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Effects of Reducing Browning on the Somatic Embryogenesis of *Coffea arabica*

Jarinya Hongwiangchanand Noppamart Lokkamlue*

Department of Science and Bioinnovation, Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

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Abstract

The browning of plant tissues is a major constraint in plant tissue culture.This is especially true for embryogenic cells grown on a culture medium to produce true-to-type hybrid coffee via somatic embryogenesis. There isvery little information in the current literature of the rate of browning intensity involved the development stage of coffee callus.We investigated the use ofthe antioxidantcysteine (Cys) and activated charcoal (AC) to induce callus formation, reduce browning of the callus, and induce somatic embryos of *Coffea arabica*.Explants were cultured on MS media supplemented with Cys and AC. The study involved varying the Cys rates in the order of 30, 35, and 40 mg/L, as well as varying the AC rates in the order of 0.008%, 0.01%, and 0.08% (w/v). The statistical analysis of the results was performed using the Statistical Package for Social Sciences (SPSS) version 14, with a significance level set at $p<0.05$. Neither anti-browningagent increased the rate of callus induction.The use ofMS+1 µM 2,4-dichlorophenoxyacetic acid (2,4-D) +5 µM 6-benzyl adenine (BA) media supplemented with 30 or 35 mg/LCys or 0.008% AC reduced the rate of tissue browning and induced a high rate of somatic embryo (SE) induction (80%, 84.21%, and 65%, respectively). SEstreated with30 mg/LCys couldbe germinated to the asynchronous developmental stages within six months.They couldbe converted to mature plantlets on MS without plant growth regulator hormones. Therefore, theuse ofCys and AC could be valuable for the propagation of *Coffee arabica*L. cv. CatimorCIFC 7963.

Keywords: Activated charcoal, Antioxidants, Callus,Somatic embryo,CatimorCIFC 7963,Phenolic compounds, Leaf explant, Cystein

1. Introduction

Coffee (*Coffea arabica* L.) is one of the main agricultural exports of Thailand. Seed propagation results in uncontrolled genetic variation due to heterozygosity ofcoffee.The conventional method ofcoffee propagation,usingvegetative grafting or rooting of cuttings,produces greater genetic uniformity, but thisapproachis the high production cost of plantletsfor large-scale production (Etienne *et al.*, 2018).Somatic embryogenesis technique is used to mass-produce hundreds of thousands of hybrid plantlets that are true-to-type, reducing production costs compared to conventional methods(Landey*et al.*, 2013; Etienne *et al.*, 2016). In*in vitro* propagation, the darkening of culture medium is a problem. Darkening of the culture medium has been attributed to phenolic compounds exuded from the excised portion of explants and accumulated in the culture medium.Phenolic compoundsare secondary metabolites possessing one or more aromatic rings, with one or more hydroxyl groups. Many plants have developed defense mechanisms to protect themselves from microbial infection. One such mechanism is the production of antibacterial compounds found in phenolic compounds of medicinal plants (de León and Montesano, 2013; Al-Ghamdi *et al.*, 2020). Phenolic compounds are exuded in tissue culture protocols that involve wounding during explant transfer and culturing in stressful environments (Jones and Saxena, 2013). The medium in which explants are grown becomescolored.The browning developed during the culture of calliis due to the quinones (browning pigments), which are toxic to plant tissues leading to the death of tissue. This process involves the polyphenol oxidase (PPO) catalyzing the oxidation of phenolic compounds by oxygen to give the quinones (Schieber, 2018; Wen *et al*., 2020).PPO activity is affected bythe type and amounts of endogenous phenolic compounds, the presence of oxygen, and the pH in the plant cell (Moon *et al*., 2020). This enzyme is most abundant in young plant cells, which are removed from young mother plants for somatic embryogenesis production (Beena *et al.*, 2014; Taranto *et al*., 2017).

