcoal	1	393.86 ± 16.36 ^{a}	210.60 ± 34.71 ^{a}
	2	347.67 ± 5.95 ^{b}	165.76 ± 24.20 ^{a}

Each value represents the mean ± S.E. from six replicates per treatment. Comparison of the mean values within a column was analyzed using the DMRT. Mean values with different superscript within columns are significantly different ($P \leq 0.05$).

* One unit of PPO activity is defined as the amount of enzyme that caused a change of 0.01 in the absorbance per min.

** One unit of PAL activity is defined as a change of 0.01 in the absorbance per hour.

C. Total phenolic content

Change in total phenolic content of proliferated callus was determined after 3 months of culture. The result showed that AA treatment at 0.01 g L^{-1} concentration gave the lowest content of total phenolic (0.155 ± 0.017 mg GAE g^{-1} FW), significant different with control. In contrast, the addition of 1 and 2 g L^{-1} AC as well as 5 g L^{-1} PVP showed slight decreases in total phenolic content (0.359 ± 0.002, 0.343 ± 0.008 and 0.351 ± 0.003 mg GAE g^{-1} FW, respectively) as compared to control (0.439 ± 0.027 mg GAE g^{-1} FW) (Table 6).

Table 6 Changes in the total phenolic content after an extended callus culture for 3 months on modified VW medium supplemented with various anti-browning agents.

Anti-browning agents	Concentrations (g L^{-1})	Total phenolic content (mg GAE g^{-1} FW ± S.E.)
Control	0	0.439 ± 0.027 ^{a}
Ascorbic acid	0.001	0.179 ± 0.022 ^{ef}
	0.01	0.155 ± 0.017 ^{f}
L-cysteine	0.01	0.237 ± 0.003 ^{d}
	0.05	0.223 ± 0.025 ^{de}
Polyvinylpyrrolidone	0.5	0.300 ± 0.026 ^{c}
	5	0.351 ± 0.003 ^{bc}
Activated charcoal	1	0.359 ± 0.002 ^{b}
	2	0.343 ± 0.008 ^{bc}

The values represent the mean ± S.E. of six replicates of each treatment. Mean values within a column with different superscript are significantly different at $P \leq 0.05$ by DMRT.

D. Lipid peroxidation in terms of MDA contents

Lipid peroxidation, measured as content of MDA, was evaluated after extended culture for 3 months (Table 7). It was found that control treatment gave the highest MDA content (96.88 ± 8.86 µmol MDA g^{-1} FW). However, MDA contents were significantly diminished by the addition of all types of anti-browning agents. Particularly, 0.01 g L^{-1} AA treatment gave the lowest content of MDA (44.32 ± 0.24 µmol MDA g^{-1} FW).

Table 7 Changes in the MDA content after an extended callus culture for 3 months on modified VW medium supplemented with various anti-browning agents.

Anti-browning agents	Concentrations (g L^{-1})	MDA content [µmol (MDA) g^{-1} FW ± S.E.]
Control	0	96.88 ± 8.86 [a]
Ascorbic acid	0.001	63.81 ± 4.55 [c]
	0.01	44.32 ± 0.24 [d]
L-cysteine	0.01	71.48 ± 1.97 [bc]
	0.05	82.89 ± 3.64 [b]
Polyvinylpyrrolidone	0.5	61.29 ± 1.95 [c]
	5	65.20 ± 5.31 [c]
Activated charcoal	1	72.79 ± 2.10 [bc]
	2	63.81 ± 4.54 [bc]

The values represent the mean ± S.E. of six replicates of each treatment. Mean values within a column with different superscript are significantly different at $P \leq 0.05$ by DMRT.

E. Chlorophyll and carotenoid contents

The content of chlorophyll and carotenoid were determined after 3 months of extended culture. The incorporation of AA, Cys and PVP to culture medium gave an increase in chlorophyll *a*, chlorophyll *b* and total chlorophyll content as compared with control. However, the amount of chlorophyll *a* (0.082 ± 0.001 mg g^{-1}FW), chlorophyll *b* (0.059 ± 0.001 mg g^{-1} FW) and total chlorophyll (0.122 ± 0.002 mg g^{-1}FW) showed a significant maximum in the presence of 0.01 g L^{-1} AA. A similar response was observed for carotenoid content showing the highest carotenoid level in 0.01 g L^{-1} AA treatment (0.036 ± 0.000 mg g^{-1} FW) with a significant difference as compared to control. In contrast, the use of AC, in particular at 1 g L^{-1}, gave the lowest amount of chlorophyll *a* (0.046 mg g^{-1} FW), chlorophyll *b* (0.028 mg g^{-1} FW) and total chlorophyll (0.063 ± 0.001 mg g^{-1} FW). Although the higher concentration of AC was used (2 g L^{-1}) but only slightly increase in chlorophyll content. Similar result was obtained in carotenoid content, AC at 1 g L^{-1} gave the lowest content at 0.025 ± 0.000 mg g^{-1} FW followed by 2 g L^{-1} AC, without significant difference with control (Table 8). .

Table 8 Changes in the pigment contents after an extended callus culture for 3 months on modified VW medium supplemented with various anti-browning agents.

Anti-browning agents	Concentrations ($g\ L^{-1}$)	Chlorophyll *a* ($mg\ g^{-1}$ FW)	Chlorophyll *b* ($mg\ g^{-1}$ FW)	Total chlorophyll ($mg\ g^{-1}$ FW)	Carotenoid ($mg\ g^{-1}$ FW)
Control	0	0.053 ± 0.001^{cd}	0.035 ± 0.002^{d}	0.076 ± 0.003^{cd}	0.030 ± 0.000^{bc}
Ascorbic acid	0.001	0.062 ± 0.005^{bc}	0.042 ± 0.003^{b}	0.090 ± 0.007^{bc}	0.030 ± 0.002^{bc}
	0.01	0.082 ± 0.001^{a}	0.059 ± 0.001^{a}	0.122 ± 0.002^{a}	0.036 ± 0.000^{a}
L-cysteine	0.01	0.064 ± 0.005^{b}	0.044 ± 0.004^{b}	0.093 ± 0.007^{b}	0.030 ± 0.002^{bc}
	0.05	0.071 ± 0.005^{b}	0.050 ± 0.003^{b}	0.104 ± 0.007^{b}	0.032 ± 0.002^{abc}
Polyvinylpyrrolidone	0.5	0.070 ± 0.001^{b}	0.046 ± 0.001^{b}	0.100 ± 0.001^{b}	0.033 ± 0.001^{ab}
	5	0.062 ± 0.003^{bc}	0.042 ± 0.001^{b}	0.089 ± 0.004^{bc}	0.029 ± 0.001^{c}
Activated charcoal	1	0.046 ± 0.001^{d}	0.028 ± 0.000^{c}	0.063 ± 0.001^{d}	0.025 ± 0.000^{d}
	2	0.063 ± 0.004^{bc}	0.042 ± 0.003^{b}	0.090 ± 0.006^{bc}	0.029 ± 0.001^{c}

Each data represents the mean ± S.E. of three replicates per treatment. Mean values with different letters in the same column indicate significant differences at $P \leq 0.05$ by DMRT.

3.3.4 Change in histological and ultrastructural features after treatment with anti-browning agents

According to the biochemical data, 0.01 g L^{-1} AA was found to be very effective in controlling the browning problem and enhancing callus growth in the callus proliferation stage.

These AA-treated calli were still green and apparently vigorous, even though the culture period was extended for 3 months without any subculture to fresh medium (Figure 40A). They consisted of small meristematic cells with large nuclei and prominent nucleoli (Figure 40B) and also showed the formation of tracheary elements (Figure 40C). Moreover, the examination at subcellular level showed that 0.01 g L^{-1} AA-treated callus cell exhibited well-defined ultrastructural appearance of plasmodesmata across the cell wall and numerous organelles within dense cytoplasm (Figure 41A). Their central vacuole with intact tonoplast contained less osmiophilic material (Figure 41A, arrow head). Several chloroplasts containing starch grain and poorly developed stacks of thylakoid membrane (Figure 41B). Rounded mitochondria with tightly packed cristae and electron-dense matrix were also present (Figure 41C). Besides, plentiful polysomes (Figure 41D) and peroxisomes showing great crystal-like structures (Figure 41E) were observed. Interestingly, clear evidence of peroxisome division was occasionally observed in this study (Figure 41F). In addition, active dictyosome-producing vesicles (Figure 41G) and clear lipid bodies (Figure 41H) were also found in this healthy callus.

Conversely, the modified VW medium added with 1 g L^{-1} AC was found to be the worst medium for preventing browning as shown by biochemical factors. These proliferated callus displayed discoloration (Figure 40D). Histological observation showed the unorganized and loosely arranged in their cell structures (Figure 40E). After a deep investigation, the ultrastructural evidence revealed severe abnormalities of their callus cells at subcellular level namely disorder in several organelles, reduction in cytoplasmic density and strong degradation of plasmalemma and cell wall (Figure 42A). Furthermore, there was no plasmodesmal connection on the adjoining electron-dense cell wall between adjacent cells (Figure 42A). Chloroplasts had an oval shape with a disorganization of thylakoid membrane system and a loss of starch grain (Figure 42B) but contained some chloroplastic lipid droplets

(Figure 42B, arrow). Moreover, numerous vesicles containing intensely dense osmiophilic material were observed and they were then gradually enlarged by the conjoining of each vesicle (Figure 42C, 42D). Mitochondria were severely deformed and showed a reduction in their cristae and matrix (Figure 42E). In addition, unusual structures including double-membrane vesicles (Figure 42E, arrow) and multivesicular bodies (Figure 42F) were obviously present.

