Propagation of Ornamental Plants
Vol. 14, № 1, 2014: 32-39

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN *OSMANTHUS FRAGRANS* LOUR.

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Abstract

A protocol for plant regeneration of sweet osmanthus (*Osmanthus fragrans* Lour.) *via* somatic embryogenesis has been developed for the first time. Radicle, hypocotyl, plumule and cotyledon regions of zygotic embryos as explants were optimized at their four developmental stages. They were cultured on Murashige and Skoog (MS) medium with a range of combinations of plant growth regulators, to optimize conditions both for callus initiation and for somatic embryo (SE) development. The highest percentage (88%) of embryogenic callus development occurred with cotyledon segments collected at an early cotyledonary stage when cultured on MS medium with the addition of 1.0 mg l^{-1} BA and 0.5 mg l^{-1} 2,4-D. The highest percentage (86.7%) of somatic embryogenesis development and SEs per explant (7.5) were obtained on MS medium supplemented with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA. Over 50% of mature SEs germinated on MS medium.

Key words: embryogenic callus, plant regeneration, zygotic embryos

INTRODUCTION

Sweet osmanthus, *Osmanthus fragrans* Lour. (Oleaceae) is an economically important woody ornamental species native to China, Japan, and the Himalayas (Hu et al. 2009). Because of its strongly fragrant flowers and graceful habit, it is often grown in landscaped gardens or planted to form an avenue of trees, especially under warmer climate in the south of China. In addition, the fresh flowers have long been used in foods, teas, wines, and other beverages (Li et al. 2009). The essential oil extracted from the flowers is commonly used in perfumes and cosmetics (Wu et al. 2009). Although sweet osmanthus has substantial ornamental and economic value, flower fragrance and other ornamental traits vary considerably between individual cultivars or genotypes; and in particular, the short flowering period (only 4-5 days) of the individual florets is a target for improvement through breeding. Thus, there has been increasing interest in the development of plant regeneration systems for sweet osmanthus, using either organogenesis or somatic embryogenesis, both for the large-scale maintenance of pure germplasm and to facilitate plant improvement through molecular genetic techniques. So far, however, an efficient system has not been achieved.

The first micropropagation study reported in sweet osmanthus used shoots and shoot tips as explants (Wang et al. 2001). A later study in our laboratory (Song et al. 2005) used zigotic embryos (ZEs) cultured on Lloyd and McCown (1980) medium with the addition of 1.0 mg l^{-1} N^6-benzyladenine (BA) and 1.0 mg l^{-1} gibberellic acid (GA_3). A number of attempts to achieve callus induction have been made, using explants of young leaves, shoot tips and ZEs, and combinations of plant growth regulators (PGRs), including BA and 2,4-Dichlorophenoxyacetic acid (2,4-D), or BA and indole-3-acatic acid (NAA) (Liang and Liu 2007, Liu and Yuan 2008); however, no successful system for further regeneration has been reported. Recently, we reported an *in vitro* protocol for adventitious shoot regeneration in sweet osmanthus *via* organogenesis from a ZE explant (Zou et al. 2013); however, although a maximum regeneration frequency of 30% was achieved, the regeneration efficiency generally remained low.

Somatic embryogenesis as an alternative approach for achieving plant regeneration is usually initiated by investigating the effects of different explants, genotypes, media, and PGRs (Merkle et al. 2010, Winkelmann et al. 2010, Wang et al. 2012). For example, immature seeds of *Fraxinus excelsior*, collected at an

Received: January 13, 2014

Accepted: March 4, 2014

early stage of cotyledon development, tended to produce somatic embryos (SEs), but only seeds obtained at an advanced stage of maturation showed organogenic phenomena (Capuana et al. 2007). In *F. angustifolia*, the most suitable explants for somatic embryogenesis were immature ZEs, obtained when the endosperm was still liquid and the cotyledons were elongating (Tonon et al. 2001). In *Olea europaea*, radicles were better explants for somatic embryogenesis than cotyledons (Mazri et al. 2011). Thus, ZEs have been used for plant regeneration across a range of species in the Oleaceae family and both the developmental stage and the region of the ZE selected for explant influence the efficiency of the plant regeneration.

