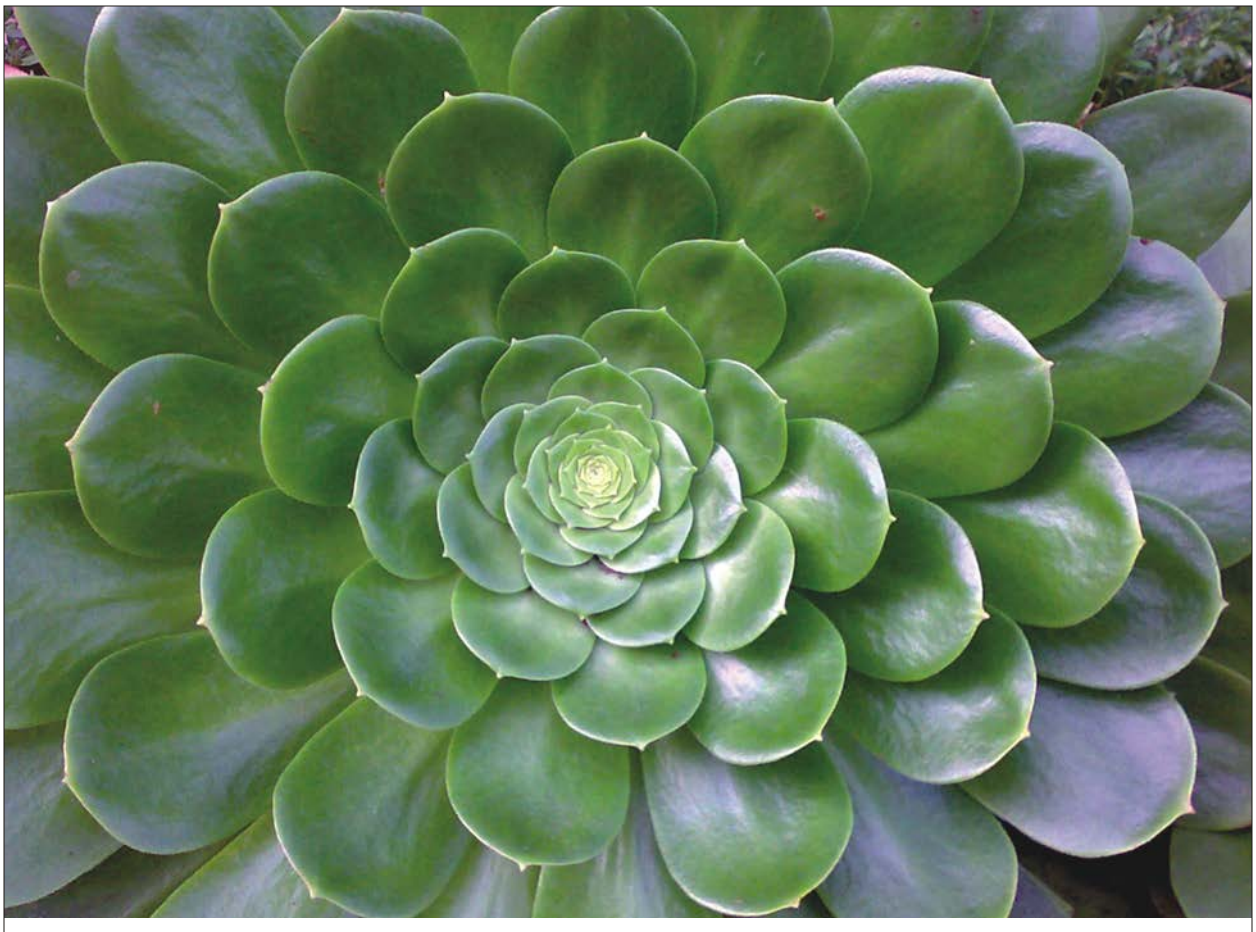

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Induction of Somatic Embryos from Different Explants of *Citrus sinensis*

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Abstract: The effects of different explants and the position of explants on the induction of callus and somatic embryos were studied on *Citrus sinensis*. The explants; cotyledon, mature zygotic embryo, *in vitro* leaf and *in vitro* stem were cultured on full-strength MS basal medium supplemented with 2,4-dichlorophenoxy-acetic acid (2,4-D), 3,6-Dichloro-2-methoxybenzoic acid (dicamba), 1-Naphthaleneacetic acid (NAA) and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram). Direct and indirect somatic embryogenesis was observed from the cotyledon and mature zygotic embryo explants. Direct somatic embryo were formed from the cotyledon and mature zygotic embryos explants cultured on the medium containing 3 and 5 mg L^{-1} NAA. Indirect somatic embryogenesis was also observed in the cotyledon explants cultured on the medium supplemented with 2 mg L^{-1} dicamba as well as the mature zygotic embryo explant cultured on the medium with 3 and 5 mg L^{-1} picloram. The somatic embryo formed through direct or indirect somatic embryogenesis contained the similar stage, namely the globular, heart, torpedo, cotyledonary before developed into mature embryo. All these mature somatic embryos were further regenerated into *in vitro* plantlet on the medium containing 5 mg L^{-1} NAA after 3 weeks culture. *In vitro* leaf and stem explants were unable to induce somatic embryo from the four types of Plant Growth Regulators (PGRs) studied. Formation of root or shoot was observed from the *in vitro* stem incubated on the medium supplemented with 1 mg L^{-1} 2,4-D; 1 and 2 mg L^{-1} picloram; 1, 2 and 5 mg L^{-1} NAA and also Murashige and Skoog (MS) medium without PGRs. Cotyledon explants which cut horizontally into three parts and cultured into medium containing 4 mg L^{-1} 2,4-D. All the explants induced non-embryogenic callus after 3 weeks culture.