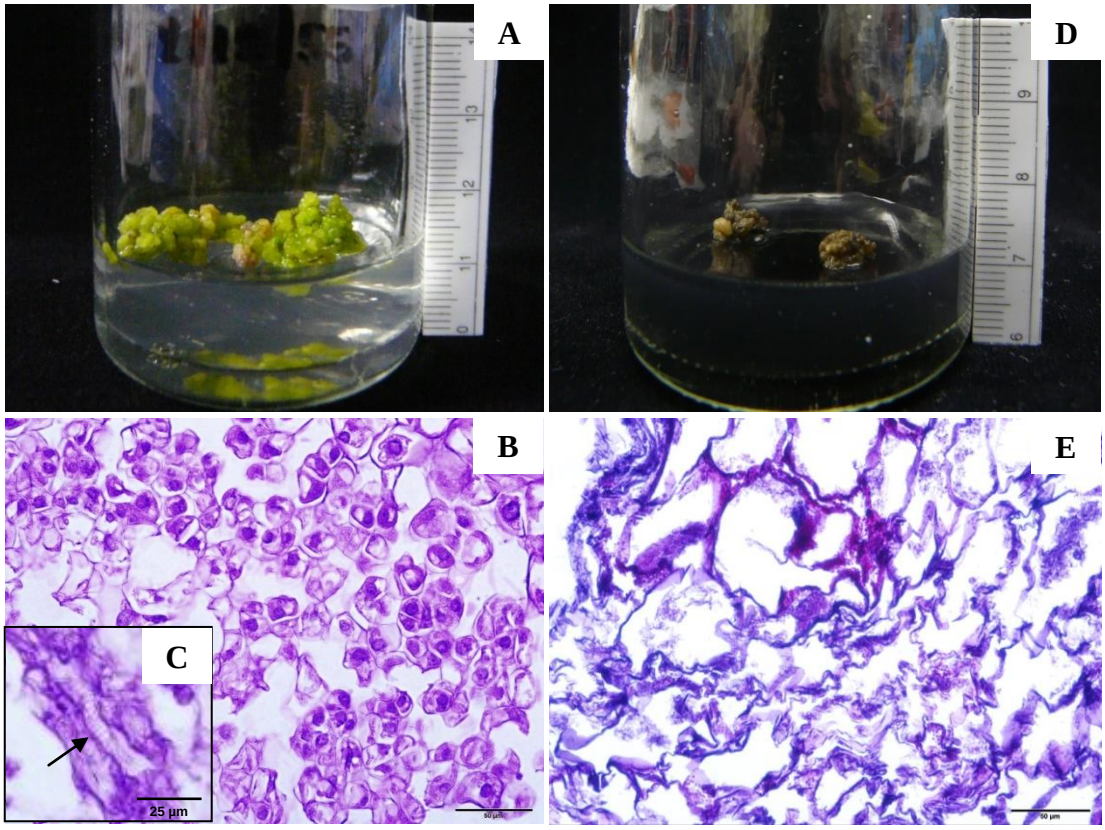


Figure 40 Proliferated callus on modified VW medium supplemented with ascorbic acid and with activated charcoal after 3 months of culture.

(**A**) Green and vigorous callus on a medium added with 0.01 g L^{-1} ascorbic acid showing (**B**) meristematic cells and (**C**) tracheary elements (*arrow*). (**D**) Brown and stunted callus on a medium incorporated with 1 g L^{-1} AC exhibiting (**E**) loosely arranged cells.

Figure 41 TEM micrographs of healthy three-month-old proliferated callus on a modified VW medium supplemented with 0.01 g L^{-1} ascorbic acid.

(**A**) Panoramic view of callus cell showing a dense cytoplasm, intact tonoplast, distinct plasmodesmata (*arrow*) and empty central vacuole (*bar* = 2 µm). (**B**-**H**) Magnified views of (**B**) chloroplast containing starch grain and simple stacked of thylakoid membrane system (*bar* = 500 nm); (**C**) mitochondria with electron-dense matrix (*bar* = 200 nm); (**D**) plentiful polysomes (*bar* = 200 nm); (**E**) numerous enlarged peroxisomes (*bar* = 500 nm); (**F**) a clear peroxisome division showing constriction (*arrow*) (*bar* = 500 nm); (**G**) well-developed dictyosome with budding vesicles (*arrow*) (*bar* = 500 nm); (**H**) numerous lipid droplets (*bar* = 500 nm).

(lipid droplet: l; mitochondria: m; peroxisome: p; polysome: ps; starch grain: s; thylakoid membrane: th; tonoplast: t; vacuole: v)

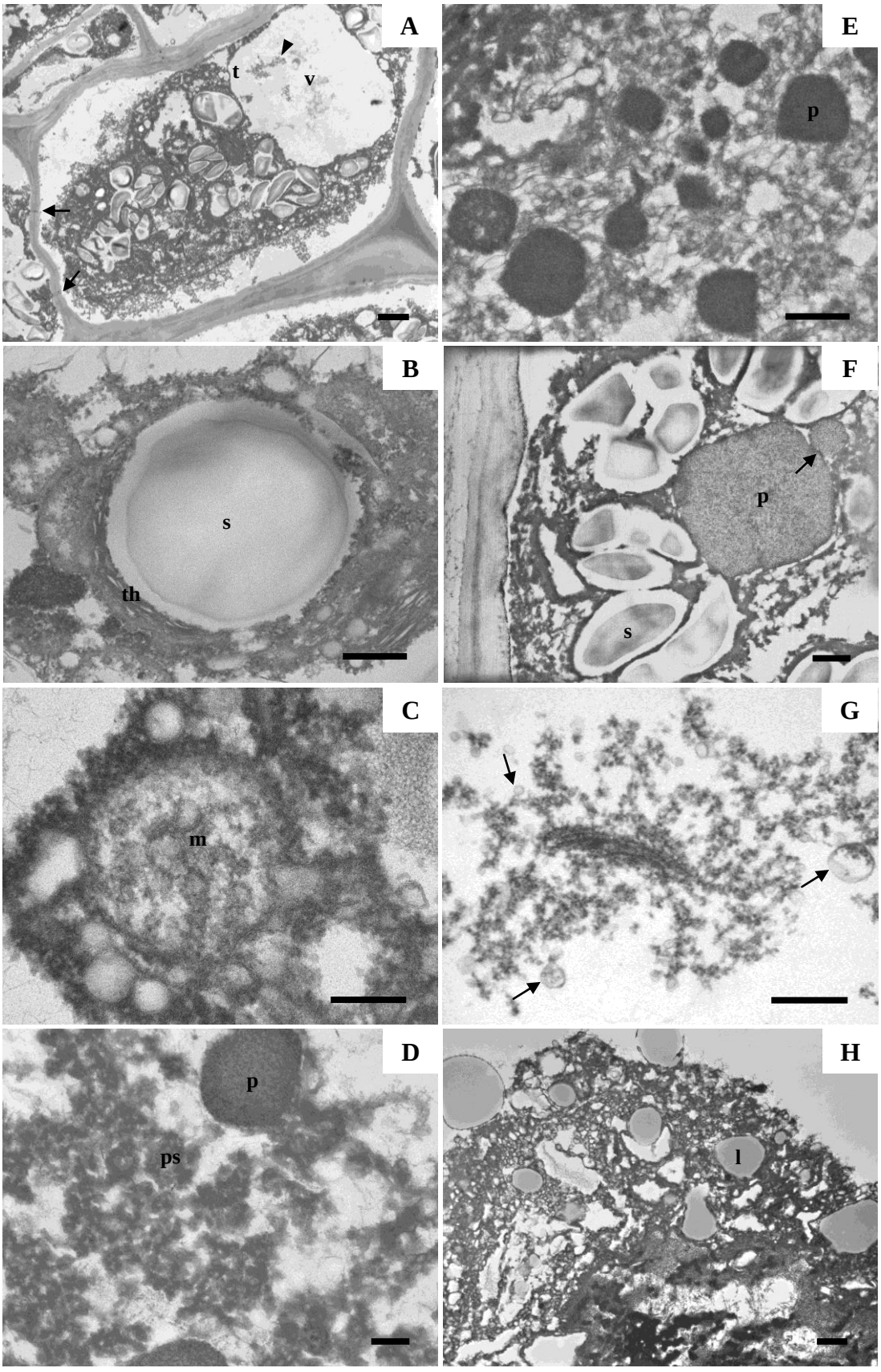


Figure 41 (see facing page for legend)

Figure 42 TEM micrographs of unhealthy three-month-old proliferated callus on a modified VW medium supplemented with 1 g L^{-1} activated charcoal.

(A) Panoramic view of callus cell showing disorganized organelles, reduced cytoplasmic density, degraded plasmalemma (asterisk) and distorted cell wall (*arrow*) (*bar* = 2 µm). **(B-F)** Magnified images of **(B)** abnormal chloroplast with swollen thylakoid membrane and lipid droplet, but lack of starch grain (*bar* = 500 nm); **(C-D)** numerous microvesicles (vs) accumulated with osmiophilic materials closely connected with the inner surface of vesicle membrane (asterisk) (*bar* = 200, 500 nm); **(E)** irregular mitochondria with deformed cristae and reduced matrix (*bar* = 250 nm) and appearance of small double-membranous vesicle (*arrow*); **(F)** multivesicular body containing internal vesicles (*arrows*) (*bar* = 100 nm).

