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THIDIAZURON ENHANCED SOMATIC EMBRYOGENESIS FROM CALLUS LINES OF ARABICA COFFEE AND SUBSEQUENT PLANT REGENERATION

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An efficient system of micropropagation via somatic embryogenesis from root-derived callus was established in Arabica coffee (*Coffea arabica* L.). Twenty-six callus lines were induced on MS (Murashige and Skoog, 1962) medium supplemented with combinations of NAA (0, 0.1, 0.5, 1 and 2 mg/L) plus BA (0, 1 and 2 mg/L), or 2,4-D (0, 0.1, 0.5, 1 and 2 mg/L) plus TDZ (0, 1 and 2 mg/L). Subsequently, two types of somatic embryos were obtained from callus cultures and named S-type and I-type embryos. The S-type embryos were obtained from an 18-month-old callus line which was induced and maintained at 2 mg/L TDZ and 0.1 mg/L 2,4-D near the end of each period of the subculture. These embryos have a developmental barrier, which did not pass through the torpedo stage and could be overcome by a supplement of 2 or 5 mg/L BA. The I-type embryos were induced from 3-month-old callus when transferred onto induction media, i.e., MS supplemented with TDZ (2 and 5 mg/L) plus 2,4-D (0 and 0.1 mg/L). The significantly highest response, i.e., 13.3 embryos per callus clump was obtained at 2 mg/L TDZ. In this study, the results reveal that TDZ has a crucial effect on embryogenic callus induction, proliferation and subsequent somatic embryogenesis.

Keywords: callus, developmental barrier, embryogenic capacity, somatic embryo

Abbreviations:
2,4-D – 2,4-Dichlorophenoxyacetic acid
BA – N^6-benzyladenine
IBA – Indole-3-butyric acid
MS medium – Murashige and Skoog (1962) medium
NAA – 1-Naphthaleneacetic acid
PGRs – Plant growth regulators
TDZ – 1-Phenyl-3-(1,2,3-thiadiazol-5-yl)-urea, thidiazuron

INTRODUCTION

Coffee is a popular drink that is prepared from roasted beans from plants which belong to the *Coffea* genus (De Los Santos-Briones and Hernández-Sotomayor, 2006). There are more than 100 species in this genus, and one of the most commercially important species is Arabica coffee (*C. arabica* L.) (Tornincasa et al., 2010). Coffee plants are cultivated primarily in the equatorial regions, and coffee beans are the top agricultural export for many countries around the world (Ahmed et al., 2013). The importance of coffee plants has led to studies on the tissue culture techniques and the requirements of *in vitro* morphogenesis, i.e., organogenesis and somatic embryogenesis from several types of explants, for improvement of varieties through genetic engineering and also for mass propagation of the elite plants

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(Berthouly et al., 1999; Fuentes-Cerda et al., 2001; Giridhar et al., 2004a; Samson et al., 2006; De-la-Peña et al., 2008; Etienne et al., 2013; Ibrahim et al., 2013). One of the most powerful techniques is production of a whole plant from a somatic cell via a process of somatic embryogenesis (SE) (Thorpe, 1994; Zimmerman, 1993; Doderman et al., 1997; Von Arnold et al., 2005). In *Coffea* genus, many *in vitro* protocols have been developed for inducing SE via direct or indirect pathways (Neuenschwabder and Baumann, 1992; Tahara et al., 1994; Quiroz-Figueroa et al., 2002; Giridhar et al., 2004b; Papanastasiou et al., 2008; Nic-Can et al., 2013). However, limited success has been achieved in direct SE of *C. arabica*, and it has been documented as a poorly direct embryogenic species (Nic-Can et al., 2015). Besides, although several reports have been proposed for indirect SE of *C. arabica*, the regeneration capacity of long-term callus and the stability of SE-derived plantlets have not been fully proven (Hermann and Hass, 1975; Yasuda et al., 1985; Papanastasiou et al., 2008).