Besides the properties of the ZE explants, PGRs are important in the induction of somatic embryogenesis. The best combination of PGRs for induction of somatic embryogenesis in *F. mandshuric*a and *F. excelsior* was, in each case, 8.8 μM 2,4-D and 4.4 μM BA (Capuana et al. 2007, Kong et al. 2012). Thidiazuron (1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea; TDZ) was a potent stimulator of both shoot organogenesis and somatic embryogenesis. Thus, in *F. americana*, shoot organogenesis was induced when ZEs were cultured on medium containing 10 μM TDZ, whereas at much lower concentrations of TDZ (0.1 and 1.0 μM), in the presence of 10 μM 2,4-D, somatic embryogenesis was observed (Bates et al. 1992).

The aim of the present study was to establish an effective regeneration protocol for sweet osmanthus by investigating the effects of: i) the regions of the ZEs selected for explants; ii) the developmental stages of ZEs; and iii) the combination of PGRs (BA and 2,4-D, or TDZ and NAA) on embryogenic callus (EC) induction and on somatic embryogenesis.

MATERIALS AND METHODS

Plant materials

Fruits, collected at four developmental stages from 20-year-old mature plants, were harvested from the campus of Huazhong Agricultural University, Wuhan, China. Seeds with the same size that maintained free from plant disease and insect pests were first obtained from the fruits by soaking, rubbing, and washing, and were then inspected to assess the extent of embryo development. The four developmental stages were as follows: Stage 1 - fruits collected on January 19th (early torpedo stage), with seeds showing young, translucent ZEs (Fig. 1A); Stage 2 - fruits collected on February 23th (late torpedo stage), with seeds showing hypocotyl elongation and cotyledon swelling (Fig. 1B); Stage 3 - fruits collected on March 20th (early cotyledonary stage), with seeds showing full hypocotyl extension and translucent cotyledons (Fig. 1C); and Stage 4 - fruits collected on May 11th (mature cotyledonary stage), with seeds showing completely mature and milky-white ZEs (Fig. 1D).

Seeds were washed under running water for 1.5-2 h. After being peeled, they were first surface-disinfected in 70% (v/v) ethanol for 30 s, washed 4-5 times with distilled water, disinfected with 1% (w/v) mercuric chloride for 10 min, and finally rinsed again 3 times with distilled water. ZE explants were excised from the disinfected seeds (categorized by developmental stage, as indicated above) and then inoculated onto culture medium, for callus induction.

Culture medium

Murashige and Skoog (1962) medium (MS), containing sucrose (30 g l^{-1}), was solidified with solid agar powder (7 g l^{-1}). The pH of the medium was adjusted to 6.0, prior to autoclaving at 121°C for 20 min. Stock cultures used for callus induction and the formation and maturation of SEs were subcultured at 30 days intervals.

Embryogenic callus induction and histological observation

In order to assess EC induction, radicle, the explants of hypocotyl, plumule, and cotyledon-segment (Fig. 1E) taken from ZEs at the four developmental stages were inoculated onto MS medium supplemented with a range of concentrations of 2,4-D (0, 0.5, 1.0, and 2.5 mg l^{-1}) and BA (0, 0.5, 1.0, and 2.5 mg l^{-1}). PGR-free MS medium was set as the control. After 30 days in darkness at 25 ± 2°C, the extent of induction of EC was recorded.

The callus tissues induced with different appearances were picked out and fixed in formalin-acetic acid-alcohol (FAA) for at least 48 h before they were stained with Ehrlich's hematoxylin. The conventional paraffin sectioning at a thickness of 8.0 μm was adopted to conduct the histological observation of the callus tissues under an optical microscope (Kong et al. 2012).

Somatic embryo induction

The callus tissues induced from each of the four ZE-explant regions (radicle, hypocotyl, plumule, and cotyledon) and from each of the four developmental stages, were then transferred to MS medium without PGRs, in order to promote SE formation. The frequency of SE induction and the number of SEs for each explant were recorded after 30 days of culture in darkness at 25 ± 2°C.