(mitochondria: m; multivesicular body: MVB; thylakoid membrane: th; vesicle: vs)

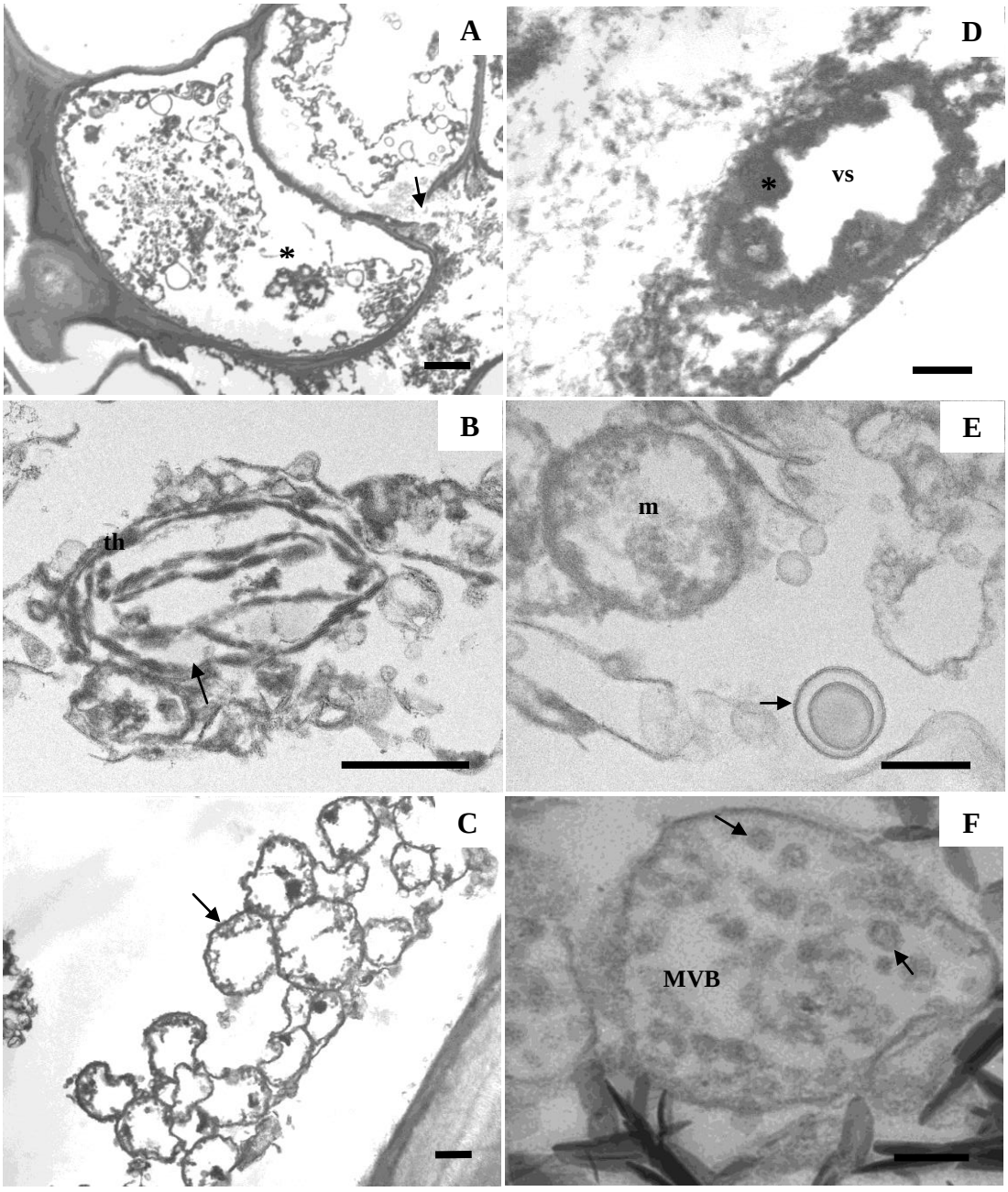


Figure 42 (see facing page for legend)

CHAPTER 4
DISCUSSION

4.1 Influence of PGRs on morphogenic responses

After bisected protocorm culture, the best callus formation was obtained from PGR combination treatment, in particular the application of exogenous cytokinin (BA) at low concentration (1 mg L^{-1}) in combination with exogenous auxin (NAA) at 0.5 mg L^{-1} indicating the importance of a low level of cytokinin on controlling callus formation. Meanwhile, the result revealed that the exogenously-applied auxin at both concentrations (0.1 and 0.5 mg L^{-1}) in PGR combination treatments did not enhance the formation of callus. This result could be attributed to the fact that there was a sufficient level of endogenous auxin within the explants for a favorable ratio of PGR leading to callus formation, in agreement with previous result reported by Khosravi et al. (2008). Thus, the application of exogenous auxin at low concentration did not have an impact on the total amount of auxin and subsequently did not influence the specific ratio of auxin to cytokinin for promoting callus formation.

Meanwhile, a high concentration of exogenous cytokinin not only increases the structural rigidity of the cell wall leading to decreasing amount of water uptake and subsequently decelerating cell division (Farooq et al., 2012), but also induces a programmed cell death process in plant cells (Vescovi et al., 2012). Thus, cytokinin at high concentration has an inhibitory effect on callus formation even combined with exogenous auxin. Accordingly, the enhancement of callus formation of *D. crumenatum* may still require a high level of exogenous auxin to balance auxin-cytokinin ratio. This finding was consistent with previous studies exhibiting the addition of auxin combined with cytokinin plays a key role to regulate cell division, an essential process for callus formation in many orchid such as *Phalaenopsis* (Tokuhara and Mii, 2001), *D. fimbriatum* Lindl. var. *oculatum* (Roy and Banerjee,

2003) and *Vanilla planifolia* (Tan et al., 2011). Auxin and cytokinin act synergistically to regulate the cell cycle, in which auxin affects DNA replication, whereas cytokinin activates mitosis (Chriqui, 2008; Machakova et al., 2008; van Staden et al., 2008).

In addition, the presence of callus on the medium without any exogenous PGRs indicated that the bisected segment of protocorm may contain an optimal balance endogenous PGRs level to induce callus as reported by Majumder (2011). However, this incidence of hormone-autonomous callus formation is an uncommon phenomenon in orchids such as *D. fimbriatum* (Roy and Banerjee, 2003), *D. chrysotoxum* Lindl. (Roy et al., 2007) and *Dendrobium* cv. Serdang Beauty. (Khosravi et al., 2008).

Meanwhile, the occurrence of a low frequency of induced callus on the medium supplied with either auxin or cytokinin alone implied that the endogenous hormone within the explants may play a role on the formation of callus (Gupta et al., 2010). Khosravi et al. (2008) also reported that the medium supplemented with NAA alone gave the PLBs-derived callus in *Dendrobium* cv. Serdang Beauty.

In the current study, the highest percentage of PLB formation was also obtained from protocorm segment after culture on the medium supplemented with NAA alone which was similar to that of the previous studies reported in *D. chrysotoxum* Lindl. (Roy et al., 2007) and *Dendrobium* Bobby Mesina Red. (Tee et al., 2010) with the medium containing only 1 µM NAA and 3 mg L^{-1} NAA, respectively. Dipti et al. (2014) noted that NAA was the crucial factor in the initiation of early and globular stages of PLB which is a prerequisite for the formation of PLB. Furthermore, the PLB formation of *D. crumenatum* on BA-alone treatment was in agreement with the observation of Kaewjampa et al. (2012) who reported that the highest rate of PLB formation of *Cymbidium* Waltz 'Idol' was achieved on the medium added with BA alone at 1 or 10 mg L^{-1}. Similarly, the increase in average number of PLBs and the percentage of PLB formation of *D. kingianum* was also obtained on medium supplemented with BA alone particularly at low concentration (0.1 g L^{-1}) (Habiba et al., 2014a).

Moreover, shoots also occurred after bisected protocorm culture. The successful shoot formation in the present study implied that the low concentration of

exogenous BA may be the most effective concentration for the shoot formation from transversely bisected protocorm. Similar results were reported by Habiba et al. (2014b), who reported that BA could enhance the direct shoot formation from the explants of *Epidendrum* ‘Rouge Star No. 8 as shown by the high percentage of shoot formation and the high average number of shoots per explant. Habiba et al. (2014b) also reported that BA is one of the cytokinins involved in cell division, shoot differentiation and modification of apical dominance.

As mentioned above, there were three types of morphological appearance including callus, PLB and shoot occurring after transversely bisected protocorm culture for 8 weeks on the medium supplemented with various concentrations of NAA and BA. Histological features showed that the bisected segment of protocorm started to form callus, called callogenesis, after culture for a week due to the occurrence of cellular origin of callus near the wound site of bisected protocorm. This result indicated that wounding may be considered as a trigger of cell dedifferentiation giving rise to the onset of callus formation, similar to that reported by Iwase et al. (2011) and Ikeuchi et al. (2013). Moreover, a single subepidermal cell containing a dense cytoplasm and large nucleus was proposed as an origin of callus mass of *D. crumenatum*. The characteristic features of this originating cell were an actively dividing cell which may be associated with the formation of callus. Similarly, Lombardi et al. (2007) reported that callus of *Passiflora cincinnata* Mast. initiated from the subepidermal cell of leaf discs. The present study also showed the originating callus cells continued to divide and produce more amounts of callus cell leading to increase the size of callus mass. This induced callus comprised small isodiametric cells with a prominent nucleus and nucleolus within densely staining cytoplasm, which was in agreement with Creemers-Molenaar et al. (1994) who reported that histological feature of callus cell could indicate a high regenerative potential of their cells due to the appearance of their meristematic characteristics. Thus, the histological observation confirmed that PGRs may be a key role related with callogenesis pathway as mentioned by previous study of Hamidvand et al. (2013).

