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Recent Advances of *In Vitro* Embryogenesis of Monocotyledon and Dicotyledon

Sun Yan-Lin^{1,2} and Hong Soon-Kwan^{2,3}

¹School of Life Sciences, Ludong University, Yantai, Shandong

²Department of Bio-Health Technology, College of Biomedical Science,
Kangwon National University, Chuncheon, Kangwon-Do,

³Institute of Bioscience and Biotechnology,
Kangwon National University, Chuncheon, Kangwon-Do,

¹China

^{2,3}Korea

1. Introduction

Plant tissue and cell culture is a rapid way of achieving plant breeding through the protoplast fusion and regeneration novel hybrid, the production of large numbers of identical individuals and disease and/or pest resistant varieties, thus indirectly increasing the crop yield. Particularly for some plant species, they cannot be improved by conventional breeding because of poor seed germination, frequency of seedling death, or/and environmental challenges such as habitat destruction and illegal and indiscriminate collection. Based on the plasticity and totipotency of plants, plant tissue and cell culture techniques offer a viable tool for mass multiplication and germplasm conservation of some plants, especially those rare and endangered medicinal plants while at the same time facilitating pharmaceutical and other commercial needs (Sahoo & Chand, 1998; Anis & Faisal, 2005). Owing to these useful applications, plant tissue culture technology has now become a remarkably important, useful tool in experimental studies.

The concept of *in vitro* plant cell culture was firstly developed by Gottlieb Haberlandt, a German scientist in 1902. He isolated single fully differentiated individual plant cells from different plant species and cultured them in a nutrient medium containing glucose, peptone, and Knop's salt solution. However, Haberlandt did not succeed to induce plant cells to divide. Later, Hanning (1904) initiated a new line of investigation involving the culture of embryogenic tissue. He excised embryogenic tissues like mature embryos from *Raphanus sativus*, *R. landra*, *R. caudatus*, and *Cochlearia donica* to culture them to maturity on mineral salts and sugar solution. Until in 1934, Gautheret (1934) found successful results on *in vitro* culture of plants. In the following few years, single somatic cells of some green plants have been induced to develop into entire individuals and eventually produce flowers and fruits (Vasil & Hilderbrandt, 1965). In addition, studies of plant tissue culture in monocotyledons were a bit later than that in dicotyledons: Loo (1945) firstly performed stem cultures *in vitro* from apical meristems of monocotyledonous *Asparagus officinalis*; until in 1951, Morel &

Wetmore (1951) successfully obtained the proliferation *in vitro* from tuber of monocotyledonous *Amorphophallus rivieri*. Based on one hundred years' investigation, plant tissue culture technologies have achieved a great progress in many aspects including the effects of plant growth regulators, auxins, and cytokinins, genotype-dependence, callus type-dependence and so on. However, plant tissue and cell cultures in medicinal plants and recalcitrant crops, especially monocotyledonous species and grass species are still deficient. In this chapter, recent advances of *in vitro* embryogenesis of monocotyledon, the halophyte *Leymus chinensis* (Trin.) Tzvel (= *Aneurolepidium chinensis* Trin. Kitag, Poaceae, LC, thereafter) and dicotyledon, the medicinal plant, *Eleutherococcus senticosus* (Rupr. et Maxim.) Harms (= *Acanthopanax senticosus*, Araliaceae, ES, thereafter) will be presented.

LC, a perennial rhizomatous grass belonging to the tribe Poaceae (Czerepanov, 2007), is widely distributed through Northern China, Mongolia and Siberia (Liu et al., 2002a). Due to its intrinsic adaptation to highly alkaline-sodic soil conditions (Jin et al., 2006), this plant species has been used to protect soil and water from loss in arid areas of Northwest of China. Combined with its fine agronomic properties such as rich productivity, high protein content, and palatable to cattle, this plant species has become a major candidate in artificial grassland construction and grassland ecological environment improvement (Jia, 1987). Despite the LC population is common in distinctive regions of China, especially in Songnen Steppe, LC grasslands are being seriously ruined owing to deteriorating environmental conditions, animal destroy, and human destructive activities (Wang et al., 2005). Moreover, the protandry in LC, which limits pollination within flowering shoots, results in self-incompatible and then causes the propagation problem in low seed-set and fecundity (Huang et al., 2004; Wang et al., 2005). Plant breeding or trait improvement in this plant species becomes important and urgently needed.