The cotyledon regions collected at Stage 3 showed the best efficiency on EC induction when cultured on MS medium with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} 2,4-D. Thus, callus tissues that had been induced by cotyledons at Stage 3, using MS medium with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} 2,4-D as the initial medium, were transferred to MS medium supplemented with a range of concentra-

tions of NAA (0 and 0.5 mg l^{-1}), BA (0, 2.5, 5.0, and 10.0 mg l^{-1}), and TDZ (0, 0.5, and 5.0 mg l^{-1}), in combination. After a further 30 days of culture, in darkness at 25 ± 2°C, both the frequency of SE induction and the number of SEs for each explant were recorded.

Somatic embryo maturation and plantlet regeneration

The calluses with formed somatic embryos were subcultured monthly onto MS medium in darkness at 25 ± 2°C for further development. When developed to the mature cotyledonary stage, SEs were isolated from callus and subcultured on MS medium, under a 14/10 h (light/dark) photoperiod with cool-white fluorescent lighting at a photosynthetic photon flux density of 40 μmol m^{-2} s^{-1}, and the germination rate was recorded 45 days thereafter.

Experimental design and data analysis

Experiments were set up according to a completely randomized design. Each treatment was performed with 20 ZEs and was replicated at least 4 times. The results were arcsine transformed before statistical analysis. All data were analyzed using SAS 8.0. Analysis of variance (ANOVA) was used to test the statistical significance and differences among means were calculated using Duncan's multiple range test at 0.05 level.

RESULTS AND DISCUSSION

EC induction

Plant regeneration is usually influenced by a series of factors, including genotypes and physiological status of explants and the type and concentration of PGRs (Castellanos et al. 2006, Nhut et al. 2007, Elhiti and Stasolla 2012). In preliminary experiments, different explants have been used to induce callus formation, such as the new leaves and shoots growing in spring, young inflorescences and seeds, but only ZE explants were effective in the production of embryogenic tissues. In the present study, both a soft-brown (Fig. 1F) and a soft-white (Fig. 1G) callus was formed readily from radicle or hypocotyl segments; in contrast, a light-yellowish, globular callus (Fig. 1H) was formed mostly from ZE cotyledon-segment explants. It was found that only the yellowish, globular callus could develop into SEs during subsequent culture on differentiation medium. The histological observation revealed that the yellowish, globular callus tissues contained the embryogenic cell masses (Fig. 1I), which had prominent dark nuclei and were closely linked. The embryogenic cell masses were then developed into the globular embryo (Fig. 1J). Cotyledons have also been used as explants for the induction of embryogenic tissues in *F. mandshurica*, *F. americana* and *F. excelsior* (Bates et al. 1992, Capuana et al. 2007, Kong et al. 2012).

The frequencies of EC induction by cotyledon segments from the four ZE developmental stages cultured in the various different initial media varied between 20% and 88%. They showed a greater degree of variation than the frequencies achieved using any of the other three ZE-explant types (radicle, hypocotyl, and plumule) from any of the ZE developmental stages (Table 1). The developmental phase had a significant influence on EC induction when plumules or cotyledons were used as explants. Thus, the highest frequencies of EC induction in the initial culture medium were all obtained using cotyledons collected at Stage 3 (early cotyledonary stage). The ZEs of seeds collected before Stage 1 (early torpedo stage), possessing a globular or heart-shaped embryo, were too young to be separated from the translucent endosperms. Explants taken from fully mature seeds since Stage 4 (mature cotyledonary stage) were not easily induced. It seems that this was a consequence of the complete organ differentiation of mature ZE explants taken at that developmental stage. At an earlier (though well-defined) developmental stage, ZEs tended to be more responsive to the induction stimulus, owing to a more favorable physiological make-up (Tonon et al. 2001, Capuana et al. 2007, Kong et al. 2012). Therefore, cotyledon segments from seeds collected at an early cotyledonary stage were the best explants for EC induction in sweet osmanthus.