Moreover, histological data also showed other pathways of morphological response. The present results revealed that PLB could form directly from the explant without any intervening callus phase, which was referred to as one

type of morphogenetic pathway called direct somatic embryogenesis as reported by Quiroz-Figueroa et al. (2006). In contrast, indirect somatic embryogenesis can be induced indirectly from callus. Apart from callus and PLB formations, shoot was also induced directly from the explants via direct organogenesis as shown in histological data.

Hence, this study proposed that the specific morphogenic response of bisected protocorm was controlled by the correct combination of PGRs, cytokinin and auxin, giving rise to various morphogenesis as confirmed by histological aspects.

4.2 Influence of an extended culture period on the alteration of callus

4.2.1 Histological and structural alterations during callus culture

The bisected protocorm-derived calli were cultured on a modified VW medium containing 2 % sucrose, 2 % peptone, 0.5 mg L^{-1} NAA and 1 mg L^{-1} BA. The green callus gradually became yellow-brown and then turned into a darker brown color during callus culture without any subculture. This discoloration was attributed to the oxidative browning which is a consequence of phenolic oxidation inducing the formation of brown pigments.

Cellular level observation of callus during culture demonstrated that the three-month-old cultured callus on a medium without any subculture displayed a loose and disordered cell arrangement, in contrast to the one-month-old callus. The present result was similar to the observation by He et al. (2009) who reported the differences between the brown and non-brown callus of *Jatropha curcas* in which non-brown callus cells were spheroid and compactly arranged while irregular and loosely arranged cells were observed in brown callus.

In addition, the observation on ergastic substances such as tannin and insoluble carbohydrate in cultured callus indicated that the accumulation of these compounds during callus culture was associated with growth capacity of their cells. One- and two-month-old callus showed a greater accumulation of insoluble carbohydrate than three-month-old cultured callus. This result corresponded to a

histological detection of carbohydrate in the embryogenic callus of *Vanilla planifolia* (Palama et al., 2010). Meanwhile, three-month-old cultured calli were accumulated the phenolic compound called tannin. Tannin is a highly polymerized substance and the product of its oxidation 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; Zamir et al., 2004; Dai and Mumper, 2010; Ahmad et al., 2013). Moreover, the present result also revealed that the browning callus had accumulated little insoluble carbohydrate indicating less energy sources required for further development which could result in a loss of totipotency in morphogenically competent cells.

4.2.2 Ultrastructural alteration during callus culture

The present study showed that the alterations of ultrastructure in many organelles of callus were associated with browning.

The one-month-old green callus, serving as control, displayed the 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. The appearance of amyloplast at the subcellular level coincided with the presence of carbohydrates revealed by PAS staining and indicated the morphogenic potential and represented active metabolism of plant cell as mentioned by Laparra et al., (1997) and Zenkteler and Kwasna, (2007). 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). Moreover, the presence of two types of starch-containing plastids (chloroplasts and amyloplast) 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). In addition, other plastids were also observed in green callus, namely globular chromoplast and chloro-chromoplast, which were contained small plastoglobuli. Bréhélin et al. (2007) reported that plastoglobuli are lipoprotein particles in plastids that contain lipid isoprenoid-derived metabolites. Plastoglobuli have also been shown to play a key

role in the chloroplast-to-chromoplast transition and carotenoid metabolism (Bian et al., 2011).

Furthermore, numerous free ribosomes, cisternae of RER and dictyosome were also presented in dense cytoplasm of one-month-old green callus. This is in agreement with earlier finding of Appezzato-da-Glória and Machado (2004) who revealed that a ribosome-rich cytoplasm, conspicuous dictyosome and RER were observed in the meristemoids 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).

In addition, the well-organized organelles in one-month-old green callus as mentioned above are consistent with observations made by others for embryogenic callus of peach palm and banana, which could develop further to form plantlets (Steinmacher et al., 2011; Ribeiro et al., 2012). Thus, the present findings may indicate the growth capacity of callus cells and reveled that these well-organized organelles might play an important role in cells' ability to regenerate from non-browning callus of *D. crumenatum*.

Moreover, the evidence of the plasmalemma and organellar membranes and the appearance of empty double-membranous vesicles also occurred in two-month-old callus. These vesicles were very similar to an autophagosome-like vesicle detected in *Arabidopsis* cells undergoing programmed cell death (PCD) (Hofius et al., 2009). 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). Moreover, the autophagosome-like vesicle was involved with autolysis in the tissue browning of bamboo shoots. This autolysis can extend from injured to uninjured cells resulting in the continued browning during culture (Huang et al., 2002).

The ultrastructural study also displayed various disorganized organelles in three-month-old callus cells such as minimal cytoplasm, degraded plastids and detached plasmalemma. A cell with shrunken membrane indicated a membrane dysfunction, an increase in cell membrane permeability and a leakage of

ions that were distinguishing features of an oxidative stress response (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 compositions, water and light can lead to enhancement of 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, prolonged culture period with nutrient deficiency, environmental stress, long-term oxidative stress and negatively impacted metabolic processes of plant cells, including photosynthesis and respiration. Such of these factors 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).

The present results also showed that three-month-old callus had distortion in thickened cell walls, 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).

Additionally, cells of three-month-old callus also contained dark osmiophilic material, presumably tannin, in numerous microvesicles and in the cytoplasm. Laukkanen et al. (2000) reported that tannin accumulated to a high level in the cytoplasm and intercellular spaces 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

three-month-old callus cells of *D. crumenatum*. In addition, numerous SER, peroxisomes with a large crystal-like structure and lipid bodies were also observed in three-month-old callus. Peroxisomes are the site of H_2O_2 scavenging in response to H_2O_2 that is formed by photorespiration and β-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 in both 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 three-month-old callus. 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).

4.2.3 Ultrastructural alteration in crucial organelles (nucleus, mitochondria and chloroplast)

Although various organelles underwent changes during callus culture, the crucial organelles including **nucleus, mitochondria** and **chloroplast** showed distinct alteration. The result revealed that the alterations of these organelles are associated with the period of inoculation-induced browning in *D. crumenatum*.

This study showed that one-month-old callus exhibited a clear nucleus. This structure is related to the characteristics of totipotent embryogenic cells that have a nucleus containing less perinuclear heterochromation but more euchromatin (Verdeil et al., 2007). Moreover, the existence of isodiametric mitochondria with a distinct double-membrane envelope in the one-month-old callus cells indicated that a high metabolic activity and respiration rate have occurred in these callus cells, as 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 are involved with the response of a plant to oxidative stress (Bi et al., 2009). Additionally, chloroplasts containing starch grain were clearly observed in the one-month-old callus. 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 as shown in this present study were also clearly observed during organogenesis like those reported in *Glycine max* (L.) Merrill and *Bauhinia forficata* Link (Appezzato-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 some plant cells, including of *Drosera spathulata* 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 which is an energy-requiring processes (Thorpe et al., 2008).

In two-month-old callus, there were some ultrastructural alterations in the important organelles including nucleus, mitochondria and chloroplast. These facets were consistent with the characteristics of plant cells after exposing to chilling stress, which is an oxidative 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 similar to three-month-old *D. crumenatum* callus. It is possible that callus browning could be a consequence of oxidative stress due to organelle disorganization and increased lipid peroxidation during callus culture without subculture.

In addition, the three-month-old callus showed the appearance of irregular nucleus with peripheral chromatin condensation-induced nuclear envelope breakdown, swollen mitochondria with distorted mitochondrial membrane and swollen chloroplasts with deformed thylakoid membrane. Peripheral nuclear chromatin was also noted both in plant cells undergoing PCD (Gunawardena et al., 2005) and in plants induced by oxidative stress (Speranza et al., 2007). Moreover, the changes in mitochondria of three-month-old callus were consistent with the typical structure of dysfunctional mitochondria in necrotic cells as a consequence of lipid peroxidation and over-generation of ROS (Lee et al., 2002; Yoshinaga et al., 2005).

In addition, the malformed chloroplasts as proposed in this study 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 as described in 4.2.4. Dilation 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. 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, nucleus and mitochondria are the primary organelles affected by oxidative stress, and alterations to these organelles subsequently indicate a sign of necrosis. These finding 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.

4.2.4 Changes in biochemical factors during callus culture

The biochemical factors including PPO, PAL activity, total phenolic, MDA content, chlorophyll and carotenoid content could explain the occurrence of browning.

The result showed a gradual increase in PPO activity during extended period of incubation as shown by a highest PPO activity in three-month-old callus. 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). PPO is closely involved in the phenolic oxidation as PPO is able to catalyze two distinct oxidative reactions, monophenol and diphenol oxidations, leading to the formation of toxic *o*-quinone and polymerization of brown pigment. Accordingly, a high level of PPO results in discoloration and eventual death of explants (Mayer, 2006). 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) as PPO is a crucial enzyme for defense against oxidative stress in plant cells (Mayer, 2006). Even though this oxidative enzyme is located in the thylakoid membrane of the chloroplast in latent state, the ROS can cause the cellular decompartmentzation resulting in activation of the latent PPO and consequent starting of the PPO-mediated phenolic oxidation (Laukkanen et al., 1999; Laukkanen et al., 2000; Wang et al., 2010).