For *in vitro* embryogenesis of LC, the first report was performed by Gao (1982), using rhizome as explants resulting in about 20% callus induction frequency and 24.2% plant regeneration frequency. Later in 1990, Cui et al. (1990) investigated young rhizome and mature seeds as explants to induce callus induction, and referred to the relationship with callus status and plant regeneration in LC for the first time. However, their callus induction and plant regeneration frequencies were still not very high. In the following few years, many scientists continued to attempt the optimal tissue culture conditions and explants for *in vitro* tissue culture of LC (Liu et al., 2002b; Liu et al., 2004; Sun & Hong 2009, 2010a, 2010b). Induction of embryogenic calli, considered as the most critical step for the success in plant regeneration, is influenced by genotype, explants type, and medium composition as well as by their interaction (Rachmawati & Anzai, 2006). In this chapter, we will summarize the factors influencing LC callus induction, embryogenesis, and plant regeneration efficiency, and focus their interaction.

ES, called Siberian ginseng, Ciwujia in Chinese and Gasiogalpi in Korean, is a woody medicinal plant, distributed in southeast Russia, northeast China, Korea, and Japan (Lee, 1979; Hahn et al., 1985). The cortical root and stem tissues of this species have long been used for medicinal properties (Umeyama et al., 1992; Davydov & Krikorian, 2000). Main active compounds such as triterpene saponins isolated from ES possess important pharmacological activities, including inhibiting histamine release, improving immune system, fighting cancer and aging, and improving adrenal function (Umeyama et al., 1992; Gaffney et al., 2001). However, the poor and/or even failed seed setting, seed dormancy and

over-exploitation always puzzle this species (Yu et al., 2003). Thus, improving its propagation efficiency on enhancing yield and quality to achieve efficient farm cultivation and considerable economic benefits has become an important issue. To achieve this goal, many investigations have been reported, including conventional propagations, habitat conditions, molecular classification, and mass production through *in vitro* tissue cultures.

Conventional propagations of ES have two means: seed propagation and stem cutting propagation. However, until now, two propagations are still considered difficult because of long-term stratification prior to the maturation of the zygotic embryos in mature seeds or difficultly rooting induction from stem cuts (Isoda & Shoji, 1994). Based on this situation, plant cell culture techniques have been applied as a new means for propagation of this species (Choi et al., 1999a, b). Compared with the rise and development of tissue culture in LC, the tissue culture studies in ES initiate relatively late. The first callus induction attempt was done in 1991, and this work reported plant regeneration could be successfully achieved through direct secondary somatic embryogenesis from immature zygotic embryos (Gui et al., 1991). Later, somatic embryos were produced directly from the surface of zygotic embryos of this species without forming an intervening callus (Choi & Soh, 1993). In this report, two kinds of somatic embryos were induced from various explants, including hypocotyls, cotyledon, radicle: one was single embryos with closed radicle mainly formed on cotyledon and radicle, the other was polyembryos mainly formed on hypocotyls. To improve the *in vitro* tissue culture conditions, Yu et al. (1997a, b) attempted to induce embryogenic callus from immature embryos, and obtained high callus formation of 83% on modified SH medium and 100% on B5 medium with 2,4-D addition. Plant regeneration capability of embryogenic callus was different depending on the mature degree of the explants, immature embryos. Choi et al. (1999a) established a high frequency of plant production *via* somatic embryogenesis from callus with cultured on MS medium with 1.0 mg/l 2,4-D for somatic embryo induction and then MS medium lacking 2,4-D before plant regeneration. In the following report by Choi et al. (1999b), various explants such as cotyledon, hypocotyl and root were investigated in plant regeneration *via* direct somatic embryogenesis, of which hypocotyls segments showed the highest somatic embryo formation frequency (75%). This report obtained the highest germination rate of 93% from somatic embryos, and thus established an efficient means for mass propagation through somatic embryogenesis of ES. As known that the somatic embryogenesis and plant regeneration in plants were genotype-specific and explants-specific (Liu et al., 2004; Sun & Hong, 2010), Li & Yu (2002) investigated somatic embryogenesis from various explants including young leaf, stem, node, petiole, peduncle, flower and root using three different genotypes of ES accession Korea, Russia, and Japan. In this report, the highest callus formation frequency was obtained from flower explants, and normal plantlets were produced from somatic embryos when transferred to 1/4 MS medium.