It has long been demonstrated that the ratio between auxin and cytokinin plays a crucial regulatory role in plant morphogenesis and development *in vivo* and *in vitro* (Skoog and Miller, 1957; Smigocki and Owens, 1989; Thorpe, 1994). It was reported that thidiazuron (TDZ) has been considered a multidimensional PGR (Murthy et al., 1998), and it may have both auxin and cytokinin effects (Guo et al., 2011). In tissue culture of many plant species, the application of TDZ could induce a diverse array of *in vitro* morphogenesis, including callogenesis, organogenesis and somatic embryogenesis (Murthy et al., 1998; Chen, 2012; Lee and Chen, 2014; Tsai et al., 2016).

The aim of this study was to establish a reliable system for inducing dedifferentiation of explants, subsequent maintaining of the long-term embryogenic callus and eventually obtaining viable SE-derived plantlets of *C. arabica*. Based on our knowledge, this study is the first to analyze the effects of TDZ, an effective and multidimensional plant growth regulator, throughout the whole regeneration pathway, i.e., callus induction, somatic embryogenesis, growth and development of somatic embryos in this species.

MATERIALS AND METHODS

PLANT MATERIALS

The seeds of *Coffea arabica* L. were purchased from a local farm located in Datun Mountain (Taipei, Taiwan). These seeds were wiped with 70% ethanol, followed by agitation for 10 min in a solution of 0.5% sodium hypochlorite with several drops of Tween 20. After washing with distilled water three times, the seeds were sown on a MS (Murashige and Skoog, 1962) basal medium supplemented with 30 g/L sucrose and 3 g/L Gelrite. The culture containers were test tubes (150 mm × 25 mm), and each contained 40 ml of the medium. The pH of the media was adjusted to 5.7 with 1M KOH or HCl prior to autoclaving for 20 min at 121°C. The cultures were incubated in a growth chamber with a 16/8 h (light/dark) photoperiod at an irradiance of 42–55 μmol m^{-2} s^{-1} (daylight fluorescent tubes FL-20BR/18, 18 W, China Electric Co., Taipei, Taiwan) and a temperature of 25 ± 2°C.

CALLUS INDUCTION AND PROLIFERATION

One-cm-long root explants harvested from 3-month-old seedlings were used in the experiment. To induce callus formation, the explants were cultured on an MS basal medium as mentioned above and supplemented with combinations of NAA (0, 0.1, 0.5, 1 and 2 mg/L) plus BA (0, 1 and 2 mg/L), or 2,4-D (0, 0.1, 0.5, 1 and 2 mg/L) plus TDZ (0, 1 and 2 mg/L). The plant growth regulators (PGRs) were added prior to autoclaving. The explants were cultured on the surface of the media in darkness and at 25 ± 2°C. The culture containers were test tubes (150 mm × 20 mm), and each contained 10 ml of the medium. After three months of culture, the parent explants were excised. Proliferation of the callus was achieved by subculturing each callus line in darkness using the same combinations of PGRs with a 1-month-long interval. The proliferation rate of the callus was calculated as the ratio of the final fresh mass to the initial fresh mass following 1 month of culture. Five replicates (each contained one callus clump) were used for each treatment. The callus lines were referred to as B_xN_y (B_x means BA at x mg/L, N_y means NAA at y mg/L, respectively) or T_xD_y (T_x means TDZ at x mg/L, D_y means 2,4-D at y mg/L, respectively).

INDUCTION OF SOMATIC EMBRYOGENESIS FROM CALLUS

Three groups of callus were used according to their texture, including soft to friable callus (lines B_0N_2, B_1N_2, $T_0D_{0.5}$, T_0D_1 and T_0D_2), granular callus (line B_0N_1) and compact callus (lines $B_0N_{0.5}$, B_2N_1, B_2N_2, T_1D_1, $T_2D_{0.1}$ and $T_2D_{0.5}$) to test their capacity of somatic embryogenesis. Three-month-old callus was cut into small clumps (about 0.1 g) and cultured on the basal medium supplemented with TDZ (2 and 5 mg/L) plus 2,4-D (0 and 0.1 mg/L). The culture condition for inducing somatic embryogenesis was in darkness and at

22 ± 1°C. The culture containers were test tubes (150 mm × 20 mm), and each contained 10 ml of the medium. Four replicates (each contained one callus clump) were used for each treatment.