In addition, the result revealed that the PAL activity was highest at the initial incubation, which may be affected by wounding, as the activity of PAL was closely related with the expression level of *PAL* gene which was regulated and elevated in response to abiotic stress including mechanical injury (MacDonald and D'Cunha, 2007; Vogt, 2010). Besides, the present study also showed an increase in PAL activity after extended culture incubation that was correlated with the occurrence of browning. This finding was consistent with previous reports of Xu et al., (2005); Xu and Li (2006) who mentioned that the activity of PAL was increased in explants browning of *Phalaenopsis*. Similarly, *PAL* gene expression was up-regulated during explants culture and was associated with browning of the explants (Xu et al., 2007). Due to the fact that PAL is the main enzyme in phenylpropanoid pathway, this PAL-mediated pathway can provide phenolic substrates for oxidative enzymes, PPO (Hisaminato et al., 2001; He and Luo, 2007). Thus, PAL is also a key enzyme associated with enzymatic browning of many plant species, including *D. crumenatum*.

This study also showed that total phenolic content was highest after 3 months of culture. The over-production of phenolics adversely influenced callus growth and a high amount of phenolic compounds resulted in tissue browning. Similar result was also 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 of browning in *Pistacia vera* L. callus gradually increased and were approximately 2- and 2.3-fold higher than the controls, respectively due to phenolic compound acting as a substrate of PPO, an oxidative enzyme. The products of their oxidation are highly reactive and toxic to the explants leading to necrosis and tissue death (Titov et al., 2006; Reis et al., 2008). Thus, the extended culture period of *D. crumenatum* callus showed a positive relation between the quantity of total phenolic and the degree of browning. This is consistent with previous reports on browning in *Phalaenopsis* tissue culture (Xu and Li, 2006; Yin et al., 2006; Xu et al., 2010).

In addition, the current study revealed that MDA content gradually increase during extended callus culture and was correlated with the occurrence of browning. Due to an increase in MDA content, the occurrence of callus browning of *D. crumenatum* may be a consequence of oxidative damage as reported in *Pinus sylvestris* L. (Laukkanen et al., 2000) and *Pinus virginiana* Mill. (Tang and Newton, 2004) since lipid peroxidation can occur as a result of oxidative stress affected by the excessive production of free radical reaction (Sharma et al., 2012). Accordingly, this present finding implies that a gradual generation of ROS may occur concomitantly with an increase in lipid peroxidation.

The contents of total chlorophyll, chlorophyll *a*, chlorophyll *b* and carotenoids in the current study decreased during extended callus culture with an increasing incidence of browning. This finding was consistent with previous report of Laukkanen et al. (2000) who mentioned that a decrease in chlorophyll and carotenoid contents is associated with the deterioration of callus caused by tissue browning. Moreover, a gradual decrease in chlorophyll and carotenoid concentrations was also observed during *J. curcas* callus culture (He et al., 2009) and shoot proliferation (Misra et al., 2010). The reduction of chlorophyll, one of the major causes of changes in colour, could lead to the arrest of photosynthesis and cell growth, since chlorophyll is an essential pigment for photosynthesis. In previous study, the loss of chlorophyll content was normally considered to be the consequence of chlorophyll degradation resulting from oxidative stress caused by peroxy radical and phenoxy radical (Yamauchi et al., 2004; Toivonen and Brummell, 2008). Moreover, a reduced accumulation of chlorophyll could be due to the inhibition of chlorophyll biosynthesis

which was consistent with earlier work of Ghozlène et al. (2013) on stressed plants. However, the degradation of chlorophyll and the inhibition of chlorophyll biosynthesis can cause not only chlorophyll reduction, but also an increase in ROS production (Misra et al., 2010; Hörtensteiner and Kräutler, 2011). Accordingly, the reduction of chlorophyll content induced by abiotic stress, which then stimulates the overproduction of ROS and increased activity of chlorophyll-degrading enzymes (Sevengor et al. 2011), 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.

However, carotenoids play an important role in overcoming oxidative stress and preventing lipid peroxidation in membranes by decreasing carotenoid content (Amirjani, 2010; Boguszewska and Zagdańska, 2012) since carotenoids can act effective scavengers that eliminate singlet oxygen, triplet state of chlorophyll and lipid radicals, thus preventing the destruction of chlorophyll (Ramel et al., 2012). This concept is fortified by study of El-Tayeb (2005) that observed a significant decrease in carotenoid content with increasing level of salt stress in barley (*Hordeum vulgare*) grains. Likewise, Yang et al. (2013) revealed that the carotenoid content in wheat (*Triticum aestivum* L.) seedlings decreased after exposure to UV-B radiation. Accordingly, the decrease in carotenoid content in *D. crumenatum* may be a result of carotenoid action on prevention of lipid peroxidation.

4.3 Influence of anti-browning agents on preventing the browning

Lethal browning has been observed during culture of *D. crumenatum* and many plant species such as Cavendish banana (Ko et al., 2009), *Phalaenopsis* (Zhou et al., 2009) and *J. curcas* L. (Misra et al., 2010). For minimizing this impediment, several researchers have employed anti-browning agents to reduce tissue browning, but the successful browning prevention has been achieved only in woody plants. In *D. crumenatum* orchid, however, no previous research has focused on the action of anti-browning agents for controlling the browning problem.

4.3.1 Effect of anti-browning agents on callus growth and browning

The increase in fresh weight of callus is considered as one of the growth parameters for determining the effect of anti-browning agent on callus growth. The present study showed that AA, Cys and PVP had no substantial effect on the enhancement of callus growth due to the non-significant increase in callus mass after an extended period of callus culture. The result was consistent with report of Khosroushahi et al. (2011), who reported that the incorporation of antioxidant showed no significant effects on callus proliferation of *Taxus brevifolia*. However, the medium containing AA was found to be an effective treatment for callus growth as represented by the maximum increase in callus fresh weight. Potters et al. (2002) reported that AA is a putative agent to regulate the plant cell cycle by controlling the G1-S transition resulting in promoting cell division and cell expansion which were the key factors for enhancement of callus growth.

In contrast, the addition of AC to culture medium was found to arrest the callus proliferation as shown by its lowest increased callus fresh weight. Similar results showing adverse effects of AC were observed in callus culture of *Nigella glandulifera* Freyn et Sint (Zhou et al., 2010), *Berberis vulgaris* var. *asperma* (Mohammadi-Nasab et al., 2011) and *Strelitzia reginae* (North et al., 2012). This is because AC can strongly adsorb phenolic compound, PGRs and nutrients leading to an impedance of the growth of plant cell attributed to the fact that AC has a high adsorptive property (Thomas, 2008).

Thus, this study indicated that the incorporation of all types of anti-browning agents in culture medium did not affect the growth and proliferation of callus, but they could reduce callus browning as described next.

4.3.2 Effect of anti-browning agents on callus browning

In this study, it is clear that AA was the most effective anti-browning agent for controlling the browning problem. Similar results as using AA for the prevention of browning were obtained by Teixeira da Silva (2013), who reported that 10 mg L^{-1} AA was an efficient treatment to minimize lethal browning of explants in hybrid *Cymbidium* Twilight Moon 'Day Light'. Poudyal et al. (2008) revealed that the adding 0.1 mg L^{-1} AA into a MS medium can be used to prevent browning

problem in Yali pear (*Pyrus bretshneideri*). Ndakidemi et al. (2014) also reported that a higher concentration of AA (200 mg L^{-1}) was effective to control tissue browning of nodal segments in *Brachylaena huillensis*. It is possible that AA, a reduced form of vitamin C and a water soluble antioxidant, acts as a free radical scavenger to inhibit the phenolic oxidation which causes enzymatic browning (Goveia, 2007; Dolatabadian and Saleh Jouneghani, 2009). In *D. crumenatum*, however, the use of AA at higher concentration (0.01 g L^{-1}) for reducing callus browning was more effective than lower AA level (0.001 g L^{-1}).

Moreover, this is the first evidence establishing the negative effect of adsorptive material, particularly AC, to prevent browning. The failure of using AC treated medium to prevent the browning of *D. crumenatum* orchid was similar to that of Cavendish banana (*Musa acuminate* Colla, AAA) (Ko et al,. 2009) and *Nigella glandulifera* Freyn et Sint (Zhou et al., 2010). The finding of the current study thus indicates that AC did not alleviate the browning occurrence, even though it has a high adsorption capacity to adsorb the phenolic compound – a key substrate of enzymatic browning reaction (Nokthai et al., 2010).

4.3.3 Effect of anti-browning agents on PPO and PAL activities

PPO and PAL have been reported to be associated with tissue browning.

PPO activity:

PPO is the key enzyme causing lethal browning due to its catalyzing the oxidation of phenolic compound to form highly-reactive *o*-quinone. These PPO-generated quinones then react further with other *o*-quinones, reducing sugars or amino group of cellular proteins to form brown-colored phytomelanins, a toxic compound to plant cells (Sapers et al., 2002; Araji et al., 2014). Ru et al. (2013) reported that the increase in PPO activity promoted the occurrence of tissue browning and was also considered as a key factor in the biosynthesis of phenolic compounds in response to oxidative damage as reported by Zhou et al. (2010) and Khosroushahi et al. (2011).