Key words: Somatic embryo, *Citrus sinensis*, tissue culture, cotyledons

INTRODUCTION

Citrus sinensis, is a member of Rutaceae family (citrus family) and has the common name like sweet orange or naval orange (Christman, 2003). The sweet orange is native originally to Vietnam, India and southern China. *C. sinensis* is one of the major commercial fruit crops that is widely consumed both as fresh fruit or juice attributed to its high vitamin C content and its antioxidant potential. Even though more than 104 million tons of citrus are produced and about 15 millions tons are traded annually (Bekele, 2007), the production of this plant has always been hindered by the long juvenility period and the breeding of citrus cultivars by conventional methods is restricted by the complication of their genetic systems (Kayim and Koe, 2006). As with other fruits, citrus is attacked by several

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pre- and/or postharvest pathogens that affect fruit quality (Bekele, 2007). The genetic improvements of this perennial woody plant often take many years using traditional plant-breeding methods (Kayim and Koe, 2006). Thus, plant tissue culture techniques can be applied as an alternative for the traditional plant-breeding methods that are time consuming. Furthermore, somatic embryogenesis is one of the efficient methods in plants tissue culture for mass propagating the plant within a short period of time. Apart from that, regeneration of plants through somatic embryogenesis has been a preferred method for genetic transformation in woody perennials because somatic embryogenesis leads to the formation of bipolar structures, possessing both a shoot and a root meristem and somatic embryos have always assumed to have a single cell origin and have closed vascular system (Rai *et al*., 2007). In view of the importance of somatic embryogenesis as well as limited research that have been conducted on somatic embryogenesis of *C. sinensis*, the present studies were carried out in order to determine the effects of different plant growth regulators at various concentrations on different explants of *C. sinensis*. Through this, the suitable explants, type and concentrations of plant growth regulators that were capable to induce somatic embryos were identified. Apart from establishing the somatic embryogenesis protocols, this study also compared the morphology of the somatic embryo formed with the zygotic embryo of *C. sinensis*.

MATERIALS AND METHODS

Plant Materials

The cotyledon and zygotic embryo explants used were derived from the seeds of *C. sinensis* obtained between October 2005 to June 2006.

Surface Sterilization

Surface sterilization of the seed explants were initiated by soaking the seeds in detergent for one minute followed by washing under running tape water for 30 min. The seeds were then immersed for 15 min in 30% (v/v) Clorox® containing 2 to 3 drops of Tween-20 (Amresco, USA). In order to remove the traces of Clorox®, the seeds were rinsed three times with sterile-distilled water for 5, 10 and 15 min, respectively.

Culture Medium

The full strength MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and various auxins (2,4-D (Sigma, USA), dicamba (Duchefa, Netherland), NAA (Sigma, USA) and picloram (Duchefa, Netherland)) at the concentrations of 1, 2, 3, 4 and 5 mg L^{-1} was used as the callus induction medium. The pH of the medium was adjusted to 5.7±0.1 with 0.1 M NaOH or 0.1 M HCl using the pH meter (Mettler Toledo) prior to autoclaving at 121°C for 15 mins. Approximately 25 mL of the sterilized medium were poured into the Petri dish. In this study, the MS medium without any plant growth regulator (MS0) was used as the control.

Embryogenic Callus Induction

Four different types of explants namely, the cotyledon, mature embryo, stems and leaf explants were used to induce the embryogenic callus from *C. sinensis*. To obtain the cotyledon and embryo explants, the seed coat was removed from the surface sterilised seeds. The embryo was separated from the cotyledon and cultured on the callus induction medium. As for the cotyledon, each of the cotyledon was further cut into approximately nine small pieces. As for the leaf and stem explants, the explants were obtained from the four-weeks-old *in vitro* plantlet cultured in MS medium containing 0.5 mg L^{-1} BAP. The *in vitro* leaf (5×5 mm) and stem explants (0.5 cm) were aseptically excised and cultured on the callus induction medium.

The cultures were incubated at 25±2°C with 16 h photoperiod with the light intensity of 2000 lux under cool-white fluorescent lamps. All the experiments were conducted with 5 replicates and repeated for three times. The percentage, day and degree of callus formation were determined after 4 weeks of culture. The morphology of the embryogenic callus and non embryogenic callus was also observed every 2 weeks after the callus formation.

Effects of Explants Position

As the preliminary studies showed that cotyledon was the most potential explant for the induction of embryogenic callus, the effects of different explants position was further examined. The zygotic embryo was removed from the seed and ouly the cotyledon was used as the explants. The cotyledon was cut horizontally into three different parts, A, B and C. Part A refers to the upper part of the cotyledon that containing the cut embryo. Part B refers to the middle part of the cotyledon and Part C refers to the lower part of the cotyledon which was away from the embryo. Parts A, B and C at the size of 7±2 cm were cultured on the MS medium supplemented with 4 mg L^{-1} 2,4-D. The cultures were maintained at the culture room at 25±2°C under the photoperiod of 16/8 (day/night) with the light intensity of 2000 lux controlled by from the white cool fluorescent lamps (Philips, Japan). Alike the previous experiments, all the treatments consisted of 5 replicates and experiments were repeated thrice.

Proliferation of Embryogenic and Non-embryogenic Callus

To multiply the callus, the embryogenic calli were separated from the non-embryogenic calli. Only the healthy embryogenic and non-embryogenic calli were subcultured into the fresh medium containing the same composition as the induction medium every two to three weeks after the callus initiation. The cultures were incubated at 25±2°C with 16 h photoperiod under fluorescent light with the light intensity of 2000 lux controlled by cool white fluorescent light tubes (Philips, Japan).

Development and Plantlet Regeneration from Somatic Embryos

In order to develop and further regenerate the somatic embryos from the embryogenic callus, the embryogenic callus which was represented by the compact, yellowish white or light green with nodular structure and slow growing callus was transferred to the medium similar to the initiation medium to prevent the culture shock. Somatic embryo was indicated by the formation of green colour, smooth and zygotic embryo shape which has different developmental stage. Meanwhile, the plantlet regeneration was indicated by the development of morphologically normal plantlets with shoots and roots.

RESULTS AND DISCUSSION

Effects of Various Auxins on the Cotyledon Explants

For the cotyledon explants, callus was not induced in the MS medium without auxin (Table 1). The explants swollen and turned to dark green in colour after two weeks of culture and subsequently turned to yellow in colour and died after 12 weeks of culture. Similar observation was recorded in Eastern redbud (Geneve and Kester, 1990) and cashew (Gogate and Nadguda, 2003) whereby explants did not show any embryogenic response or callus formation in medium that was lacked of plant growth regulators.