At present, *C. arabica*cv. Catimor CIFC 7963has been reported exhibiting the characteristics of leaf rust resistance in Thailand (Rittisang and Lokkamlue, 2020). However, coffee has a long seed–breeding cycle of up to five years and the tissue culture method allows easy maintenance of the desirable traits of coffee. The somatic embryogenesis might be the most effective technique because of its potential for producing the highest rate of multiplication. In a previous experiment, we found that most coffee calli were dark brown during culture, resulting in a 61% rate of somatic embryo (SE) induction (Rittisang

* Corresponding author. e-mail: faasnmlo@ku.ac.th.

and Lokkamlue, 2020). Therefore, phenolic compound accumulation is a serious concern for coffee cell development if a callus with minimized phenolic compound accumulation is necessary for the successful induction of coffee SE.

Most strategies for controlling tissue browning involve either the inhibition ofPPO activity orthe conversion of quinones to colorless adducts. The chemicals used to inhibit PPO activity areclassified ascompetitive inhibitors, chelating agents, or acidulants, while the antioxidants prevent the formation of browning pigment by binding to intermediates (Brütsch *et al.*, 2018).

Cysteine (2-amino-3-mercaptopropionic acid), a thiol compound,has beeninvestigated as an anti-browning agent, which reduces quinones to their phenol precursors or colorless adducts, and is dependent on environment factors (Ioannou and Ghol, 2013; Ali *et al.*, 2015; Ali *et al.*, 2016). The cysteinyl-catechol conjugate directly inhibits PPO and produces some browning inhibition by forming peptide side chains (Ali *et al.*, 2015). Therefore, cysteine may act as a PPOinhibitor, ormay react with quinonesto give a colorless adduct (Cabezas-Serrano *et al.*, 2013; Ali *et al.*, 2015). Cysteine acts as acompetitive PPO inhibitor at low concentrations, while at high concentrations it can react with the assay product, quinone, to producea colorless adduct (Ali *et al.*, 2015).

Activated charcoal (AC) is frequently added to culture medium to reduce the concentration of toxic compounds or improve the morphogenic responses of explants. The use of AC as a component of the culture medium reduces the incidence of tissue browning, which can lead to loss of explants, and its omission has been shown to seriously compromise the efficiency of the process. Furthermore, the excised explants significantly increased phenolic compoundexcretion (Jones and Saxena, 2013). ACalsoabsorbs the phenolic compounds, which initially leach into the medium from the excised portions of the explants (Sashikesh *et al.*, 2023).

Coffee contains a high concentration of phenolic compounds that cause the browning process (Ahmad *et al.*, 2013). Therefore, understanding the processes contributing to the oxidation of phenolic compounds, and the way in which these processes can be minimized when initiating explants is critical for successful induction ofsomatic embryogenesis.Gallego Rúa*et al.* (2016)found thatSEs were able to regenerate effectively in a medium-low levelof polyphenols, and polyphenols were distributed in the periphery of the embryo epidermal layer. In the non-regenerating variety, embryogenic calli were compact, and contained polyphenols randomly distributed across all tissues. Therefore, theembryogenic capacity seems to be associated with a balanced concentration and distribution of polyphenols (Gallego Rúa*et al*., 2016). At the end of the embryo induction step, high concentrations of polyphenols lead to a non-regenerating response of SEs (Gallego Rúa *et al.*, 2016). The occurrence of browning in plant tissue culture is unpredictable due to genotypic variation (Jones and Saxena, 2013).There are few reports related to *C. arabica*cv. Catimor CIFC 7963 grown in *in vitro* culture. Moreover, considering that the biochemical alterations during cell differentiation have not yet been studied, very little information is available on whether the optimum accumulation of phenolics under low light intensity is associated with the developmental stage of *C. arabica*cv. Catimor CIFC 7963. The objective of this study was to determine the concentration of AC required for the adsorption of phenolic compounds and cysteine to reduce the browning of callus in*C. arabica*cv. Catimor CIFC 7963, and evaluate its effects on callus formation and somatic embryogenesis.