DEVELOPMENT OF SOMATIC EMBRYOS

Globular stage embryos from 3-month-old and 18-month-old callus line $T_2D_{0.1}$ were used to test their capacity to develop further at 2 and 5 mg/L BA or TDZ. The culture containers were test tubes (150 mm × 20 mm), and each contained 10 ml of the medium. The pH of the media was 5.7. The cultures were incubated in a growth chamber with a 16/8 h (light/dark) photoperiod at an irradiance of 42–55 μmol m^{-2} s^{-1} and a temperature of 22 ± 1°C. Five replicates (each contained four embryos) were used for each treatment.

PLANTLET CONVERSION FROM SOMATIC EMBRYOS AND ACCLIMATIZATION

Cotyledonary stage embryos from 3-month-old callus line $T_2D_{0.1}$ were used to test their capacity to convert into plantlets at 0, 0.1, 0.5 and 1 mg/L IBA. Except for the PGRs, the culture condition was the same as mentioned above. Four replicates (each contained one embryo) were used for each treatment. Six-month-old plantlets were transplanted into 3-in. plastic pots with a mixture of peat moss and vermiculite (1:1) for acclimatization in a shaded house.

STATISTICAL ANALYSIS

All the experiments were designed with a randomized complete block design, and each treatment contained at least 4 replicates. Analysis of variance (ANOVA) was used for data evaluation. The significant differences among the treatments were compared using the Duncan multiple range test (Duncan, 1955) with a 0.05 level of probability.

RESULTS

EFFECTS OF BA AND NAA ON CALLUS INDUCTION AND MAINTENANCE

No callus could be obtained from explants at PGR-free medium or at BA-containing medium (Table 1). By contrast, callus was formed in the presence of NAA or at combinations of NAA plus BA (Table 1). Twelve lines of callus were selected for subculturing, and the proliferation rate was from 4.7 to 10.8 after 3 month of culture (Table 1). During subculture, three groups of callus were indentified according to their color and texture, white to yellow, granular structures (including lines $B_0N_{0.1}$, B_0N_1 and $B_2N_{0.1}$), white to yellow or yellow, compact masses (including lines $B_0N_{0.5}$, $B_1N_{0.1}$, $B_1N_{0.5}$, B_1N_1, $B_2N_{0.5}$, B_2N_1 and B_2N_2), transparent to white, soft to friable textures (including lines B_0N_2 and B_1N_2). After 18 months of long-term subculture, the viability of callus was apparently reduced and its appearance, i.e., color and texture, was altered (Table 1). There were only 6 lines of callus which could be maintained for 18 months, and their proliferation rate was 0.2 to 3.1 (Table 1). During 18 months of subculture or when transferred onto media supplemented with TDZ (2 and 5 mg/L) plus 2,4-D (0 and 0.1 mg/L), none of the callus lines could form somatic embryos (data not shown).

EFFECTS OF TDZ AND 2,4-D ON CALLUS INDUCTION AND MAINTENANCE

Except for the PGR-free medium, all combinations of TDZ and 2,4-D induced visible callus. However, callus could not be proliferated at four combinations, including the medium supplemented with the lowest dosage of 2,4-D (i.e., 0.1 mg/L 2,4-D), the media supplemented with TDZ alone (i.e., 1 mg/L TDZ, 2 mg/L TDZ) and the medium supplemented with a combination of 2,4-D and TDZ (i.e., 0.1 mg/L 2,4-D plus 1 mg/L TDZ) (Table 2). By contrast, 10 callus lines could be proliferated in the presence of 2,4-D or combined with TDZ (Table 2). The proliferation rate of these callus lines was 3.5 to 5.4 (Table 2). During subculture, three groups of callus were indentified according to their color and texture, transparent to white, soft to friable texture (including lines $T_0D_{0.5}$, T_0D_1 and T_0D_2), light yellow, soft texture (including lines $T_1D_{0.5}$), yellow, compact masses (including lines T_1D_1, T_1D_2, $T_2D_{0.1}$, $T_2D_{0.5}$, T_2D_1 and T_2D_2). After 18 months of long-term subculture, only 6 lines of callus, including $T_0D_{0.5}$, T_0D_1, T_0D_2, T_1D_1, $T_2D_{0.1}$ and $T_2D_{0.5}$, could be maintained (Table 2). The significantly lowest proliferation rate was found in callus lines T_1D_1, $T_2D_{0.1}$, and $T_2D_{0.5}$ (Table 2). During subculture, only line $T_2D_{0.1}$ callus could form somatic embryos (Table 2). After 18 months of subculture, the appearance of line $T_2D_{0.1}$ callus which was yellow initially turned into light to dark brown.