In citrus, 2,4-D has been mostly used for callus induction from albedo tissue of fruits (Murashige and Tucker, 1969; Amo-Macro and Picazo, 1994), leaf (Goh *et al*., 1995) immature seed (Ling and Iwamasa, 1997) and undeveloped ovules (Gmitter and Moore, 1986). Similarly, in this study, 2,4-D was found to be the most suitable auxin for callus induction from the cotyledon explants. After 2 weeks of culture, the explants incubated at the higher concentrations (4 and 5 mg L^{-1}) were swollen,

Table 1: Effects of different auxins at various concentrations on the callus induction from cotyledon explants of *C. sinensis* after 12 weeks of culture in MS medium

Treatments	Conc. (mg L^{-1})	Day of callus formation (week)	Degree of callus formation	Callus appearance	Explants with callus (%)	No. of somatic embryos
MS0		—	—	—	—	—
2,4-D	1	5	+	Friable, yellowish	80±20	—
	2	5	++	Friable, yellowish	93.3±11.6	—
	3	4	+++	Friable, yellowish	100	—
	4	3	+++++	Friable, yellowish	100	—
	5	4	+++++	Friable, yellowish Spongy callus	100	—
Dicamba	1	7	+	Friable, yellowish	66.7±11.6	—
	2	7	++	Friable, yellowish	93.4	—
				Compact, yellowish	6.7	38
	3	6	+++	Friable, yellowish	100	—
	4	6	++++	Friable, yellowish	100	—
	5	6	+	Friable, yellowish	26.7±11.6	
NAA	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	—
	4	—	—	—	—	—
	5	—	—	—	—	13
Picloram	1	5	+	Friable, yellowish	86.7±11.6	—
	2	5	+++	Friable, yellowish	93.3±11.6	—
	3	5	+++	Friable, yellowish Spongy callus	100	—
	4	4	++++	Friable, yellowish Spongy callus	100	—
	5	4	+++	Friable, yellowish Spongy callus	93.3±11.6	—

+: Very weak; ++: Weak; +++: Moderate; ++++: Profuse; +++++: Very profuse

turned to dark green in colour and ruptured. The callus formation was observed after the explants was cultured for 3 weeks on MS medium supplemented with 4 and 5 mg L^{-1} of 2,4-D. The callus formation was also observed when the cotyledon explants were cultured in the MS medium containing 2,4-D at the concentrations lower than 4 mg L^{-1}. However, a longer period (4 weeks of culture) was required for the explants treated with lower concentrations of 2,4-D to form the callus. The degree and percentage of callus formation in the MS medium containing 1 mg L^{-1} of 2,4-D was the lowest among all the 2,4-D concentrations tested. Most of the explants in the MS medium supplemented with 1 mg L^{-1} 2,4-D remained swollen and dark green in colour after 3 weeks of culture. In terms of the morphology and colour of the callus formed within the 2,4-D treatments, it was found that the profuse and healthy callus formation was obtained from explants treated with 4 mg L^{-1} 2,4-D. In the treatment using 5 mg L^{-1} 2,4-D, two types of calli were induced. The calli induced in 5 mg L^{-1} 2,4-D was either friable or spongy callus. Explants treated with other concentrations of 2,4-D only induced the friable yellowish callus.

Although a significantly high percentage of callus induction was obtained in all the treatments using 2,4-D, but none of the callus formed managed to form the somatic embryo. In *Eragrostis tef* (Zucc.) Trotter, 2,4-D was able to induce the soft and undifferentiated callus with embryogenic capacity compared to the soft, watery, amorphous and grew very poorly callus in the medium supplemented with dicamba (Kebebew *et al*., 1998). According to Fujimura and Komamine (1980), 2,4-D inhibited somatic embryo development at an early stage. Kamada and Harada (1979) also mentioned the inhibitory effect of 2,4-D on the somatic embryogenesis of carrot.

In the treatment using dicamba as one of the auxins supply revealed that the earliest callus formation was observed when the cotyledon explants were cultured in medium containing dicamba at the concentrations higher than 3 mg L^{-1}. In these treatments, 6 weeks of culture was required for callus induction. The duration for callus induction was significantly longer than the 4 weeks period observed

in the 2,4-D treatments. At the concentration as high as 5 mg L^{-1} dicamba, ouly 26.7% of the explants formed callus (Table 1). The calli induced in all these concentrations (3 to 5 mg L^{-1}) were friable and yellow in colour. Similarly, the cotyledon explants treated with lower concentration of dicamba (1 mg L^{-1}) induced the friable callus after 6 weeks of culture. However, only about 66.7% of the explants treated with 1 mg L^{-1} dicamba formed the callus. The explants that could not induce any callus were swollen and turned to dark green in colour after 6 weeks of culture. In the treatment using 2 mg L^{-1} dicamba, 2 types of calli were initiated. A total of 93.3% of the explants formed friable yellowish calli whilst 6.7% formed the compact yellowish calli. In this study, about 6.7% of these compact and light green calli formed in 2 mg L^{-1} dicamba productively induced the first somatic embryo (38 somatic embryos) after 6 weeks of culture. In this study, cotyledon explants were able to induce somatic embryo in the medium containing 2 mg L^{-1} of dicamba. Similar observations have also been reported by Hegde *et al.* (1994) and Onay *et al.* (1995) in which the somatic embryo were grow from cotyledon explants. The light yellow compact, glossy with knobbly callus was induced from cotyledon explants resembles morphology to the embryogenic callus induced from *Eragrostis tef* (Zucc.) Trotter (Kebebew *et al.*, 1998) yet is different from embryogenic callus induced from stigma and style explants of *C. sienesis* which was friable yellowish (Carimi *et al.*, 1998).

As for the treatment using picloram, the degree of the callus induction was comparable to those observed in the dicamba treatment. In the MS medium supplemented with lower concentrations of picloram (1 and 2 mg L^{-1}), only about 50% of the explants managed to induce the friable and yellowish callus. MS medium with 3 to 5 mg L^{-1} of picloram induced two types of callus; friable, yellowish callus and the spongy callus simultaneously about 5 weeks of culture. Similar to the treatment using 2,4-D, none of the callus form was able to formed the somatic embryo.