The results show that AA was found to be the best treatment for controlling the enzymatic browning through decreasing PPO activity. This is in agreement with the previous studies on bamboo (Huang et al., 2002), *Phalaenopsis*

(Lai et al., 2010) and *Taxus brevifolia* (Khosroushahi et al., 2011) showing the remarkable reduction in PPO activity by using AA as a PPO inhibitor. Barry-Ryan et al. (2007) and Ko et al. (2009) mentioned that AA can be absorbed by plant cell and then translocated to a target site for inhibiting the phenolic oxidation.

Conversely, the present result reveals that Cys showed a slight inhibition of PPO activity, even it is known as an effective PPO inhibitor in *Lactuca sativa* (Altunkaya and Gökmen, 2008). The failure of Cys to inhibit PPO activity of *D. crumenatum* callus is in agreement with the previous report in *Taxus brevifolia* L. callus (Khosoushahi et al., 2011).

Meanwhile, AC-treated callus showed the highest level of PPO activity. This result clearly indicates that AC did not significantly inhibit PPO activity, even though AC showed a positive effect on browning prevention in *Juglans regia* L. (Ehteshamnia and Gholami, 2014) and tissue browning can be aggravated by an increase in PPO activity (Wu and Lin, 2002). Thus, AC was found to be the worst anti-browning agent for preventing callus browning.

PAL activity:

PAL is also closely involved in enzymatic browning due to play an important role in the phenolic biosynthesis (Yin et al., 2008; Ghasemzadeh and Ghasemzadeh, 2011; Nadernejad et al., 2012). Vogt (2010) also mentioned that PAL is an extremely sensitive indicator of stress conditions and it is commonly considered as a biochemical marker indicating the synthesis of both structural and protective compounds. The present finding reveals that AA had a potent effect on the inhibition of PAL activity as demonstrated by the lowest PAL activity. However, this result was inconsistent with the finding of Roura et al. (2008), who reported that the addition of AA caused an increase in PAL activity. Meanwhile, Cys and PVP also presented a decrease in PAL activity. However, Cys and PVP may not be effective enough for inhibiting the PAL-catalyzed synthesis of phenolics as compared to AA.

In contrast, the present study reveals that the incorporation of AC into the culture medium seems to have negative effects on the reduction in PAL activity after extended culture for 3 months, implying that AC has failed completely to suppress enzymatic browning. Although the content of phenolics appeared to be low at the early stage of culture, it was proposed that this low content acted as a positive

feedback regulation of PAL synthesis (Peiser et al., 1998). The increase in the level of PAL may later elevate PAL activity leading to the activation of phenolic biosynthesis.

Thus, this research confirmed that the alleviation of browning can be achieved by decreasing PPO and PAL activities. However, the inhibitory effect of anti-browning agent on PPO and PAL activity depended on the type of anti-browning agent and plant species.

4.3.4 Effect of anti-browning agents on total phenolic content

The excessive production and accumulation of phenolic content showed an adverse influence on the growth of callus and could cause a browning problem (Dubravina et al., 2005; Naz et al., 2008).

Of the four anti-browning agents tested, AA was found to significantly reduce the amount of total phenolic content, which was consistent with the previous reports by Hoque and Arima (2002), Strosse et al. (2004), Uchendu et al. (2011) and Teixeira da Silva (2013). The reduction in total phenolic content may be a consequence of the inhibition on PAL-catalyzed biosynthesis, which was proposed to be a main factor to solve the enzymatic browning (Hisaminato et al., 2001; He and Luo, 2007).

The addition of AC into the medium led to a slight decrease in the total phenolic content which was correlated with the activity of PAL as previous described in 2.3.2. Although AC has been reported to diminish the tissue browning in many plant species such as *Anacardium occidentale* L. (Aliyu, 2005), *Rubus idaeus* L. (Wang et al., 2005), *Vicia faba* L. (Abdelwahd et al., 2008), *Zingiber officinale* Rosc. (Guo et al., 2007) and *Strelitzia reginae* (North et al., 2011), the successful adsorption of phenolic compound occurred only after the early stage of culture or initial damage of the explants. The present study thus pointed out that the AC was not an effective adsorbent in controlling phenolic accumulation because it was unable to completely adsorb the phenolic compound after extended culture on the same medium since the capacity of adsorption was also affected by pH of the medium and impurity of AC (van Winkle, 2000; Thomas, 2008). Moreover, the non-selective adsorptive property of AC also caused the undesirable result in browning prevention, thus the

concentration of AC should be carefully investigated. In addition, Reyes et al. (2007) also suggested that the correlation between the synthesis rate and the decrease rate of phenolic compounds, in which the synthetic rate was higher than the decrease rate, can cause the increase of total phenolic content.

4.3.5 Effect of anti-browning agents on lipid peroxidation

Typically, MDA is one of the most specific biomarkers of lipid peroxidation, which can point to the excessive production of free radicals causing adverse effect on membrane lipid (Blokhina et al., 2003; Sharma et al., 2012). The present study showed that MDA content was strongly reduced by 0.01 g L^{-1} AA, which was similar to the application of exogenous AA having an inhibitory effect on lipid peroxidation (Venkatesh and Park, 2014). As a result of the reduction in MDA content, the low MDA content in AA-treated callus could indicate a stress tolerance of callus cells and consequently lead to low oxidative damage of cellular and organellar membranes as reported by Wang and Han (2009) and Ashraf et al. (2010). AA acts as a reducing agent participating in defense mechanism against oxidative stress by increasing the antioxidant metabolism and directly scavenging ROS, then AA itself is altered to form dehydro-ascorbic acid resulting in protection of the cell from oxidative injury-induced lipid peroxidation (Noctor and Foyer, 1998; Davey et al., 2000; Misra et al., 2010; Karuppanapandian et al., 2011).

Meanwhile, a high level of MDA content was found in the control treatment without any anti-browning agents representing severe membrane injury. A similar finding has been reported in other plant species such as *Pinus sylvestris* L. (Laukkanen et al., 2000) and *Pinus virginiana* Mill. (Tang and Newton, 2004). These studies indicated that the MDA level appeared to be associated with the occurrence of oxidative browning, supporting the current observation in control treatment of *D. crumenatum*. The present study revealed that Cys and AC had no effect on the reduction in lipid peroxidation even though Cys and AC can detoxify free radicals (Benson, 1990; Altunkaya and Gökmen, 2008). According to the report of Tripathy and Oelmüller (2012), an increase in lipid peroxidation was attributed to an imbalance between ROS generation and antioxidative enzymes.

4.3.6 Effect of anti-browning agents on chlorophyll and carotenoid contents

The alteration in chlorophyll and carotenoid content could explain the color change of callus attributed to enzymatic browning (He et al., 2009). For that reason, understanding the effect of anti-browning agents on the loss of chlorophyll and carotenoid is necessary for controlling callus browning. The study showed that the significantly increased levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid content were obtained from 0.01 g L^{-1} AA treatment. A similar result was observed by Arab and Ehsanpour (2006) who reported that its incorporation of AA to the medium increased the chlorophyll content of *Medicago sativa* L. Due to its antioxidant property, AA can potentially lead to an increase in chlorophyll content by indirectly reduced the ROS-caused chlorophyll degradation (Ashraf, 2009; Ahmad et al., 2013). Moreover, the increase in content of carotenoid in this AA treatment may imply the reduction of ROS possibly by the complete scavenging action of AA. Accordingly, this result indicates the ability of callus cell to cope with stressful condition during prolonged culture on the presence of AA. Thus, AA treatment can be proposed as the best treatment for preventing the loss of photosynthetic pigments and consequently controlling the browning problem.

In contrast, AC treatment at 1 g L^{-1} resulted in decreased levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid contents. The change of chlorophyll content was used as a reliable indicator for chloroplast damage which was the physiological response under oxidative stress condition (Campos et al., 2012; Sharma et al., 2012). Chloroplast degradation can lead to the excessive generation of ROS and increase the activity of chlorophyll-degrading enzymes resulting in chlorophyll breakdown and inhibition of chlorophyll synthesis (Eckhardt et al., 2004; Santos, 2004; Kariola et al., 2005; Sevengor et al., 2011). Accordingly, this result indicates that AC did not prevent the destruction of chloroplast membrane and thus results in the reduction of chlorophyll. Besides, the result on carotenoid content in AC treatment was also consistent to the previous reports showing that the decreased carotenoid content had an important role in overcoming the oxidative stress conditions since carotenoid can prevent chlorophyll degradation by eliminating the singlet state of oxygen, triplet state of chlorophyll and other free radical

(Boguszewska and Zagdańska, 2012; Ramel et al., 2012; Sharma et al., 2012). However, the reduction in carotenoid content has negative effect on the rate of photosynthesis due to the fact that carotenoid content has a direct relationship with the photosynthesis rate (El-Tayeb, 2005; Lobato et al., 2010; Yang et al., 2013).

4.3.7 Change in histological and ultrastructural features after treatment with anti-browning agents

Previously, several researchers have reported the impact of anti-browning agent on ultrastructure to prevent enzymatic browning in vegetable and fruit (Li-Qin et al., 2009). However, there was no research focused on the effect of antioxidant or absorbent on lethal browning at the ultrastructural level in orchid, particularly *D. crumenatum*.