INDUCTION OF INDIRECT SOMATIC EMBRYOGENESIS

Two types, S-type and I-type, of somatic embryos were obtained in this study. The S-type embryos was derived from long-term line $T_2D_{0.1}$ callus near the end of each period of subculture, and each 0.1 g callus clump produced 12.6 embryos (data not shown). The formation of S-type embryos on the long-term callus directly caused a large decrease of the callus. It was found that most of the callus underwent redifferentiation and did not proliferate any more.

TABLE 1. Effects of BA and NAA on callus induction, proliferation and morphology of *Coffea arabica*.

PGRs (mg/L)		Callus formation	3-month-old callus		18-month-old callus	
BA	NAA		Proliferation rate**	Characteristics	Proliferation rate	Characteristics
0	0	–*	–	–	–	–
0	0.1	+ (line $B_0N_{0.1}$)	6.1 de***	White to yellow, granular structure	–	Necrotic
0	0.5	+ (line $B_0N_{0.5}$)	8.7 b	Yellow, compact masses	1.1 ab	Yellow, compact masses
0	1	+ (line B_0N_1)	6.4 cde	White to yellow, granular structure	2.3 ab	Yellow, granular structure
0	2	+ (line B_0N_2)	4.7 e	Transparent to white, soft to friable texture	1.1 ab	Yellow, compact masses
1	0	–	–	–	–	–
1	0.1	+ (line $B_1N_{0.1}$)	8.2 bc	White to yellow, compact masses	–	Necrotic
1	0.5	+ (line $B_1N_{0.5}$)	9.2 ab	Yellow, compact masses	–	Necrotic
1	1	+ (line B_1N_1)	8.1 bc	White to yellow, compact masses	–	Necrotic
1	2	+ (line B_1N_2)	5.8 de	Transparent to white, soft to friable texture	3.2 a	Light brown, soft texture
2	0	–	–	–	–	–
2	0.1	+ (line $B_2N_{0.1}$)	6.4 cde	White to yellow, granular structure	–	–
2	0.5	+ (line $B_2N_{0.5}$)	10.6 a	Yellow and compact masses	–	–
2	1	+ (line B_2N_1)	10.8 a	Yellow, compact masses	0.2 b	Dark brown, soft texture, partial necrotic
2	2	+ (line B_2N_2)	10.5 a	Yellow, compact masses	3.1 a	Light brown, soft texture

* "+" explants with viable callus, "–" explants with no viable callus

** The proliferation rate of callus was calculated as the ratio of the final fresh mass to the initial fresh mass after one month of culture.

*** Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

The I-type embryos were obtained from the short-term callus, i.e., 3-month-old, when transferred onto an induction medium. When all the callus lines were transferred onto TDZ and 2,4-D-containing medium, only lines $T_2D_{0.1}$ and $T_2D_{0.5}$ could form somatic embryos which were named the I-type embryos (Table 3). Three textures of callus were used to induce formation of the I-type embryos, including soft to friable callus (lines B_0N_2, B_1N_2, $T_0D_{0.5}$, T_0D_1 and T_0D_2), granular callus (line B_0N_1) and compact callus (lines $B_0N_{0.5}$, B_2N_1, B_2N_2, T_1D_1, $T_2D_{0.1}$ and $T_2D_{0.5}$), but only two lines of compact callus ($T_2D_{0.1}$ and $T_2D_{0.5}$) resulted in somatic embryogenesis. The significantly highest response was obtained at 2 mg/L TDZ, and an average of 13.3 embryos could be obtained from a callus clump of line $T_2D_{0.1}$ (Table 3). In the presence of 0.1 mg/L 2,4-D or 5 mg/L TDZ, the embryogenic response of line $T_2D_{0.1}$ callus was significantly retarded and no embryos were obtained (Table 3). Except for 5 mg/L TDZ, the embryogenic capacity of line $T_2D_{0.1}$ callus was

TABLE 2. Effects of TDZ and 2,4-D on callus induction, proliferation and morphology of *Coffea arabica*.