Studies were also carried out using NAA as one of the auxins supply. However, all the explants cultured on the MS medium supplemented with NAA failed to induce the callus. All the explants were swollen and turned to light, yellowish colour after 4 weeks of culture. Even though NAA did not induce any callus, 6.7% of the explants cultured on the MS medium containing 5 mg L^{-1} NAA induced green and globular somatic embryos after 12 weeks of culture through direct somatic embryogenesis. All these somatic embryos were isolated and multiplied in the fresh same medium (Table 1).

Effects of Various Auxins on the Mature Zygotic Embryo Explants

In this study, the mature zygotic embryos were found more suitable than the immature embryo as preliminary studies showed that the immature embryos turned to white in colour and died after three weeks of culture in the plant tissue culture medium. Most of the mature zygotic embryos cultured in the low concentration (1 and 2 mg L^{-1}) of PGRs germinated to from the *in vitro* plantlets except in the treatment of 2,4-D which could induced the callus. Kebebew *et al.* (1998) stated that the use of mature seeds as explants for plant regeneration is the simplest and is potentially more efficient method to be applied in a breeding programme. Ouly at the higher concentration of PGRs, all the mature embryos induced callus. All the explants in the treatment of 2,4-D induced callus (Table 2). At a lower concentration (1 and 2 mg L^{-1}), roots formation were observed. However, the roots did not elongated but it ruptured to induce callus. This phenomenon were reported by Kebebew *et al.* (1998) that the callus was induced from the germinating embryos of the seeds in the medium supplemented with 2 mg L^{-1} of 2,4-D.

In the medium with lower concentrations (1 and 2 mg L^{-1}) of dicamba, all the mature embryos germinated and formed the *in vitro* plantlets. However, with the increase concentrations of dicamba from 3 to 5 mg L^{-1}, the phenomenon of germination was reduced. Consequently, the percentage of callus formation was also increased from 44.4% in to 65.2% in 4 mg L^{-1} and 88.9% in 5 mg L^{-1} dicamba. In terms of the morphology of the callus, in MS medium supplemented with 3 mg L^{-1} of dicamba, all the calli induced were friable yellowish callus whereas in concentrations higher than 3 mg L^{-1} (4 and 5 mg L^{-1} dicamba), the calli induced were either friable yellowish or spongy.

Table 2: Effects of different auxins at various concentrations on the callus induction from mature zygotic embryo explants of *C. sinensis* after 14 weeks of culture in MS medium

Treatments	Conc. (mg L^{-1})	Day of callus formation (week)	Degree of callus formation	Callus appearance	Explants with callus (%)	No. of somatic embryos
MS0		—	—	—	—	—
2,4-D	1#	7	+ +	Friable, yellowish	74.1±6.1	—
	2#	7	+ + +	Friable, yellowish	88.9±11.1	—
	3	6	+ + +	Spongy callus	100	—
	4	3	+ + + + +	Spongy callus	81.5	—
				Compact, yellowish	18.5	
	5	4	+ + + + +	Spongy callus	100	—
Dicamba	1#	—	—	—	—	—
	2#	—	—	—	—	—
	3#	10	+ +	Friable, yellowish	44.4±33.3	—
	4	8	+ + + + +	Friable, yellowish	85.2±12.8	—
				Spongy callus		—
	5	8	+ + + + +	Friable, yellowish	88.9±11.1	—
				Spongy callus		—
NAA#	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	32
	4	—	—	—	—	—
	5	—	—	—	—	—
Picloram	1#	—	—	—	—	—
	2#	—	—	—	—	—
	3	8	+ + + +	Spongy callus	70.7±6.4	—
				Compact, light green	7.4	25
				Spongy callus		—
	4	6	+ + + + +	Compact, yellowish	88.9	—
				Spongy callus	11.1	—
	5	6	+ + + + +	Compact, yellowish	96.3	4
					3.7	

+: Very weak; + +: Weak; + + +: Moderate; + + + +: Profuse; + + + + +: Very profuse; #: Root or shoot formation

Callus has also been induced from the explants cultured on MS medium supplemented with various concentrations of picloram. Within the picloram treatments, it was found that callus was only successfully induced in the treatments using 3 to 5 mg L^{-1} of picloram. The highest percentage of callus formation (100%) was observed in 5 mg L^{-1} picloram whilst the lowest (78%) was gained in 3 mg L^{-1} picloram. Few of the explants in 3 mg L^{-1} picloram formed roots prior to friable yellowish callus formation. At the concentrations of 3 to 5 mg L^{-1} picloram, the calli induced were either spongy, compact white, light green or compact yellowish. A total of 25 somatic embryos were noted on the compact, light green callus induced in 3 mg L^{-1} picloram. Meanwhile, none of the explants cultured in 1 and 2 mg L^{-1} picloram managed to induce any callus. However, the germination and formation of *in vitro* plantlets was observed (Fig. 1a-f).

Similar to the cotyledon explants, mature embryos cultured on MS medium containing various concentrations of NAA did not induce any callus. Most of the explants treated with NAA germinated and formed the *in vitro* plantlets. However, about 32 of the embryos cultured on MS medium supplemented with 3 mg L^{-1} NAA formed the somatic embryo directly in the absence of callus phase. The interplay of several signal pathways, endogenenous or exogenous factors and growth regulators closely coordinate and regulate the events of cell proliferation, elongation and differentiation that ultimately build up the embryo body (Baldan *et al*., 2003). Baldan *et al*. (2003) stated that exogenous auxin is required for cultured cell to acquire embryogenic competence; nevertheless, the involvement of endogenous auxin in morphogenetic changes during delevopmental process had been recently established both in zygotic and somatic embryo (Thomas *et al*., 2002). Thus, in this experiment, explants in the treatment using NAA did not induce any callus. All the explants were regenerated even in the high concentration.