A specific concentration of 2,4-D is required for the initiation of cell programming *via* somatic embryogenesis (Da Silva et al. 2009). In this study, both on PGR-free MS medium (control) and on MS medium supplemented with 0.5 mg l^{-1} BA (without 2,4-D), no callus formation was observed to occur during the culture period (Table 1), although some degree of swelling occurred in the explants and finally died. Similarly, in *Olea europaea*, the addition of BA alone was found to be unable to induce callus (Olivicoltura 1988). We observed a much higher frequency of EC induction (> 40%) with cotyledon explants on MS medium when 0.5 mg l^{-1} BA and 0.5 mg l^{-1} 2,4-D were supplied together. When the concentration of BA was set at 0.5 mg l^{-1}, the frequency of EC induction was not significantly affected by increasing the concentration of 2,4-D, indicating that a concentration of 2,4-D greater than 0.5 mg l^{-1} was unnecessary (Table 1). It has been proposed that the addition of BA increases the compactness of callus tissues and that this is necessary for regeneration to occur (Vengadesan et al. 2002). In the present study, no EC was obtained on MS medium supplemented with 0.5 mg l^{-1} 2,4-D alone (Table 1); however, when this concentration of 2,4-D was supplemented with up to 1.0 mg l^{-1} BA, both the frequency of induction and the quantity of EC that was produced increased significantly, from 52.5% to 88% (Table 1). In both *F. mandshuric*a and *F. excelsior*, the best combination of 2,4-D and BA to promote embryogenic tissue induction has been found to be 8.8 μM 2,4-D and 4.4 μM BA, i.e. a 2 : 1 ratio