To achieve *in vitro* mass propagation of ES, cell suspension cultures using hypocotyls-derived callus have been firstly conducted by Choi et al. (1999a). However, the somatic embryo formation capacity of suspension cultured cells was significantly lower compared to that from callus cultures. Later, improved cell suspension cultures were observed that 35 g cotyledonary embryos (about 12,000) were converted to 567 g fresh mass of plantlets with initially culture in 500-ml flask, followed by culture in 10-l plastic tank, and then low-

strength MS medium (Choi et al., 2002). This report established an efficient protocol for the mass production of ES plantlet from tank culture of somatic embryos. In the year 2003, the *in vitro* mass propagation conditions were further improved by shortening the maturation time from immature zygotic embryos to somatic embryos within one month (Han & Choi, 2003). Based on the above results, it indicated that *in vitro* mass propagation could be practically applicable for systematic procedure of plant production of ES, and the *in vitro* plantlets could be satisfied as a source of medicinal raw materials, just like *Panax ginseng* (Furuya et al., 1983). Due to no comprehensive review of *in vitro* embryogenesis and plant regeneration on ES to date, we here, summarize the currently available scientific information on ES, aiming to provide the basis of further understanding this species.

2. *In vitro* embryogenesis of monocotyledon

The halophyte forage grass, LC was used as the model monocotyledonous plant for understanding embryogenic callus induction and plant regeneration. The factors affecting embryogenic callus induction efficiency and plant regeneration potential would be summarized as follow:

2.1 Explants type

Plant tissue culture of LC has been investigated using nearly all readily available explants such as mature embryos (Liu et al., 2002b; Kim et al., 2005), mature seeds (Cui et al., 1990; Qu et al., 2004; Kim et al., 2005; Wei et al., 2005; Kong et al., 2008; Sun & Hong, 2009, 2010a), leaf base segments (Liu et al., 2002b; Kim et al., 2005; Sun & Hong, 2009, 2010a), rhizoma (Gao, 1982; Lu et al., 2009), immature inflorescence (Liu et al., 2004), immature spikes (Liu et al., 2002b; Zhang et al., 2007), and root segments (Sun & Hong, 2009), shown in Table 1. In our previous studies (Sun & Hong, 2009; 2010a), mature seed is considered as the optimal explants to induce embryogenic callus, with 56.4 ~ 88.3% of callus induction frequencies. Similar results have been observed in reports of Cui et al. (1990) and Kim et al. (2005) that found mature seeds could produced the highest callus induction frequencies among young rhizome, embryos and leaves as explants, respectively. Using mature seeds as explants to induce callus, it is not only due to the highest callus induction efficiency, but also several advantages such as convenient acquisition and easy conservation in bulk quantities. Except using mature seeds as explants, Liu et al. (2002) suggested that immature stacys were the optimal explants for callus induction with compared to mature embryos and leaf sections, and only calli from immature stacys could regenerate plants. Lu et al. (2009) investigated roots, rhizoma and leaves as explants to induce callus, and found rhizoma are the optimal explants among these three explants. Sun & Hong (2009) have further attempted root segments as explants for callus induction, and increased callus induction frequencies to 71.0 ~ 75.0 %, respectively. However, because the status of calli derived from root segments was less efficient to regenerate shoots or plantlets than that from mature seeds followed by that from leaf base segments, root segments did not use as the optimal explants in further experiments. And in later studies, Sun & Hong (2010a) continued to use mature seeds as the optimal explants and obtained high callus induction frequencies, and authors have also successfully transformed some genes into this grass using this system (data not published).