2. Materials and Methods

2.1. Culture of explants oncallus induction medium

Somatic embryogenesis of *Coffea arabica*cv. Catimor CIFC 7963purchased from Chiang Mai Royal Agricultural Research Center,Chiang Mai, Thailand, was induced from second leaves derived from the apices of the coffee plant *in vitro*. The leaves were cut into 40 pieces of 1cm^2 and cultured on callus induction medium (CIM):MS medium (Calibre scientific company, Kansas, Lenexa, USA):1 µM 2,4-D(Plant research institute Inc., Delaware, USA), 5 µM BA(Plant research institute Inc, Delaware, USA)solidified with 10 mg/L gelrite(Calibre scientific company, Kansas, Lenexa, USA). Before autoclaving, the pH was adjusted to a range of 5.7 to 5.8. Six treatments were used to evaluate the effect of concentrations of cysteine(Merck KGaA, Darmstadt, Germany) and activated charcoal(Calibre scientific company, Kansas, Lenexa, USA): 30mg/LCys, 35mg/LCys, 40 mg/L Cys, 0.008% w/v AC, 0.01% w/v AC, and 0.08% w/v AC. All media were adjusted to pH 5.7 to 5.8 before autoclaving for 15 min at 394.15K. Petri dishes with 20 mL of culture media containing ten explants and five replicates were used. The cultures were incubated in the dark at 298.15K ± 275.15K and subcultured every four weeks for three months.

2.2. Evaluation of callus browning

After two months of culture, the calli were evaluated for browning using a protocol modified from Modeste *et al.* (2017). The browning intensity was assessed on an arbitrary scale of 1 to 4, where 1 = white callus without browning; 2 = 1% to 33% white callus higher than browning (low level); 3 = 34% to 66% browning callus higher than white callus (moderate to high level); 4 = 67 % to 100% browning callus (highest level). The average value of browning rate was determined as equation (1)

Average scale of browning = sum of browning rating/total number of calli (1)

2.3. Evaluation of callus and SEinduction

After two months of culturing, the calli were counted to calculate the rate of callus induction (RCI). For SE induction, the calli were subsequently cultured on CIM for one month. The rate of SE induction was determined and calculated for the rate of a somatic embryo (RSE). The RCI and RSE were calculated usingequation (1) (Modeste*et al.*, 2017), andequation (2),respectively.

RCI = (no. of the explants that induced calli/total no. of the explants cultured) × 100 (1)

RSE = (no. of the explant with SE/ total no. of the explant with callus)×100 (2)

2.4. SEdevelopment of C. arabica cv.Catimor CIFC 7963

During the development of SE, we transferred the embryogenic calli to embryogenic callus induction medium (ECIM): half-strength MS medium (1/2MS),30

mg/L sucrose (Bio Basic Inc., Toronto, Canada), 20 mg/L thymine (Ajax Finechem Pty. Ltd., Taren Point, Australia), 20 mg/L glycine (Ajax Finechem Pty. Ltd., Taren Point, Australia), 1 mg/L niacin (Ajax Finechem Pty. Ltd., Taren Point, Australia), 200 mg/L myo-inositol (Himedia Laboratories Pvt. Ltd., Maharashtra, India), 40 mg/L L-cysteine, 200 mg/L casein hydrolysate(Himedia Laboratories Pvt. Ltd., Maharashtra, India), 800 mg/L malt extract (Becton, Dickinson and Company,Sparks, MD, USA) 60 mg/L adenine sulfate (Johnson Matthey Company, Istanbul, Turkey),0.1 µM BA, and solidified with 8 mg/L gelrite, for three months.

2.5. Germination and conversion of SEs into plantlets

The six-month-old 105 cotyledonary-stage embryos were cultured on MS medium without plant growth regulators (PGRs). All cultures were placed in a growth room for six months at 298.15K±275.15Kunder light radiation of 30 µmol.m^{-2}.s^{-1} for a 16-hr photoperiod.

2.6. Statistical analysis

The results were analyzed using analysis of variance (ANOVA) with the Statistica 14.0 software. For unequal numbers, analysis of variance was performed using a generalized linear model. When a significant difference was observed between treatments, multiple range tests using one way ANOVAs at a 5% ($p<0.05$) threshold were used to separate the averages.

3. Results

3.1. Callus induction in C. arabica cv. Catimor CIFC 7963 leaf explants

One week after the leaf explant culture, cell division was induced in the leaf sectionin groups of small cells. The groups of small cells showed continuous development to callus after one month on MS media supplemented with 1 µM 2,4-D and 5 µM BA (CIM, control medium) with cysteine application of 30, 35, and 40 mg/L,and AC application of 0.008% and 0.01% (w/v).We obtained two-month-old compact and friable calli. However, it was observed that the CIM produced compact, rather than friable callus, while only CIM containing cysteine and AC improved friable callus induction (Figs. 1A–1C).