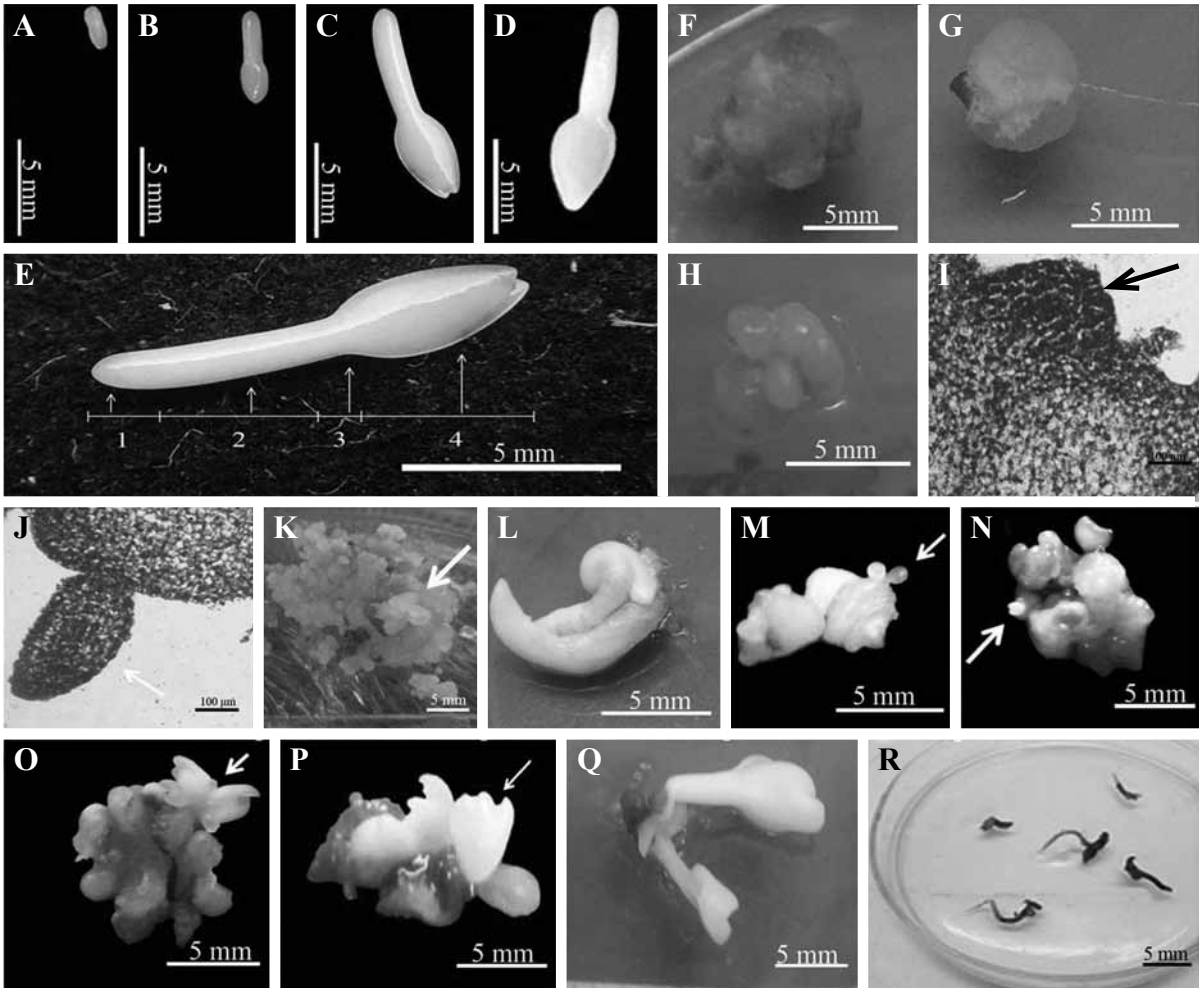

Fig. 1. Plant regeneration from ZE explants *via* somatic embryogenesis. A-E) ZE developmental stages and structure. A) Stage 1: seeds with young and translucent ZE (collected on January 19th), B) Stage 2: seeds with elongated hypocotyl (collected on February 23th), C) Stage 3: seeds with fully extended hypocotyl and translucent cotyledons (collected on March 20th), D) Stage 4: seeds with mature and milky-white ZE (collected on May 11th), E) Types of ZE taken at Stage 3 (arrow indicate, from left to right: 1. radicle; 2. hypocotyl; 3. plumule; 4. cotyledon), F) Soft-brown callus, G) Soft-white callus, H) Light-yellowish, globular callus, I) The embryogenic cell masses (arrow indicate, Bars = 100 µm), J) The globular embryo derived by embryogenic cell masses (arrow indicate, Bars = 100 µm), K) The formation and proliferation of SEs (arrow indicate), L) Abnormal SE, M-P) Development of SEs (arrow indicate), M) Globular stage of SE, N) Heart stage of SE, O) Torpedo stage of SE, P) Cotyledonary stage of SE, Q) Maturation of SE, R) Germination of SE. Bars = 5 mm.

(Capuana et al. 2007, Kong et al. 2012). Similarly, an appropriate combination of BA and 2,4-D is essential for EC initiation in sweet osmanthus.

SE production

It has been reported that radicles are better explants than cotyledons for the induction of somatic embryogenesis in *Olea europaea* (Mazri et al. 2011). In contrast, in the present study, no SEs were induced from radicle explants. Consistent with the literature on both common ash and manchurian ash (Capuana et al. 2007, Kong et al. 2012), our study revealed that cotyledon segments showed a much higher potential for SE induction than any other explants, and the frequency of SE induction was significantly greater than that obtained using either plumule or hypocotyl explants (Table 2). Somatic embryogenesis has been reported to occur only from explants taken when the endosperm is still liquid and the cotyledons are elongating (Tonon et al. 2001). In our study, both the frequency of SE induction and the number of SEs obtained were significantly greater using explants collected at stage 3 than using explants collected at any of the other three developmental stages (Table 2). At stage 3, the seeds were developing fast, but the cotyledons were still translucent and immature. With seeds that were fully mature, the frequency of SE induction from cotyledon explants decreased to the level obtained using explants taken at earlier developmental

Table 1. Effect of combinations of BA and 2,4-D upon the induction of embryogenic callus (%).