PGRs (mg/L)		Callus formation	3-month-old callus		18-month-old callus	
TDZ	2,4-D		Proliferation rate**	Characteristics	Proliferation rate	Characteristics
0	0	–*	–	–	–	–
0	0.1	+ (line $T_0D_{0.1}$)	–	Necrotic	–	–
0	0.5	+ (line $T_0D_{0.5}$)	5.4 a	Transparent to white, soft to friable texture	3.5 b	Yellow, soft to friable texture
0	1	+ (line T_0D_1)	4.3 bc	Transparent to white, soft to friable texture	6.8 a	Transparent to white, soft to friable texture
0	2	+ (line T_0D_2)	3.8 c	Transparent to white, soft to friable texture	5.4 a	Transparent to white, soft to friable texture
1	0	+ (line T_1D_0)	–	Necrotic	–	–
1	0.1	+ (line $T_1D_{0.1}$)	–	Necrotic	–	–
1	0.5	+ (line $T_1D_{0.5}$)	4.8 b	Light yellow, soft texture	–	Necrotic
1	1	+ (line T_1D_1)	4.7 b	Yellow, compact masses	1.1 c	Yellow, soft texture
1	2	+ (line T_1D_2)	4.3 bc	Yellow, compact masses	–	Necrotic
2	0	+ (line T_2D_0)	–	Necrotic	–	–
2	0.1	+ (line $T_2D_{0.1}$)	3.8 c	Yellow, compact masses	0.2 c	Light to dark brown, compact masses, formed somatic embryos
2	0.5	+ (line $T_2D_{0.5}$)	3.6 c	Yellow, compact masses	0.2 c	Light to dark brown, compact masses, partial necrotic
2	1	+ (line T_2D_1)	3.5 c	Yellow, compact masses	–	Necrotic
2	2	+ (line T_2D_2)	3.6 c	Yellow, compact masses	–	Necrotic

* "+" explants with viable callus, "–" explants with no viable callus

** The proliferation rate of callus was calculated as the ratio of the final fresh mass to the initial fresh mass after one month of culture.

*** Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

significantly higher than line $T_2D_{0.5}$ callus in the same PGR treatment (Table 3).

DEVELOPMENT OF SOMATIC EMBRYOS

The S-type embryos have a developmental barrier, i.e., the globular stage embryos developed into torpedo stage embryos but could not develop further. In the presence of 2 and 5 mg/L TDZ for one month of culture, all the torpedo stage embryos remained their state and no embryos developed into the next stage, i.e., cotyledonary stage (Table 4). By contrast, in the presence of 2 and 5 mg/L BA, the globular stage embryos successfully developed into cotyledonary stage embryos; the success rate was 100% (Table 4).

PLANTLET CONVERSION AND ACCLIMATIZATION

When the cotyledonary stage embryos were transferred onto IBA-containing medium, well-rooted plantlets were obtained (Table 5). In the

TABLE 3. Effects of TDZ and 2,4-D on somatic embryogenesis from the 3-month-old callus of *Coffea arabica*.

Callus lines	PGRs for inducing somatic embryogenesis (mg/L)		No. of somatic embryos per callus (0.1 g)
	TDZ	2,4-D	
$T_2D_{0.1}$	2	0	13.3 a
	2	0.1	4.3 b
	5	0	3.0 c
	5	0.1	2.0 c
$T_2D_{0.5}$	2	0	2.5 c
	2	0.1	0 d
	5	0	3.3 bc
	5	0.1	0 d

Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

TABLE 4. Effects of BA and TDZ on growth and development of globular stage embryos (GSEs) derived from 3-month-old and 18-month-old line $T_2D_{0.1}$ callus of *Coffea arabica* after one month of culture.