In the CIM containing 0.08% ACnone of the groups of small cells showed development to callus (Fig. 1D). The experimental values of callus induction rate,addition of cysteine, and AC, showed no significant differences among the different concentrations or the CIM, but did have a significant effect on the 0.08% AC (Table 1).Increasing the concentration of AC beyond 0.01% resulted in decreased callus formation. AC appears to eliminate both undesirable compounds, thereby facilitating adsorption of hormones through the culture media. The highest induction rate (100%) was significantly improved over the rate derived from different media (37.5%) (Table 1).

Table 1. Effect of cysteine and AC on two-month-old callus in *C. arabica*cv.Catimor CIFC 7963 leaf explants

Supplements	Concentration	Rates of callus induction (%RCI)
Control(CIM)	0	100[a]
Cysteine	30 mg/L	100[a]
	35 mg/L	100[a]
	40 mg/L	100[a]
AC	0.008%	100[a]
	0.01%	100[a]
	0.08%	37.5[b*]

Note: * Groups of small cells. [a,b] represent differences among treatment means within the same column ($p<0.05$).

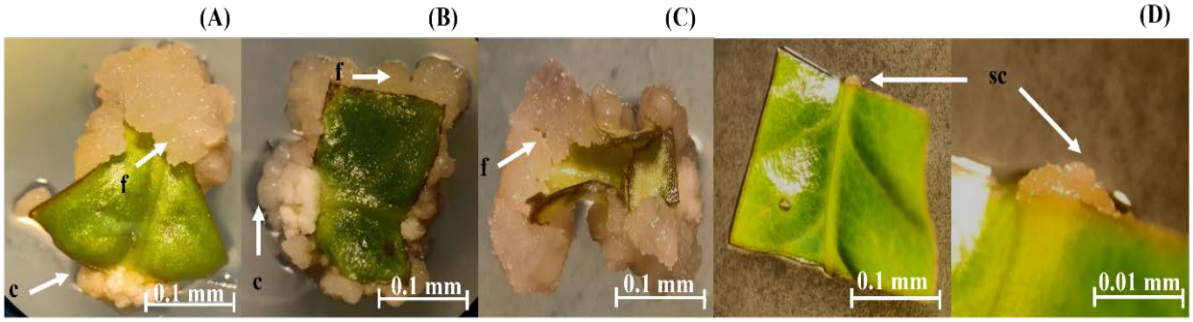

Figure1. Friable callus (f), compact callus (c) and groups of small cells (sc) in *C. arabica*cv. Catimor CIFC 7963leaf explants on media after two months. (A) CIM (B) CIM + 30 mg/L cysteine (C) CIM + 0.008% AC (D) CIM + 0.08% AC.

3.2. Tissue browning intensity in C. arabica cv. Catimor CIFC 7963callus from leaf explants

All calli were cultured on media for two to three months. At the end of the second month, some yellowish calli hada brownish color,and green colored leaves presented as yellowish to brownish in the different types and concentrations of supplements (Fig. 2). The scale of browning of tissue was classified as 1 to 4, as described in Fig. 2. The reduction of callus browning depended on the type and the concentration of supplements. We found that the two-month-old calli derived from *C. arabica*cv.Catimor CIFC 7963leaf explants produced on CIM containing no supplements exhibited the highest intensity of browning, and thecallus browning rate was recorded as3 on the scale discussed above. Concentrations of30 and 35 mg/L cysteine and 0.008% AC produceda significant reduction of the callus browning rate, to scale 2.Among the concentrations of cysteine used,40 mg/L resulted in an increase of callus browning intensity to scale 3. Similarly, the increased concentration of AC from0.01% to 0.08%, had no effect on the reduction of browning callus. CIM containing 0.08% AC had the highest effect on the calli, reaching a scale of 4 (Table 2). High phenolic compounds may minimize the growth and stop embryonic development in non-differentiating callus. The number of

declining and dying cells increased in parallel with the increase in browning.