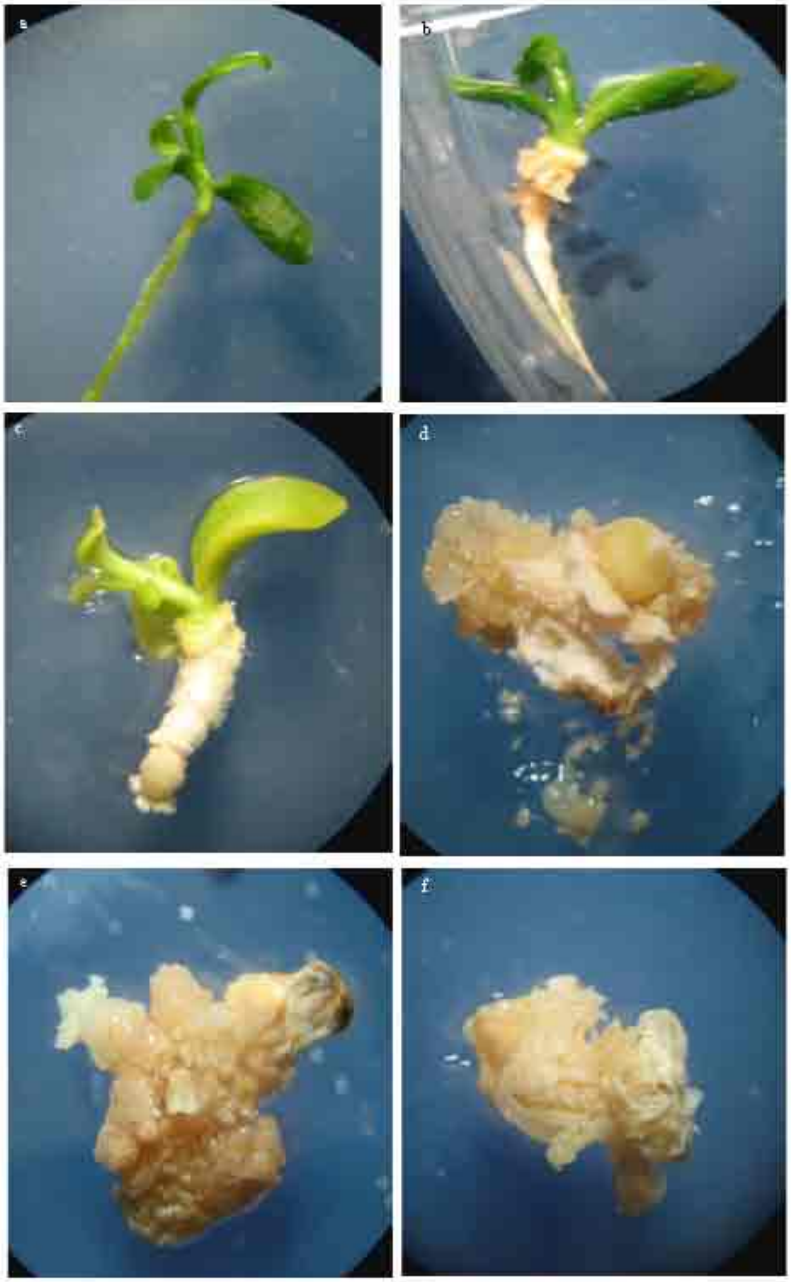

Fig. 1: Effects of picloram at various concentrations on mature zygotic embryo explants of *C. sinensis* after 6 weeks of culture in MS medium. (a) 0 mg L^{-1} (b) 1 mg L^{-1} (c) 2 mg L^{-1} (d) 3 mg L^{-1} (e) 4 mg L^{-1} and (f) 5 mg L^{-1}

Embryogenic callus were obtained from embryo explants. The capability of mature zygotic embryo explants induced the embryogenic callus is because zygotic embryo explants are already close to the embryonic state which are induced to maintain a program of repetitive embryo production (Merkle *et al*., 1998). Nevertheless, zygotic embryo is not considered as a good source for embryogenic callus. Thus, zygotic embryo explants actually is induced to replicate itself indefinitely (Merkle *et al*., 1998). According to Sharp *et al.* (1980), this type of embryogenic has been described as coming from pre-embryogenic determined cell and represents a relatively straightforward process. Even though Merkle *et al*. (1998) and Sharp (1980) concluded that zygotic embryo is not suitable explants for somatic embryogenesis, many researchers defined it differently. Tu *et al.* (1996) reported that the zygotic embryo is a good explant source for somatic embryogenesis and plants regeneration in *Hyoscyamus niger*.

Effects of Various Auxins on *in vitro* Leaf Explants

The *in vitro* leaf explants of *C. sinensis* did not induce any somatic embryo from the friable yellowish callus. The friable yellowish callus was induced from the treatments of 2,4-D and at higher concentrations of dicamba (3 to 5 mg L^{-1}) (Table 3). NAA and picloram were unable to induce any callus. Furthermore, leaf explants in the treatment of picloram turned to yellow in colour and dead after 10 weeks of culture. Similarity, the leaf explants of different *Dianthus* cultivars failed to induce somatic embryogenesis in the treatment of NAA, 2,4-D and picloram (Nakano and Mii, 1993). Meanwhile, leaf explants from rose were cultured in the medium containing 2,4-D for callus induction (Rout *et al*., 1999). Nevertheless, the callus was subcultured to medium with gibberellic acid and benzyladenine to generate friable embryogenic callus and somatic embryo.