BA (mg/L)	TDZ (mg/L)	Formation of torpedo stage embryos (%)		Formation of cotyledonary stage embryos (%)	
		GSEs from 3-month-old callus	GSEs from 18-month-old callus	GSEs from 3-month-old callus	GSEs from 18-month-old callus
2	0	0 a	0 b	100 a	100 a
5	0	0 a	0 b	100 a	100 a
0	2	0 a	100 a	100 a	0 b
0	5	0 a	100 a	100 a	0 b

Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

TABLE 5. Effect of IBA on growth and development of cotyledonary stage embryos derived from 3-month-old line $T_2D_{0.1}$ callus of *Coffea arabica* after two months of culture.

IBA (mg/L)	No. of leaves per plantlet	No. of roots per plantlet
0	2.5 b	0.8 c
0.1	4.0 a	3.3 a
0.5	3.5 ab	2.3 b
1	3.5 ab	2.5 ab

Means within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

presence of 0.1 mg/L IBA for two months of culture, an average of 4.0 leaves and 3.3 roots were produced per plantlet (Table 5). Twenty plantlets were used for acclimatization, and it was found that all the plants grew well with normal morphology and resulted in 100% survival rate after 2 months of culture in plastic pots.

THE REGENERATION PATHWAY

The typical embryogenic callus showed yellow and compact masses initially and maintained the appearance during the period of a short-term subculture (Fig. 1a). After a long-term subculture for 18 months, the callus gradually turned into light to dark brown in color, and somatic embryos formed spontaneously near the end of each interval of subculture (Fig. 1b). The embryos developed further following the typical progress of somatic embryogenesis in dicot plants, i.e., globular stage (Fig. 1c), heart stage (Fig. 1c), torpedo stage (Fig. 1c), cotyledonary stage (Fig. 1d) and eventually plantlets (Fig. 1e).

DISCUSSION

In a previous report, *C. arabica* leaf explants cultured on medium with 5 μM BA formed white friable callus and subsequently formed somatic embryos which proved that the long-term callus still has the ability to produce somatic embryos (Yasuda et al., 1985). However, dealing with the root explants in this study, BA could not be the sole plant growth regulator to induce embryogenic callus as well as the subsequent embryo induction.

In *Coffea* spp., TDZ could induce the explants to form somatic embryogenesis directly (Giridhar et al., 2004c; Ibrahim et al., 2013). However, Arabica coffee has been considered a poorly direct embryogenic species (Ibrahim et al., 2013; Nic-Can et al., 2015). Giridhar et al. (2003c) reported that TDZ induced direct somatic embryogenesis and subsequent secondary embryogenesis from the cotyledon leaf, the first leaf and stalk segments of *C. arabica* and *C. canephora*. However, in this study, TDZ could not induce root explants of *C. arabica* to directly form somatic embryos. It is suggested that the ability to directly form somatic embryos is highly affected by the genotype and explant type. In *C. arabica*, TDZ gave a positive effect on callus induction when combined with 2,4-D and also played a key role in subsequent embryo induction.

In the presence of an adequate dosage of NAA or kinetin plus IBA, the embryogenic callus of *C. arabica* was white to yellow and showed friable texture (Ahmed et al., 2013). At 0.5–2 mg/L 2,4-D,