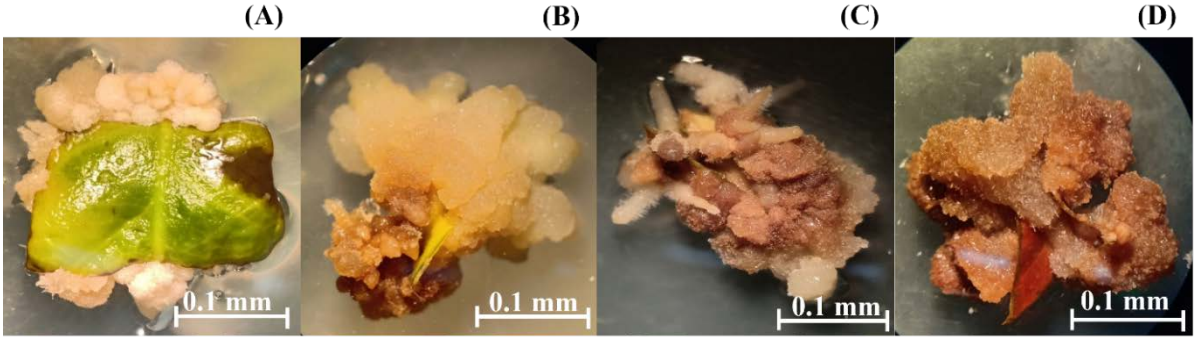

Figure 2. Two-month-old calli of *C. arabica*cv. Catimor CIFC 7963 leaf used for browning intensity assessment. (A) Without browning. (B) Browning intensities 1% to 33%. (C) Browning intensities 34% to 66%. (D) Browning intensities 67% to 100%.

Table 2. Browning intensity of two-month-old callus in *C. arabica*cv. Catimor CIFC 7963 leaf explants

Supplements	Concentration	Callus browning scale*
Control(CIM)	0	3
Cysteine	30 mg/L	2
	35 mg/L	2
	40 mg/L	3
AC	0.008%	2
	0.01%	3
	0.08%	4

*In column three, 2 corresponds to calli with 1% to 33% browning, 3 corresponds to calli with 34% to 66% browning, and 4 corresponds to calli with 67% to 100 % browning.

3.3. Effect of cysteine and AC on SEinduction in C. arabica cv. Catimor CIFC 7963calli

Three months after explant culture, the induction of SEs was observed on CIM supplemented with anti-browning agents, followed by subsequent development of SEs on the ECIM**.** The results showed that after three to six months of culture, the SEs were characterized by asynchronous developmental stages (globular, heart, torpedo, and cotyledonary stages) and some calliproduced roots(Figs. 3A and 3B). The highest percentage of SE induction (84.21%) was observed on CIM supplemented with 35 mg/L cysteine,followed by CIM supplemented with 30 mg/L cysteine (80%). However, there was no significant difference in the RSE (Table 3). CIM and CIM supplementedwith0.008% AC also produced SE, but did inducelower percentages of RSE, at 61.54 and 65, respectively.AC appears to have been included as a component of the culture medium to prevent high incidences of tissue browning, which consequently leads to loss of cells in callus. Furthermore, it was shown that its omission mainly compromised the efficiency of the SE process. Increased concentrations of cysteine beyond 40 mg/L cysteine and 0.01% AC decreased the percentage of RSE (Table 3).We found that only SEsfrom CIM supplementedwith30 mg/L cysteine developed into differential sizes of cotyledonary embryos on ECIM after six months (Fig. 1B). The six-month-old cotyledonary embryos germinated, and 50 out of 105embryos produced roots on MS without PGRs within one month (Fig. 1C), and then produced true leaves (Fig. 1D). We found that about half of the embryos lacked roots. This result indicated that most cotyledonary embryos were monopolar.