According to the biochemical data, the current findings show that treatment with 0.01 g L^{-1} AA was selected as the best medium for preventing callus browning. For confirmation of the prior findings on biochemical study, the histological and ultrastructural analyses were performed. The present results demonstrated that the three-month-old callus treated with AA at 0.01 g L^{-1} was friable in texture and had a well-organized appearance of many organelles, including a distinct plasmodesmata, clear organellar membranes, chloroplast containing starch, normal mitochondria, plentiful polysomes, obvious peroxisomes, active dictyosome and numerous lipid bodies.

The ultrastructural study could indicate the characteristic features and metabolic activity of healthy callus cell. Particularly, starch-containing chloroplasts could provide a large-scale energy source which is essential for embryogenic potential and organogenic potential of plant cells (Thorpe et al., 2008). Likewise, normal mitochondria are an important organelle for maintaining cellular life due to their producing ATP by cellular respiration (Dimmer and Rapaport, 2008). Moreover, Bi et al. (2009) and Vanlerberghe (2013) mentioned that mitochondria are also involved in a plant response to oxidative stress by controlling ROS generation as reported by Pastore et al. (2007). In addition, Moghaddam and Taha (2006) reported that the incidence of plasmodesmata play an important role in the cell-to-cell communication which is required for growth and development of plant cells. Moreover, Pilarska et al.

(2013) mentioned that the presence of obvious plasmalemma and organellar membrane revealed the good-compartmentation between oxidative enzymes and their substrates which is consistent with the low level of lipid peroxidation as shown in the present study. The clear evidence of membrane confirmed that AA could be involved in the inhibition of lipid peroxidation by directly scavenging free radicals resulting in protection of the membrane and then prevention of phenolic oxidation as reported previously (Gill and Tuteja, 2010). In addition, the plant cell containing high level of polysomes and dictysomes showed high cellular activity according to the ability of these organelles to synthesize protein (Berjak et al., 2000) and typically involving cell wall biosynthesis (Pihakaski-Maunsbach et al., 1993). Moreover, peroxisomes also play a part of many metabolic pathways in particular scavenging hydrogen peroxide (H_2O_2) resulting in the protection of cell from the destructive effect of H_2O_2 (Hu et al., 2012). Lodish et al. (2004) reported that peroxisomes contain several oxidative enzymes such as oxidase, catalase, ascorbate peroxidase or superoxide dismutase which are involved in the oxidation of fatty acid and the degradation of H_2O_2. In the pathway of H_2O_2 detoxification, ascorbate acts as an electron donor for the destruction of H_2O_2 which is catalyzed by ascorbate peroxidase (Karyotou and Donaldson, 2005; Gallie, 2012). Furthermore, peroxisomes are also dynamic organelles that can degrade or increase their number by peroxisome division in response to environmental stress (Yan et al., 2005; Castillo et al., 2008; Aung et al., 2010; Fransen, 2012). Likewise, the formation of lipid bodies within the cytoplasm also known as an indicator of plant cell response to oxidative stress (Chapman et al., 2012).

Thus, the present ultrastructural studies confirmed the biochemical findings, where the effectiveness of AA to enhance the pathway involved in defense response against stress was observed. For this reason, the oxidative stress-induced cellular decompartmentation and callus browning of *D. crumenatum* could be alleviated by AA.

Meanwhile, three-month-old cultured callus on the medium supplemented with 1 g L^{-1} AC showed several ultrastructural alterations including decreased cytoplasmic density, distorted membrane, swollen chloroplast, dense osmiophilic material within vesicles, irregular mitochondria, membranous vesicles

and multivesicular bodies which were no different from browning callus (control group).

The appearance of malformed organelles in AC-treated callus exhibited a sign of cellular injury during extended culture as verified by the occurred of ultrastructural damage. These changes of organelles may be a consequence of oxidative damage that was consistent with the high level of lipid peroxidation as reported above. Gill and Tuteja (2010) proposed that the destruction of cellular and organellar membranes is an indicator for determining the level of lipid degradation under stress conditions, as high concentration of ROS can cause destruction on many cell components such as protein, lipid and nucleic acid resulting in oxidative damage (Benson, 2000; Sharma et al., 2012). Several studies have shown that the ultrastructural alterations in organelles were strongly associated with the ROS-induced cell damage leading to diminished cellular competence and subsequent loss of totipotency (Benson, 2000; Sharma et al., 2012). The irregular chloroplast with an abnormal thylakoid membrane system is an important sign of plant cell under oxidative stress, which has the effect of on decreasing carbon assimilation and biomass accumulation (Ibrahim and Mostafa, 2007; Ali et al., 2014). Moreover, the swollen chloroplast was also a key organelle related with the ontogeny of tannosome (Brillouet et al., 2013). The morphological change of mitochondria as observed in this study was similar to dysfunctional mitochondria in oxidative stress-induced cell death of *Arabidopsis thaliana* (Yoshinaga et al., 2005).

Furthermore, the appearance of unusual organelles such as double-membranous vesicle and multivesicular body in treatment supplemented with AC indicates that these callus cells may respond to ROS-induced severe oxidative stress as found in the plant response to chilling stress (Lee et al., 2002) and biotic stress (Hofius et al., 2009). Double-membranous vesicle is similar to autophagosome-like vesicles which are involved in the degradation of cytoplasmic components (van Doorn and Papini, 2013). Besides, multivesicular body known as endosomal carrier vesicles occurred in response to abiotic stress before the degeneration of cytoplasm and some organelles (Olmos et al., 2006; An et al., 2007; Białońska et al., 2007). Numerous vesicles with osmiophilic material were also observed in callus cells treated with AC. This osmiophilic deposit was probably phenolic compounds that

were precipitated by caffeine in TEM fixative solution (Mueller and Greenwood, 1978). The deposition of phenolic compound was proposed as the main cause of callus browning due to the free radical-dependent phenolic oxidation (Stefanowska et al., 2002).

In this case, the ultrastructural observation clearly confirmed the adverse effects of AC in controlling browning as previous mentioned in biochemical assay. Accordingly AC was not appropriate for preventing membranous organelles from high threshold ROS levels-induced stress after extended culture resulting in the occurrence of callus browning. Although, AC has been reported to diminish the oxidative browning in many plant species such as *Strelitzia reginae* (North et al., 2012), it was effective at the contact point between wounded tissue and medium in the initial incubation.

CHAPTER 5
CONCLUSIONS

5.1 Major finding of the thesis

5.1.1 Effect of plant growth regulators on morphogenic responses

The successful formation of callus was achieved on the modified VW medium containing 0.5 mg L^{-1} NAA in combination with 1 mg L^{-1} BA. Callus originated from subepidermal cells of transversely bisected protocorm segment that divided in anticlinal orientation, then underwent peri- and anticlinal division within 4 weeks of culture. Induced callus continued to grow and gave rise to the nodular compact callus after 8 weeks of culture.

5.1.2 Effect of incubation periods on anatomical and biochemical alterations during callus culture without any subculture

Bisected *D. crumenatum* protocorm-derived callus gradually turned brown as the culture period was prolonged. Accordingly, 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. This browning crisis resulted in alterations to the shape and appearance of many cellular structures and organelles, in particular nucleus, mitochondria and chloroplast. Moreover, the changes in biochemical factors were found to be related to the occurrence of browning. For instance, an increase in PPO activity, PAL activity, MDA content and total phenolic content was observed. Meanwhile, chlorophyll and carotenoid contents were found to be decreased. Early tissue browning can also serve as a sign for the need to be subcultured, before being dead of the callus. Understanding the causes of observed changes during callus browning can provide important clues for predicting the onset of browning.

5.1.3 Effect of anti-browning agents for preventing browning problem on callus proliferation

The alleviation of callus browning in *D. crumanatum* is essential to enhance prolong subculture and regeneration capacity of callus cells. To achieve this, 0.01 g L^{-1} AA as antioxidant was proposed as the best suitable anti-browning agent to control browning problem, primarily due to the reduction of enzymatic activities of PPO and PAL, a reduction in content of total phenolic and MDA and an increase in content of chlorophyll and carotenoid. Moreover, the AA-treated callus remained vigorous and green with well-organized organelles and the characteristics of meristematic cell still present.

However, this study proved herein that absorbent (1 g L^{-1} AC) had an undesirable effect on the prevention of browning problem by causing excessive activity of oxidative enzymes and consequently increasing the production of total phenolic content, but decreasing in chlorophyll and carotenoid contents. Ultrastructural appearance also revealed the malformed organelles in AC-treated callus cell exhibiting a sign of cellular injury during culture as verified by the occurrence of ultrastructural damage and the negative result in biochemical tests.

For *D. crumenatum*, therefore, antioxidant can prevent the browning problem better than absorbent in the following order: AA>Cys>PVP>AC. Besides, the most effective browning inhibitor may depend on the several factors such as plant species and types and concentrations of anti-browning agents.

5.2 Suggestion

The well-established callus culture in orchid is still very difficult and limited only to a few species, due to the slow growth and a tendency to undergo browning. Accordingly, this present study provided valuable information for understanding the occurrence of browning and also established the effective way to prevent browning. However, further in-depth research in the precise role of browning

inhibitor, particularly AA, on browning prevention at the molecular level needs to be done.