Effects of Various Auxins on *in vitro* Stem Explants

For those four treatments for the *in vitro* stem explants, none of them induced the embryogeneic callus. Three types of PGRs except dicamba were induced either friable yellowish callus or the spongy callus. In the lower concentration (1 mg L^{-1}) of PGRs and MS0, the *in vitro* stem explants formed roots or shoot. Similarity roots or shoots formation also found in the treatments of 2 and 5 mg L^{-1} of NAA. NAA was found to be good in inducing callus when increase the concentration from 1 to

Table 3: Effects of different auxins at various concentrations on the callus induction from *in vitro* leaf explants of *C. sinensis* after 10 weeks of culture in MS medium

Treatments	Conc. (mg L^{-1})	Day of callus formation (week)	Degree of callus formation	Callus appearance	Explants with callus (%)	No. of somatic embryos
MS0		—	—	—	—	—
2,4-D	1	9	+ + +	Friable, yellowish	46.7±30.6	—
	2	8	+ + + +	Friable, yellowish	53.3±23.1	—
	3	8	+ + + +	Friable, yellowish	53.3±11.6	—
	4	10	+ +	Friable, yellowish	80±20	—
	5	10	+	Friable, yellowish	26.7±11.6	—
Dicamba	1	—	—	—	—	—
	2	—	—	—	—	—
	3	10	+ +	Friable, yellowish	26.7±11.6	—
	4	10	+	Friable, yellowish	13.3±11.6	—
	5	9	+ + +	Friable, yellowish	33.3±23.1	—
NAA	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	—
	4	—	—	—	—	—
	5	—	—	—	—	—
Picloram	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	—
	4	—	—	—	—	—
	5	—	—	—	—	—

+: Very weak; + +: Weak; + + +: Moderate; + + + +: Profuse; + + + + +: Very profuse

Table 4: Effects of different auxins at various concentrations on the callus induction from *in vitro* stem explants of *C. sinensis* after 10 weeks of culture in MS medium

Treatments	Conc. (mg L^{-1})	Day of callus formation (week)	Degree of callus formation	Callus appearance	Explants with callus (%)	No. of somatic embryos
MS0		—	—	—	—	—
2,4-D	1#	5	+ + + +	Friable, yellowish	100	—
	2	5	+ + + +	Friable, yellowish	100	—
	3	4	+ + + + +	Friable, yellowish	100	—
	4	4	+ + + + +	Spongy callus	100	—
	5	4	+ + + + +	Spongy callus	100	—
Dicamba	1	5	+ +	Friable, yellowish	33.3±11.6	—
	2	5	+ + +	Friable, yellowish	46.7±23.1	—
	3	5	+ +	Friable, yellowish	60±20	—
	4	4	+ + + +	Spongy callus	100	—
	5	4	+ + + + +	Spongy callus	100	—
NAA	1#	—	—	—	—	—
	2#	—	—	—	—	—
	3	—	—	—	—	—
	4	—	—	—	—	—
	5#	—	—	—	—	—
Picloram	1#	6	+ +	Friable, yellowish	46.7±11.6	—
	2#	6	+ + +	Friable, yellowish	80±20	—
	3	5	+ + +	Friable, yellowish	93.3±11.5	—
	4	5	+ + + + +	Friable, yellowish	±50	—
				Spongy callus	±50	
	5	5	+ + + +	Friable, yellowish	±50	—
				Spongy callus	±50	

+: Very weak; + +: Weak; + + +: Moderate ; + + + +: Profuse; + + + + +: Very profuse; #: Root or shoot formation

5 mg L^{-1} (Table 4) (Huang *et al*., 2002). Huang *et al*. (2002) also concluded that picloram ouly induced rooting at low concentration as found in this study. According to Hudson *et al*. (2002), 2,4-D is one of the important phenolic compounds with auxinic effect at low concentrations and acts as a rooting cofactor and prevents breaking of endogenous auxin by oxidase enzyme results in rooting. In most of the studies of micropropagation, NAA is one of the auxins that normally used in combination with cytokinins. For example in clonal propagation of *Acacia catechu* Willd. by using shoot tip culture (Kaur and Kant, 2000) and micropropagation of *Spilanthes acmella* Murr. (Saritha *et al*., 2002). Edriss and Burger (1984) obtained a 100% of rooting in shoots from epicotyl segments of Troyer citrange cultivated in 2.0 mg L^{-1} NAA.

Effects of Explants Position on Callus Induction

From all the four explants examined in this study, cotyledon explant was found to be the most potential explant for high percentage of callus induction as well as somatic embryos production. Thus, further study was carried out to determine the effects of different explants position on the callus induction from the cotyledon explants. Although there was no significant difference in terms of the percentage, day of callus formation and the morphology of the callus formed, the degree of callus formation was affected by the explant position (Table 5). It was observed that Part C of the cotyledon explants induced a profuse callus compared to part A and B cultured on the same medium composition. Callus is generally produced as a result of wounding (Thomas and Maseena, 2006). Thus, the number of the cut edges on the explants was able to help in increasing the degree of callus formation. However, in this study, the part B (from the middle part of cotyledon) which contained two cutting edges did not induce more callus compared to part C, which ouly has a cutting edge. Part A which also contained a cutting edge produced the lowest amount of callus among the three parts studied. This could be due to the fact that the distributions of the endogenous auxins were different for different parts of the explants. Endogenous or exogenous factors and growth regulators closely coordinate and regulate the events of cell proliferation, elongation and differentiation that ultimately build up the embryo body

Table 5: Effect of different positions of the cotyledon explants of *C. sinensis* on the callus induction

Explant position	Degree	Explants with callus (%)	Callus appearance
Part A	+ + +	100	Spongy callus Friable, yellow
Part B	+ + + +	100	Spongy callus Friable, yellow
Part C	+ + + + +	100	Spongy callus Friable, yellow

+: Very weak; + +: Weak; + + +: Moderate; + + + +: Profuse; + + + + +: Very profuse

(Baldan *et al*., 2003). The involvement of endogenous auxin in morphogenetic changes during developmental processes has been recently established both in zygotic and somatic embryogenesis (Aderkas *et al*., 2001; Thomas *et al*., 2002). Endogenous levels of growth regulators vary over the course of embryo maturation.

Proliferation of Embryogenic and Non-embryogenic Callus

Both the embryogenic and non-embryogenic calli derived from all the explants were subcultured into fresh, new medium similar to the callus initiation medium after 2 or 3 weeks of culture. As for the embryogenic callus, it was further proliferated by isolating it from the non-embryogenic callus and cultured in the fresh, new initiation medium. Most of the calli increased in size, grew healthily with not much morphological differences when transferred to the fresh new medium. However, the mature zygotic embryo-derived callus which was initially yellowish and spongy turned to be compact and white in colour when maintained in the MS medium supplemented with 4 mg L^{-1} of 2,4-D. Similarly, the compact, yellowish callus was also formed when the spongy callus from the mature embryo was continuously maintained in 4 mg L^{-1} picloram after 4 weeks subculture into new, fresh initiation medium.