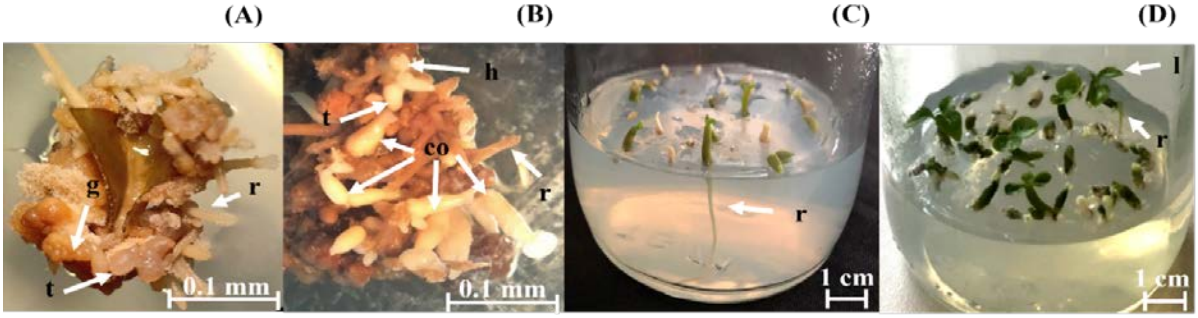

Figure 3. Somatic embryogenesis of *C. arabica*cv. Catimor CIFC 7963 embryogenic calli with roots (r). (A) stages of embryo: globular (g) and torpedo (t) embryos at three months. (B) Development of somatic embryos: torpedo (t), hart (h), and cotyledonary (co) embryos at six months. (C) Germination of six-month-old cotyledonary embryos at one month. (D) Regenerated plantlet with root and leaf (l) at two months.

Table 3. Rate of somatic embryo induction in *C. arabica*cv. Catimor CIFC 7963 calli at three months

Supplements	Concentration	Rate of somatic embryo (% RSE)
Control(CIM)	0	61.54^{ab}
Cysteine	30 mg/L	80^{a}
	35 mg/L	84.21^{a}
	40 mg/L	51.28^{b}
AC	0.008%	65^{ab}
	0.01%	12.5^{c}
	0.08%	0^{c}

Note: a,ab,b,c represent differences among treatment means within the same column ($p < 0.05$).

3.4. Conversion of SEs into plantlets in C. arabica cv. Catimor CIFC 7963

In the present study, six-month-old cotyledonary embryos were converted into mature plantlets on MS medium without PGRs after six months. Our results showed that*C. arabica*cv. Catimor CIFC 7963mature plantlets had well-developed shoot and root systems,and showed normal leaves(Fig. 4A). The plantlets showed varied growth due to the size of the cotyledonary embryos.The plantlets were cultured for a further six months, and the 12-month-old plantlets showed different developmental stages (Fig. 4B).

Figure 4. Mature plants of *C. arabica*cv. Catimor CIFC 7963 on MS medium from six-month-old cotyledonary embryos. (A) At six months. (B) At 12 months.

4. Discussion

We investigated total friable callus induction on culture medium with 0.008% AC. Adding 0.08% AC to the medium did not significantly promote the formation of callus. The addition of 0.01 % to 0.08% AC decreased the number of SEs. It seems that the effect of AC is mainly due to the adsorption of substances such as 2,4-D and BA from the medium. Although AC has been reported to have a positive effect in other works (Abdulwahed, 2013), its presence may reduce the effectiveness of the medium for *in vitro* growth of the jojoba plant (*Simmondsia chinensis* L.) (Hassanein *et al.*, 2015).

There are also other factors that influence the SE response to AC concentration, such as plant species, cell density, cell cluster size, and cell lines used that modify the effect of AC adsorbing nutrient components from the culture medium (Das and Srivastav, 2015). However, AC also adsorbs high phenolic compounds (Gholizadeh *et al.*, 2013) produced by plant tissues that may inhibit embryogenesis and further embryo development. These results indicated that the embryogenesis of *C. arabica*cv. Catimor CIFC 7963was dependent on the AC concentration in the medium.