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APPENDICES

APPENDIX A

Vacin and Went (VW) medium

Components	Quantity
Macroelements	
Potassium nitrate, KNO_3	525 mg
Potassium phosphate, KH_2PO_4	250 mg
Magnesium sulfate, $MgSO_4.7H_2O$	250 mg
Ammonium sulfate, $(NH_4)_2SO_4$	500 mg
Tricalcium phosphate, $Ca_3(PO_4)_2$	200 mg
Microelements	
Manganese sulfate, $MnSO_4.H_2O$	6.8 mg
Iron	
Ferrous sulfate, $FeSO_4.7H_2O$	27.8 mg
Disodium ethylene diaminetetraacetate, Na_2EDTA	37.3 mg
Sugar	
Sucrose	20 g

(Source: Vacin and Went, 1949)

APPENDIX B

Components of FAA II solution

Formaldehyde	5 % (v/v)
Glacial acetic acid	5 % (v/v)
70 % alcohol	90 % (v/v)

Dehydration solution

No.	Total alcohol (%)	Composition (mL)				
			Ethanol			
		TBA	95 % alcohol	100 % alcohol	Water	Other
1	5	-	5	-	95	
2	10	-	10	-	90	
3	20	-	20	-	80	
4	30	-	30	-	70	
5	50	10	40	-	50	
6	70	20	50	-	30	-
7	85	35	50	-	15	-
8	95	55	40	-	5	-
9	100	75	-	25	-	-
10	-	100	-	-	-	Eosin
11	-	100	-	-	-	-
12*	-	100	-	-	-	Paraffin oil 100

TBA: tert-butyl alcohol

* Equal of TBA:paraffin oil = 1:1

Deparaffinization process

1.	xylene substitute I	3	min
2.	xylene substitute II	3	min
3.	absolute alcohol : xylene substitute	3	min
4.	absolute alcohol I	2	min
5.	absolute alcohol II	2	min
6.	95 % alcohol I	2	min
7.	95 % alcohol II	2	min
8.	70 % alcohol I	2	min
9.	70 % alcohol II	2	min
10.	50 % alcohol I (optional)	2	min
11.	50 % alcohol II (optional)	2	min

Delafied's hematoxylin and safranin staining

Reagents:

1). Delafied's hematoxylin

Hematoxylin	8	g
95 % ethanol	250	mL
Ammonium alum	16	g
Distilled water	800	mL
Glycerin	400	mL
Potassium permanganate	0.4	g

2). Safranin O

Safranin O	2.5	g
95 % ethanol	10	mL
Distilled water	90	mL

3). 0.1 % lithium carbonate

Lithium carbonate	0.1	g
Distilled water	100	mL

4). Acidulated water

Concentrated HCl	1-2	drops
Distilled water	100	mL

Staining procedure:

1.	Deparaffinization and bring to distilled water		
2.	Stain in Delafied's hematoxylin	25	min
3.	Rinse in tap water	2	min
4.	Destain in acidulate water	6	dips
5.	Rinse with tap water	1-2	dips
6.	Place in 0.1 % lithium carbonate	2	min
7.	Rinse in tap water	1-2	dips
8.	Counterstain with safranin	3	min
9.	Wash in tap water	3-4	dips
10.	Treat with acidulate water	1-2	dips
11.	Immerse in 0.1 % lithium carbonate	2-3	sec
12.	Dehydrate through graded alcohol		
13.	Clear in Xylene		
14.	Mount in Hi-mo		

Result:

Nucleus	Blue
Cytoplasm	Light pink

1 % toluidine blue O staining

Reagents:

1 % toluidine blue O

Toluidine blue O (TBO)	1	g
Sodium borate	1	g
Distilled water	100	mL

Staining procedure:

1.	Deparaffinization and bring to distilled water		
2.	Stain in 1 % TBO	1-2	min
3.	Rinse with distilled water		
4.	Dehydrate quickly through two changes of 95 % and 100 % alcohol	10	dips each
5.	Clear in xylene		

6. Mount in Hi-mo

Result:

Nucleus	Blue
Phenolic compound	Green

Periodic acid-Schiff's reaction

Reagents:

1). 1 % periodic acid

Periodic acid	1	g
Distilled	100	mL

2). Schiff's reagent

3). Harris's hemaoxylin

Hematoxylin	5	g
Absoloute alcohol	50	mL
Aluminum Ammonium Sulfate	100	g
Distilled water	1000	mL
Mercuric oxide	2.5	g

4). 1 % acid alcohol

70 % Ethanol	99	mL
Concentrated HCl	1	mL

Staining procedure:

1.	Deparaffinization and bring to distilled water		
2.	Place section in 1 % periodic acid	15	min
3.	Wash in tab water	3	min
4.	Wash in distilled water	1	min
5.	Treat in Schiff's reagent	15	min
6.	Wash in tab water	10	min
7.	Counterstain in Harris's hematoxylin	10	min
8.	Wash in distilled water	5	min
9.	Differentiate in 1 % acid alcohol	1-2	dips
10.	Wash in tap water	1-2	min
11.	Dehydrate through graded alcohol		

12. Clear in Xylene
13. Mount in Hi-mo

Result:

Insoluble carbohydrate	Magenta
Nucleus	Blue

Scanning electron microscopy observation

Fixative reagents:

SEM fixative solution

Formaldehyde	10 % (v/v)
Acetic acid	5 % (v/v)
100% ethanol	45 % (v/v)
Triton X-100	1 % (v/v)

Transmission electron microscopy observation

Fixation reagents:

Pre-fixed solution

Glutaraldehyde	2.5 % (v/v)
Caffeine	1 % (w/v)
Phosphate buffer (pH 7.2)	0.1 M

Post-fixed solution

Osmium tetroxide	1 %
Phosphate buffer (pH 7.2)	0.2 M

APPENDIX C

1. List of solutions

1.1 Solution for extraction

Sodium phosphate buffer (pH 7.2)	0.1 M
Sodium dodecyl sulfate	0.1 % (w/v)

1.2 Solution for PPO activity assay

Sodium phosphate buffer (pH 7.2)	0.1 M
Catechol	0.5 M

1.3 Solution for PAL activity assay

Tris-HCl buffer (pH 8.5)	0.1M
β-mercaptoethanol	1 mM
L-phenylalanine	0.1 M
HCl	6 N

1.4 Solution for determination of phenolic content

Folin-ciocalteu phenol reagent	10 % (v/v)
Sodium carbonate	10 % (w/v)

1.5 Solution for determination of chlorophyll and carotenoid content

Acetone	80 % (v/v)

1.6 Solution for lipid peroxidation assay

Trichloroacetic acid (TCA)	20 % (w/v)
Thiobarbituric acid (TBA)	0.5 % (w/v)
Distilled water	100 mL

2. Preparation of 0.1 M Sodium phosphate buffer (pH 7.2)

Stock solution

1). Solution A

Sodium phosphate dibasic (Na_2HPO_4)	2.84	g
Distilled water	100	mL

2). Solution B

Sodium phosphate monobasic ($NaH_2PO_4.2H_2O$)	3.12	g
Distilled water	100	mL

0.2 M Sodium phosphate buffer

Mixing solution A with solution B

Solution A	72	mL
Solution B	28	mL

0.1 M Sodium phosphate buffer

0.2 M Sodium phosphate buffer	50	mL
Distilled water	50	mL

3. Preparation of 0.1M Tris-HCl buffer (pH 8.5)

Tris-HCl (mw = 121.14) 12.14 g was dissolved in distilled water, adjusted pH to 8.5 with 1 M HCl and adjusted to 1000 mL with distilled water.

4. Standard cure of gallic acid

Stock solution of gallic acid

800 mg L^{-1} gallic acid

Gallic acid	20	mg
0.1 M Sodium phosphate buffer	25	mL

Gallic acid stock solution was diluted to 100, 200, 300, 400 and 500 mg L^{-1} and measured using spectrophotometric method as described in 2.5.4.

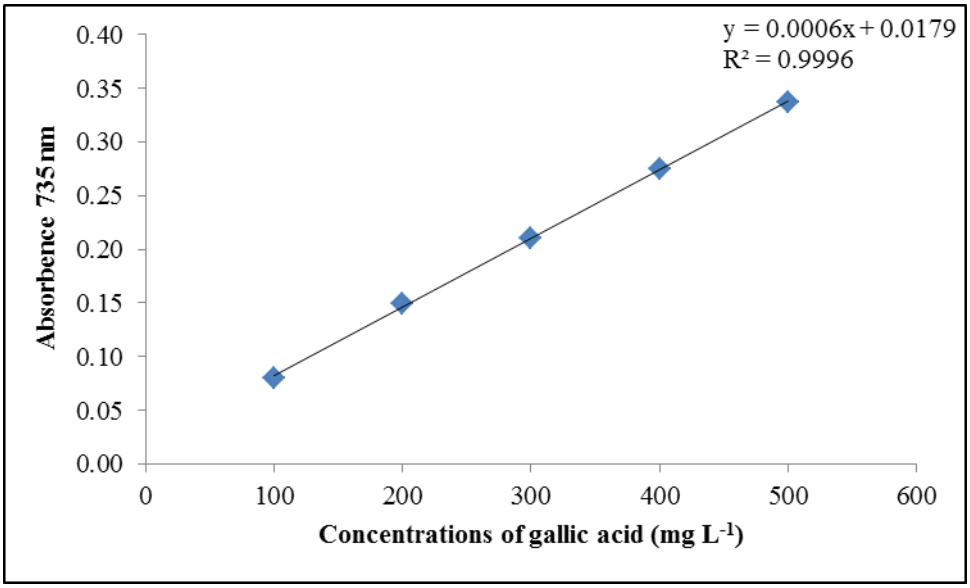

Figure 43 Standard calibration curve of gallic acid solution at absorbance 735 nm.