Development and Plantlet Regeneration from Somatic Embryos

According to Arnold *et al*. (2002), somatic embryo is defined as the bipolar and bear typical embryonic organ, the radicle, hypocotyls and cotyledon. Somatic embryo is developed and differentiated from the somatic cell without vascular connection with the original tissue (Arnold *et al*., 2002). Meanwhile, somatic embryogenesis is the process by which the formation of an embryo from a cell other than a gamete or the product of gametic fusion (Merkle *et al*., 1998). In this study, two types of somatic embryogenesis, direct and indirect somatic embryogenesis were observed. Direct somatic embryogenesis is defined as the formation somatic embryo directly without the callus phase while indirect somatic embryogenesis is the formation of somatic embryo indirectly following callus formation from explants (Tanaka *et al*., 2000).

In this study, it was fonnd that only cotyledon and mature zygotic derived calli cultured in suitable auxin and its concentration managed to develop into somatic embryos. As for the cotyledon-derived callus, somatic embryo was produced when the calli was cultured for 6 weeks in MS medium supplemented with 2 mg L^{-1} of dicamba. The studies in *Eragratis tef* (Zucc.) Trotter by Kebebew *et al*. (1998) used dicamba as the PGRs for callus proliferation, even though the callus was induced from the medium containing 2,4-D. Somatic embryogenesis was obtained successfully from the isolated shoot apices prickly-pear (*Opuntia ficus-indica* L. Mill.) in dark condition (Gomes *et al*., 2006). The involvement of endogenous auxin in morphogenetic changes during developmental processes has been recently established both in zygotic and somatic embryogenesis (Thomas *et al*., 2002). Besides, Thomas *et al*. (2002) also suggested that endogenous levels of growth regulators vary over the course of embryo maturation. Transferred of the somatic embryo into the new fresh initiation medium is for maintenance and proliferation purposes (Arnold *et al*., 2002) besides to prevent culture shock of the somatic embryos although most of the studies suggested the somatic embryo should be maintained in medium lack of auxins (Choi *et al*., 1999).

The development of the somatic embryos was highly depended on the morphology of the embryogenic callus. The development of somatic embryos was remarked by the formation of green colour with the smooth surface structure on the compact, yellowish callus. When viewed under the microscope, the cotyledonary stage (Fig. 2a) and the multiplication of somatic embryo (Fig. 2b) was observed. In the mature zygotic embryo-derived callus, different auxin as well as a significant longer period was essential for the somatic embryos production compared to the cotyledon-derived callus. The somatic embryos were produced from the mature zygotic embryo denved calli cultured in the treatments using 3 and 5 mg L^{-1} picloram after, 13 and 10 weeks of culture, respectively (Fig. 3a). All the somatic embryos formed were the globular stage somatic embryos. After isolated and subcultured into the new, fresh initiation medium for 3 weeks, the number of globular stage somatic embryo increased and further developed into the heart shaped embryo (Fig. 3b).

In this study, somatic embryo was also produced through the direct somatic embryogenesis process. The direct somatic embryogenesis was observed when the cotyledon and mature zygotic embryo explants were cultured on MS medium containing NAA. For the cotyledon explants, somatic embryos were developed in the medium containing 5 mg L^{-1} NAA after 12 weeks (Fig. 4) whereas in the mature zygotic embryo explants, somatic embryos were produced when the explants were cultured in 3 mg L^{-1} NAA after 14 weeks of culture (Fig. 5a). Some distinguish stages, the globular, heart shaped stage were also observed in the somatic embryos formed through direct somatic embryogenesis. Those somatic embryos were isolated from the explants and further proliferated in the fresh, same medium. After 3 weeks of culture, it was found that the somatic embryos derived from mature zygotic embryo went through regeneration by forming shoots and roots in the MS medium containing 3 mg L^{-1} NAA (Fig. 5b).

Basically, there are five developmental stages of the somatic embryo, namely the globular stage, heart stage, torpedo stage, cotyledonary stage and the mature embryo stage. In this study, the five developmental stages were also observed. Choi *et al.* (1999) also reported that *in vitro* developed somatic embryos are liable to form embryogenic callus or secondary embryo in many cases. Similar observations have also been reported in *Psopocarpus tetragonolobus* (L.) DC. (Gupta *et al.*, 1997) in which the sequential development of somatic embryo from the globular stages to the heart-shaped stage and finally to the cotyledonary stage. According to Handley *et al.* (1995), the limitations due to low initiation frequencies and the genetic specificity of explants are important problems

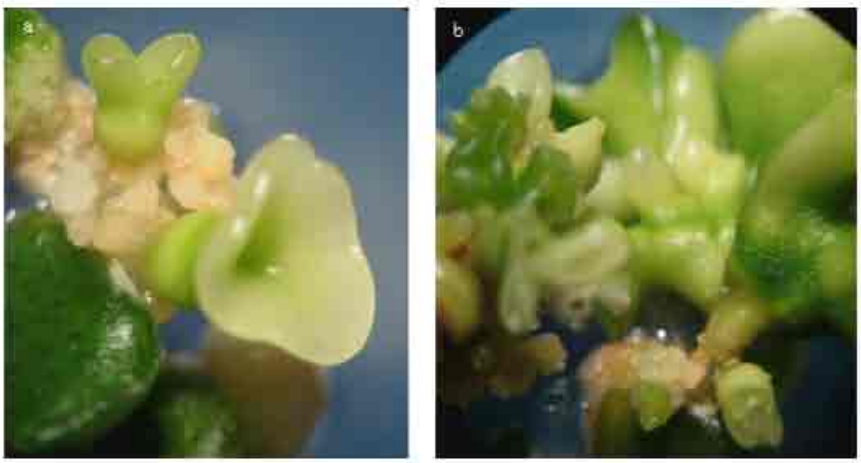

Fig. 2: Developmental stages of somatic embryogenesis of *C. sinesis*. (a) cotyledonary shape somatic embryo from cotyledon explants after 6 weeks of culture in the treatment of 2 mg L^{-1} of dicamba and (b) Multiplication of somatic embryo formed through indirect somatic embryogenesis