We found both compact and friable callus induction on culture medium with cysteine. The culture medium supplemented with 35 mg/L cysteine had abalanced phenolic compound concentration,and produced thehighest number of SEs. The total phenolic compound content was found to be highest in light roasted arabica coffee beans (de Souza *et al.*, 2020). However, the phenolic compounds produced from young leaf tissue showed browning of the callus, and the different stages of leaves showed significant differences in total phenolic compounds (Chen *et al.*, 2018; Ngamsuk *et al.*, 2019).The possible role of these compounds in plant improvement via somatic embryogenesis has been examined in various plants, such as *Theobroma cacao*L. (Modeste *et al.*, 2017)and *Salacca sumatrana* Becc. (Elimasni *et al.*, 2020).The differential accumulation of phenolic compounds, in the culture medium of *C. arabica* could be a major cause of the interruption to the cell division pattern, affecting cellular proliferation and the process of somatic embryogenesis (Nic-Can *et al.*, 2015).Thebalanced concentration and distribution of polyphenols induces embryogenesis (Gallego Rúa *et al.*, 2016). Therefore, the reduced browning of coffee callus resulted from the secretion of phenolic compounds in scale 2 (callus with 1% to 33% of browning) affectedSE induction (Tables 2 and 3). This result is consistent with those of Modeste*et al*. (2017),who reported that cocoa (*Theobroma cacao* L.) callus could be derived frompetals. They also observed that somatic embryogenesis was more successful when calli were cultured in media supplemented with 8, 16, or24 mg/L cysteine.We found that the browning rate of coffee callus treated with 35mg/L cysteine appeared to reduce in comparison with that of the untreated control. Therefore, although the effect of 35mg/L cysteine may be inhibiting the browning reaction caused by the formation of a colorless compound, it did not involve a direct inhibition on the PPO active site. Consistent with the report, cysteine's nucleophilic reactivity towards quinones inhibits enzymatic browning reactions, resulting in colorless products (Ali *et al.*, 2015). Furthermore, in the previous experiment, we discovered that secondary SEs developed from primary SEs. This is a valuable technique for regenerating embryo cells during plant gene transformation (Rittisang and Lokkamlue, 2020). During the gene transformation process in plant cells, wounding

occurs which leads to exudation of phenolic compounds. This phenomenon is a concern for transgenic cell development as accumulation of phenolic compounds can hinder the successful induction of transgenic coffee SEs and further study of genes involved in SE. Therefore, it is important to minimize phenolic compound accumulation in callus during the gene transformation protocol to achieve successful transgenic SE induction.

We found that only treatment with 30 mg/L cysteine resulted in the conversion of SEs to plantlets. We found that half of the embryos lacked roots, a lower rate than the two-thirds found in previous research (Rittisang and Lokkamlue, 2020). We should further culture these embryos on a suitable rooting medium to produce more coffee plants. The rooting process of SEs in *C. arabica* cv. Catimor CIFC 7963 can be improved by using an optimum concentration of cysteine. The conversion of six-month-old cotyledonary embryos into mature plantlets takes about six months, which is in line with previous research that also found a conversion time of around seven months (Rittisang and Lokkamlue, 2020).We identified different developmental stages of plantlets, with more leave and roots, after culture at 12 months as all embryonic stages were observed simultaneously in the same callus since the differentiation occurred asynchronously (Figs. 3A and 3B). This result suggested that plantlets with well-developed leaves and roots had more efficient photosynthesis and a better supply of water and nutrients from the environment under such drought conditions(Yao*et al.*, 2017; Saghaiesh and Souri, 2018).

5. Conclusion

The addition of the anti-browning agents cysteine and ACtoMS + 1 µM 2,4-D + 5 µMBA medium had no significant effect on callus induction in*C. arabica*cv. Catimor CIFC 7963.Callus browning was reduced in the presence of anti-browning agents, to different extents. The best results for reducing the browning intensity of callus at scale 2 (amount of brown callus< that of white callus) were obtained at concentrations of cysteine of30 or 35 mg/L and 0.008% AC. This intensity level of browning releases a balanced amount of phenolic compounds, promoting somatic embryogenesis. Moreover, treatment with 30 mg/L cysteine improved the rate of somatic embryogenesis and produced embryos of different stages.This protocol could improve*in vitro* propagation and reduce the cost of producing*C. arabica*cv. Catimor CIFC 7963plantlets by increasing the reliability of the rooting process in coffee SEs without rooting hormones on culture medium.

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