PGRs (mg l^{-1})		Region of ZE	Frequency of EC induction (%)			
BA	2,4-D		Stage 1	Stage 2	Stage 3	Stage 4
0 (control)	0	Radicle	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Hypocotyl	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Plumule	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Cotyledon	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
0.5	0	Radicle	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Hypocotyl	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Plumule	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Cotyledon	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
0.5	0.5	Radicle	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Hypocotyl	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Plumule	0.0 ± 0.0 c C	5.0 ± 3.3 cd BC	17.5 ± 4.5 de A	15.0 ± 5.0 c AB
		Cotyledon	22.5 ± 5.9 b B	27.5 ± 6.5 b B	52.5 ± 3.7 b A	30.0 ± 3.8 b B
0.5	1.0	Radicle	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Hypocotyl	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Plumule	0.0 ± 0.0 c B	0.0 ± 0.0 d B	15.0 ± 5.0 def A	12.5 ± 5.3 c A
		Cotyledon	25.0 ± 5.0 b B	32.5 ± 6.5 b B	50.0 ± 3.8 b A	27.5 ± 3.7 b B
0.5	2.5	Radicle	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Hypocotyl	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Plumule	0.0 ± 0.0 c B	0.0 ± 0.0 d B	15.0 ± 5.0 def A	10.0 ± 5.8 c AB
		Cotyledon	20.0 ± 0.0 b B	25.0 ± 5.0 b B	40.0 ± 0.0 bc A	30.0 ± 5.8 b AB
0	0.5	Radicle	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Hypocotyl	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Plumule	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Cotyledon	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
1.0	0.5	Radicle	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Hypocotyl	0.0 ± 0.0 c A	4.4 ± 3.0 cd A	6.7 ± 3.2 fg A	0.0 ± 0.0 d A
		Plumule	15.6 ± 5.6 b A	26.7 ± 4.7 b A	29.3 ± 5.1 cd A	20.0 ± 6.3 bc A
		Cotyledon	64.4 ± 5.6 a B	68.9 ± 5.9 a B	88.0 ± 3.3 a A	68.0 ± 4.9 a B
2.5	0.5	Radicle	0.0 ± 0.0 c A	0.0 ± 0.0 d A	0.0 ± 0.0 g A	0.0 ± 0.0 d A
		Hypocotyl	0.0 ± 0.0 c B	0.0 ± 0.0 d B	10.0 ± 5.8 efg A	0.0 ± 0.0 d B
		Plumule	5.0 ± 5.0 c A	15.0 ± 9.6 c A	20.0 ± 8.2 de A	10.0 ± 5.8 c A
		Cotyledon	20.0 ± 8.2 b B	25.0 ± 5.0 b AB	45.0 ± 5.0 bc A	30.0 ± 5.8 b AB

The means ± standard errors within a column followed by the same small letters are not significantly different at $p \leq 0.05$, according to Duncan's multiple range tests. Means within a row followed by the same capital letters are not significantly different at $p \leq 0.05$.

stages (no higher than 60%). Similar relationship to developmental stage was found for SE induction in both *Olea europea* (Olivicoltura 1988) and *Fraxinus excelsior* (Capuana et al. 2007).

Although it is required for the induction of EC, 2,4-D is inhibitory for the development of SEs (Rai et al. 2007, Zhang et al. 2011). Less than 15% of callus tissues could develop into SEs when they were maintained on the initial MS medium with the addition of 1.0 mg l^{-1} BA and 0.5 mg l^{-1} 2,4-D (Table 3, treatment G). The highest frequency of induction of SEs (86.7%) was obtained on MS medium supplemented with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA (Table 3, treatment E); on this medium, SEs proliferated well (Fig. 1K), producing a average of 7.5 SEs per callus. PGR-free MS medium, on which a frequency of SE induction of 80% was obtained (Table 3, treatment A), and MS medium supplemented with 0.5 mg l^{-1} NAA, on which a frequency of 74.3% was obtained (Table 3, treatment F), were also effective in promoting somatic embryogenesis, but the numbers of SEs resulting from either of these two treatments were much lower than from MS medium supplemented with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA (Table 3, treatment E). In *Olea europaea*, somatic embryogenesis occurred only when NAA was used as the auxin and was inhibited if 2,4-D was used (Olivicoltura 1988). In some species, embryogenic tissues have been found to be able to regenerate new plants *via* both shoot organogenesis

Table 2. Effects of zygotic embryo (ZE) region and developmental stage upon somatic embryogenesis.