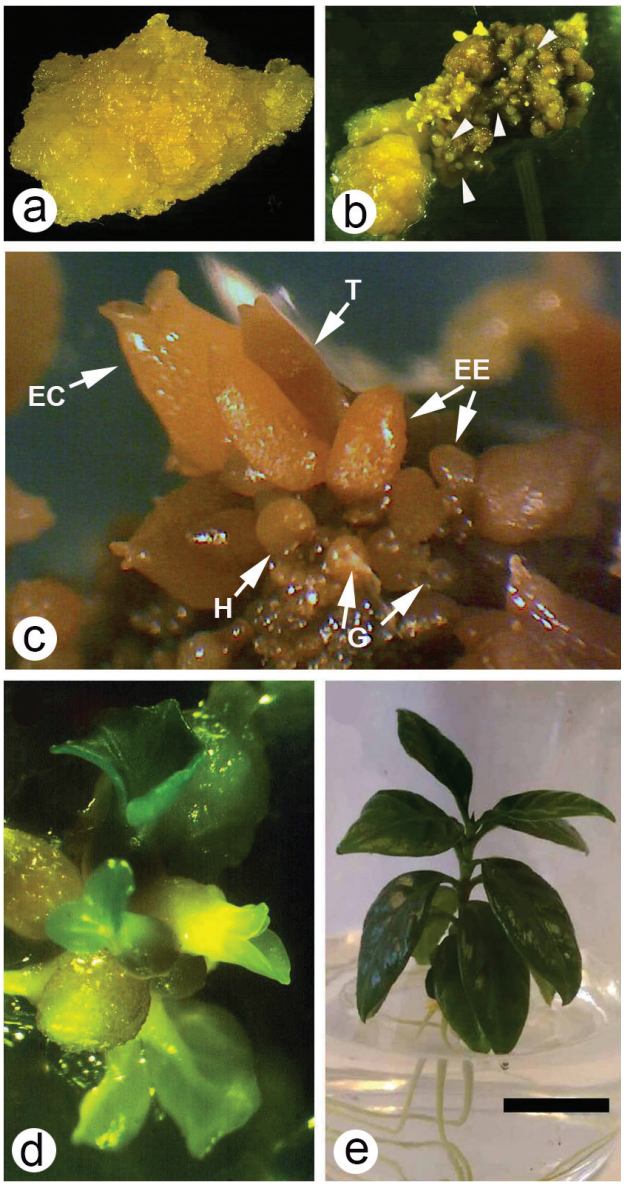

Fig. 1. Plant regeneration via indirect somatic embryogenesis of *Coffea arabica* L. (The bar in Fig. 1e applies to all panels, bar = 2.5 mm for Fig. 1a, 3 mm for Fig. 1b, 5 mm for Fig. 1c, and 1d, 1.7 cm for Fig. 1e). (**a**) Three-month-old line $T_2D_{0.1}$ callus showed yellow color and compact masses when subcultured at 2 mg/L TDZ and 0.1 mg/L 2,4-D, (**b**) Eighteen-month-old line $T_2D_{0.1}$ callus showed brown color, compact masses and formed somatic embryos (arrowheads) when subcultured at 2 mg/L TDZ and 0.1 mg/L 2,4-D, (**c**) Globular stage embryos (G), heart stage embryos (H), elongating embryos, i.e., heart-torpedo transition (EE), torpedo stage embryos (T) and early cotyledonary stage embryo, i.e., torpedo-cotyledonary transition (EC), (**d**) Cotyledonary stage embryos, (**e**) Regenerated plantlets.

the callus of *C. arabica* was transparent to white and its texture was soft to friable. With regard to their regeneration ability, these callus lines did not

produce somatic embryos in this study. During subculture, the addition of 2 mg/L TDZ to combine with an adequate dosage of 2,4-D (0.1 and 0.5 mg/L) produced yellow compact embryogenic callus initially, and then it became light to dark brown with eventually S-type embryos after a long-term culture. Therefore, it is suggested that TDZ may play a key role in inducing the callus to turn brown gradually during subculture and consequently change the physiological state as well as gain the embryogenic capacity in the long-term callus.

In tissue culture of some plant species, embryogenic callus could be subcultured for a prolonged period, and still retain a highly regeneration potential, i.e., embryogenesis or organogenesis capacity (Bajaj and Rajam, 1995; Zheng et al., 1999; Von Arnold et al., 2005; Chen, 2012). It has been reported that the embryogenic potential of *C. canephora* callus could be maintained for over 2 years, but the procedure was less responsive in *C. arabica* (van Boxtel and Berthouly, 1996). In the present study, the embryogenic calli of *C. arabica* colud be maintained for at least 18 months and they still retained their regeneration capacity which formed S-type embryos in the presence of low auxin/cytokinin ratio (i.e., 0.1 mg/L 2,4-D plus 2 mg/L TDZ).