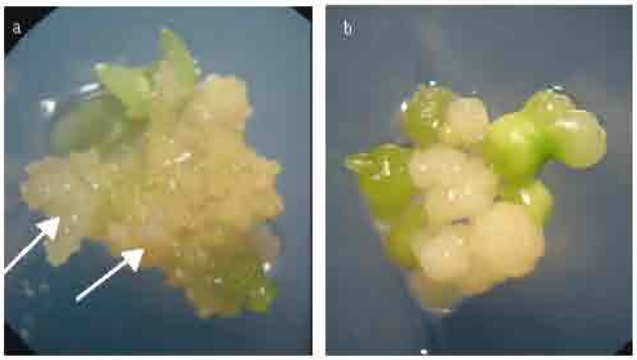

Fig. 3: Developmental stages of somatic embryogenesis of *C. sinensis*.(a) Globular stage somatic embryo in the treatment using 3 mg L^{-1} of picloram after 10 weeks of culture and (b) Heart-stage somatic embryo of *C. sinensis*

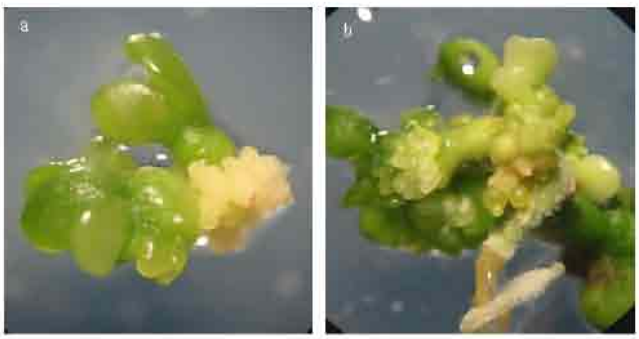

Fig. 4: Developmental stages of somatic embryogenesis of *C. sinesis*. (a) Heart shape and torpedo stage of somatic embryo from cotyledon explants of *C. sinensis* after 12 weeks of culture in 5 mg L^{-1} of NAA and (b) Multiplication of the somatic embryo

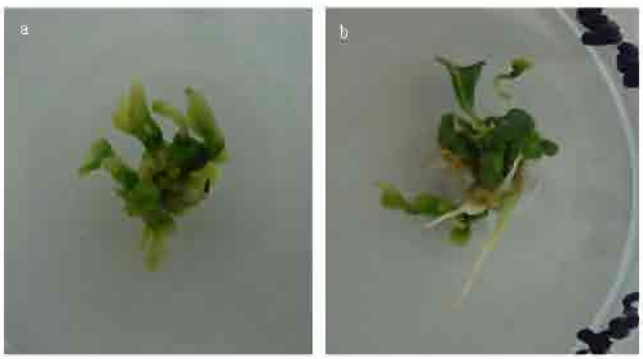

Fig. 5: Developmental stages of somatic embryogenesis of *C. sinesis*. (a) Mature somatic embryo and combination of torpedo and cotyledon stage of somatic embryo after 14 weeks of culture in medium containing 3 mg L^{-1} of NAA and (b) Regeneration (root formation) from somatic embryo of *C. sinensis*

associated with embryogenesis. However, by using the commercialized embryogenic cell suspension culture which also known as bioreactors can help to increase the production of somatic embryos (Asaka *et al*., 1993).

Despite the production of somatic embryos through indirect somatic embryogenesis, cotyledon and mature zygotic embryo explants treated with 5 and 3 mg L^{-1} NAA, respectively also formed the somatic embryos directly without the formation of callus. Direct somatic embryogenesis is a way to produce large number of somatic embryos from which clonal plants can be regenerated (Pareek and Kothari, 2003). This could be due to the fact that the explants were close to the embryonic state and developed the pre-embryogenic determine cells (PEDCs) (William and Maheshwaran, 1986). In the study by Gogate and Nadguda (2003), direct somatic embryo was obtained from the tips of the developing radical of cashew nut. Gupta *et al*. (1997) also induced somatic embryo from the edge of the cut ends of leaf explants of *Psophocarpus tetragonolobus* (L.) DC through direct somatic embryogenesis.

Direct somatic embryo also developed through the 5 developmental stages (globular-, heart shaped-, torpedo-, cotyledonary- and mature embryo). After isolated for 3 weeks and transferred into the new fresh initiation medium, the somatic embryos regenerated to form the plantlet. According to Arnold *et al*. (2002), ouly the mature embryo with normal morphology, have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation developed into normal plants. The percentage of regeneration from somatic embryo in this study was low and required a long period. This possibly due to the medium used. Arnold *et al*. (2002) also stated that regeneration of somatic embryo into plantlets required culture medium lacking PGRs even though auxin and cytokinin stimulate germination in some cases. Besides, the percentage of regeneration can be improved by transferring the somatic embryos into the medium supplemented with 1 mg L^{-1} of gibberellic acid (Kebebew *et al*., 1998).

In this study, the somatic embryo was determined based on its morphology and colour. The somatic embryo can be further confirmed through biochemical markers and histological studies. Biochemical markers for somatic embryogenesis such as the protein and isozyme help to establish embryogenic potential in plant cells for obtaining reasonable regeneration frequencies (Tchorbadjieva, 2005). Meanwhile, the histological studies managed to elucidate the origin and the developmental process of somatic embryo (Nonohay *et al*., 1999). Besides, somatic embryo can be mass propagated using the bioreactor technology. By using the bioreactor technology, unlimited numbers of somatic embryo and artificial seeds can be produced (Malabadi *et al*., 2004). In addition, somatic embryogenesis is the most attractive plant regeneration system for genetic transformation. Genetic transformation is one of the tools expected for introducing traits for instance disease and virus resistance and tolerance to temperature stress which has been difficult through conventional breeding techniques based on sexual crossing (Mishiba *et al*., 2005). Thus, genetic transformation could be one of the alternative tools to increase the yield of *C. sinensis* which greatly affected by greening disease.

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