Region of ZE	Frequency of SE induction (%)			
	Stage 1	Stage 2	Stage 3	Stage 4
Radicle	0.0 ± 0.0 b A	0.0 ± 0.0 c A	0.0 ± 0.0 c A	0.0 ± 0.0 c A
Hypocotyl	0.0 ± 0.0 b A	0.0 ± 0.0 c A	2.7 ± 1.8 c A	0.0 ± 0.0 c A
Plumule	0.0 ± 0.0 b B	12.0 ± 4.9 b AB	17.3 ± 4.7 b A	16.0 ± 4.0 b A
Cotyledon	33.3 ± 5.8 a C	55.6 ± 5.6 a B	80.0 ± 2.0 a A	56.0 ± 4.0 aB
Region of ZE	No. of SE			
	Stage 1	Stage 2	Stage 3	Stage 4
Radicle	0.0 ± 0.0 b A	0.0 ± 0.0 c A	0.0 ± 0.0 c A	0.0 ± 0.0 c A
Hypocotyl	0.0 ± 0.0 b A	0.0 ± 0.0 c A	0.5 ± 0.4 bc A	0.0 ± 0.0 c A
Plumule	0.0 ± 0.0 b B	1.0 ± 0.6 b AB	1.4 ± 0.4 b A	1.2 ± 0.6 b AB
Cotyledon	2.6 ± 0.2 a B	3.0 ± 0.3 a B	4.3 ± 0.3 a A	3.2 ± 0.2 a B

The means ± standard errors within a column followed by the same small letters are not significantly different at $p \leq 0.05$, according to Duncan's multiple range tests. Means within a row followed by the same capital letters are not significantly different at $p \leq 0.05$.

and somatic embryogenesis pathways (El-Mahrouk et al. 2010). In white ash (*F. americana*), TDZ was effective in inducing both axillary shoot production and SE formation (Bates et al. 1992). In the present study, however, in medium supplemented with either high (Table 3, treatment B,C) or moderate (Table 3, treatment D) concentrations of cytokinins, embryogenic tissues seemed inclined to form SEs rather than adventitious shoots, although adventitious shoot organogenesis occasionally occurred on MS medium supplemented with 2.5 mg l^{-1} BA, 0.5 mg l^{-1} TDZ, and 0.5 mg l^{-1} NAA. Abnormal SEs (Fig. 1L), at a frequency of 40%, were produced on medium that was supplemented with a high concentration of cytokinin (Table 3, treatment B, C). In summary, the findings indicated that SEs, rather than adventitious shoots, were more likely to be induced from cotyledon explants of sweet osmanthus.

Maturation of somatic embryogenesis and plantlet regeneration

Somatic embryogenesis occurs through patterns of histodifferentiation that resemble those that are involved in the formation of ZEs (Thuzar et al. 2011). In the present study, the SEs derived from the yellowish embryogenic calli subsequently developed into the stages of globular embryo (Fig. 1M), heart-shaped embryo (Fig. 1N), torpedo embryo (Fig. 1O), and cotyledonary embryo (Fig. 1P). When they had developed as far as the mature cotyledonary stage (Fig. 1Q), they were placed under illumination to allow germination to occur. Sixty somatic embryos were subjected to germination, and 53.3% of the SEs germinated (Fig. 1R) following 45 days of culture on MS medium in the light.

Acknowledgements: This work was supported the PhD Program Foundation of the Ministry of Educa-

Table 3. Effects of plant growth regulators on somatic embryo formation and proliferation from cotyledon explants.

PGRs (mg l^{-1})					SE production	
Treatment	NAA	BA	TDZ	2,4-D	Induction frequency (%)	No. of SEs
A (control)	-	-	-	-	80.0 ± 2.0 ab	4.3 ± 0.3 b
B	0.5	10.0	5.0	-	48.6 ± 4.0 c	2.3 ± 0.3 c
C	0.5	5.0	0.5	-	40.0 ± 0.0 c	2.1 ± 0.3 c
D	0.5	2.5	0.5	-	55.0 ± 9.6 c	2.1 ± 0.1 c
E	0.5	1.0	-	-	86.7 ± 4.2 a	7.5 ± 0.8 a
F	0.5	-	-	-	74.3 ± 3.7 b	4.6 ± 0.4 b
G	-	1.0	-	0.5	12.0 ± 4.9 d	0.8 ± 0.4 d

The means ± standard errors within a column followed by the same letters are not significantly different at $p \leq 0.05$ according to Duncan's multiple range tests.

tion of China (No. 20130146110022) and the National Natural Science Foundation of China (No. 31070623).

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