Theoretically, somatic cells could regain the ability to form embryos (when the suitable stimulus is applied) by passing through a process of dedifferentiation, i. e., callogenesis (Campos et al., 2017). In tissue culture of *Coffea*, the appearance and texture of embryogenic callus were variable which may depend on several factors (including the explant type, medium composition, culture period and PGRs) for inducing or subculturing the culture (Quiroz-Figueroa et al., 2002; Ahmed et al., 2013; Ibrahim et al., 2013; Nic-Can et al., 2015). The color of the embryogenic callus was documented as white, creamy, yellow, brown and brownish black, and its texture could be soft, friable or compact. A dynamic nature was found in the embryogenic callus of *C. arabica*, i.e., it appeared as yellow and compact masses initially, gradually turned into light brown after several months, and then became light to dark brownish masses with a low proliferation rate after a long-term culture. In this study, three textures of callus were identified and applied in inducing the formation of I-type embryos. However, only two lines of compact calli resulted in embryo formation. Therefore, it is suggested that callus texture has a profound effect on the ability to form I-type embryos.

At 2 mg/L BA, the addition of an adequate dosage of gibberellic acid (GA_3) could significantly enhance the development of somatic embryos of *C. arabica* (Ahmed et al., 2013). However, on the GA_3-free medium, a rise of BA dosage from 2 mg/L to 4 mg/L could also give a similar significant enhancement to embryo development. In this study, somatic embryos (S-type embryos) formed spontaneously during subculture of long-term callus. It is suggested that an accumulation of effects that were induced by low ratios of auxin to cytokinin may turn on the switch of embryo formation from the long-term callus. Interestingly, the resulting S-type embryos have a developmental barrier. In a previous study, BA was found to be an inducer of embryogenic differentiation in *C. arabica* (Papanastasiou et al., 2008). However, it was found that application of 2 or 5 mg/L BA could promote the progress of embryo development. Another type of somatic embryos, I-type embryos, which were induced from the short-term callus of *C. arabica* did not have a developmental barrier (Table 6). Although there were no obvious differences in the morphogenetic performance between the two types of embryos, their physiological state and the requirement for embryo development from the topedo stage to the cotyledonary stage were quite different.

Plants which regenerate from *in vitro* culture were expected to keep their intrinsic characteristics, and generally, the severity of somaclonal variation and physiological disorders increased with the culture period (Etienne and Bertrand, 2003; Campos et al., 2017). Somaclonal variation has been found in cell suspension of *C. arabica*, and the frequency and phenotype of variants was affected by the genotype and age of embryogenic cell suspension (Etienne and Bertrand, 2003). In this study, it showed a normal growth and development of regenerated plantlets which derived from 18-month-old callus cultures.

CONCLUSIONS

The present study established a reliable protocol for screening and identification of embryogenic callus of Arabica coffee via morphological observation and long-term selection. Two types of somatic embryos were obtained from the short-term and the long-term callus (Table 6). Although the requirements for the regeneration pathway were more or less the same, their physiological state, especially a developmental barrier, was quite different. Adenine-type cytokinin, BA, has a profund effect on embryo development which overcame the developmental barrier of the S-type embryos. It was also found that both urea-type cytokinin, TDZ, and texture of callus play a crucial role in inducing indirect somatic embryogenesis. In this study, the resulting somatic embryos developed following the typical progress of somatic embryogenesis in dicot plants and eventually morphological normal plantlets were obtained.

TABLE 6. The requirements for regeneration pathways of somatic embryos in *Coffea arabica*.

Regeneration pathways	Characteristics and requirements	
	S-type embryos	I-type embryos
Initial callus	long-term callus $T_2D_{0.1}$ (18-month-old)	short-term-callus lines $T_2D_{0.1}$ and $T_2D_{0.5}$ (3-month-old)
PGR requirements for inducing somatic embryogenesis from callus	spontaneously formed near the end of subculture (12.6 embryos/callus)	line $T_2D_{0.1}$ resulted in the highest number of embryos per callus (13.3 embryo/callus) at 2 mg/L TDZ
Developmental barrier into the cotyledonary stage	Yes, could be overcome by adding 2 or 5 mg/L BA	No
PGR requirements for rooting of the cotyledonary stage embryos	0.1 mg/L IBA (data not shown)	0.1 mg/L IBA (3.3 roots/embryo, the highest)
Ability to convert into plantlets	Yes	Yes
Survival rate in acclimatization	100% (data not shown)	100%

AUTHORS' CONTRIBUTIONS

JT Chen designed and performed experiments, analyzed partial data and wrote the paper; YC Wang, MZ Lin, HH Chung and B Huang performed partial experiments and analyzed partial data.

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