Plant species	Isolate	Explants	Reference
<i>Aneurolepidium chinensis</i>	---	Rhizoma	Gao 1982
<i>Aneurolepidium chinensis</i> (Trin.) Kitag	Wild-type collected from Jilin, China	Young rhizoma	Cui et al. (1990)
	Wild-type collected from Nei Mongolia, China	Mature seeds	
<i>Leymus chinensis</i> (Trin.) Tzvel.	NM-1	Immature stacys	Liu et al. (2002)
		Mature embryos	
		Leaf sections	
<i>Leymus chinensis</i> (Trin.)	Wild-type collected from Jilin, China in 2001	Mature seeds	Qu et al. (2004)
<i>Leymus chinensis</i>	Nongmu 1	Immature inflorescence	Liu et al. (2004)
	Jisheng 1		
	C-5		
	C-4		
	C-3		
	W4		
	C-2		
C-6			
<i>Leymus chinensis</i>	Wild-type collected from Jilin, China in 2002	Mature seeds	Qu et al. (2005)
<i>Leymus chinensis</i> (Trin.)	Wild-type collected from Anda, Heilongjiang, China in 2003	Embryos	Kim et al. (2005)
		Seeds	
		Leaves	
<i>Aneurolepidium chinensis</i> (Trin.) Kitag	A (grey-green leaf) collected from Daqing, Heilongjiang, China	Mature seeds	Wei et al. (2005)
	B (yellow-green leaf) collected from Daqing, Heilongjiang, China		
	C (grey leaf) collected from Daqing, Heilongjiang, China		
<i>Leymus chinensis</i>	Zaipei-3	Young spikes	Zhang et al. (2007)
<i>Leymus chinensis</i>	Wild-type collected from Daan, China in July, 2004	Mature seeds	Kong et al. (2008)
<i>Leymus chinensis</i>	---	Roots	Lu et al. (2009)
		Rhizoma	
		Leaves	
<i>Leymus chinensis</i> (Trin.) Tzvel.	WT, wild-type collected from Siping, Jilin, China	Mature seeds	Sun & Hong (2009)
		Leaf base segments	
		Root segments	
	JS, a new variety collected from Jisheng Wildrye Excellent Seed Station, Changchun, Jilin, China	Mature seeds	
		Leaf base segments	
		Root segments	
<i>Leymus chinensis</i> (Trin.)	WT, wild-type collected from Siping, Jilin, China	Mature seeds	Sun & Hong (2010a)
		Leaf base segments	
	JS, a new variety collected from Jisheng Wildrye Excellent Seed Station, Changchun, Jilin, China	Mature seeds	
		Leaf base segments	

Table 1. Summary of different isolates and explants of *Leymus chinensis* (Trin.) Tzvel. or *Aneurolepidium chinensis* (Trin.) Kitag., used in different tissue culture systems. --- means undefined in the relevant reference

2.2 Genotypes

Tissue culture capacities are estimated by callus induction and plant regeneration efficiency. For LC, the tissue culture capacities according to different genotypes are shown in Table 2. Cui et al. (1990) only could induce 29.05% of explants into calli and 23.68% of calli into shoots or plantlets using wild-type collected from Inner Mongolia of China, while in the following few years, Liu et al. (2002) have obtained nearly 3 times of callus induction frequency (88%) using NM-1 collected from Inner Mongolia of China compared to that in the study of Cui et al. (1990). In the report of Liu et al. (2002), they suggested that only embryogenic calli derived from immature stacys could be used for plant regeneration, but not other explants; NM-1 had the highest plant regeneration frequency (38%) among all ten genotypes such as WZMQY, SL, and JIS-1. Of them, WZMQY could only induce 3% of embryogenic calli into shoots or plantlets, YHT-w had obtained just 20% of plant regeneration frequency, and JIS-1 as a new variety from Jisheng Chinese Wildrye Excellent Seed Station, Jilin, China, had only resulted in 5% of plant regeneration frequency. Liu et al. (2004) optimized further the tissue culture systems of this grass, suggested that all eight genotypes including Nongmu 1 [the same as NM-1 in Liu et al. (2002)], C-2 ~ 6 (populations derived from Nongmu 1), Jisheng 1 [the same as JIS-1 in Liu et al. (2002)] and W4, had relatively high callus induction frequencies and plant regeneration frequencies, especially in Nongmu 1 and Jisheng 1. Nongmu 1 showed 90.29% of callus induction frequency, and C-6, one of its populations showed 93.21% of callus induction frequency, while the plant regeneration frequencies of Nongmu 1 and C-6 reached 43.66% and 9.46%, respectively. Qu et al. (2004) investigated mature seeds of wild-type collected in Jilin of China as explants and obtained 24% of callus induction frequency and 26.67% of plant regeneration frequency. Kim et al. (2005) used various explants of wild-type collected from Heilongjiang of China and found seeds as the optimal explants for callus induction had 68% of callus induction frequency and 36% of plant regeneration frequency; Wei et al. (2005) used mature seeds of wild-type plants collected from Heilongjiang of China and obtained relatively low callus induction and plant regeneration frequencies, of which A with grey-green leaves had the highest callus induction frequency (20%), but relatively low plant regeneration frequency (2%), B with yellow-green leaves had the lowest callus induction frequency (6%), but the highest plant regeneration frequency (4%). Kong et al. (2008) optimized the tissue culture conditions using mature seeds of wild-type collected from Heilongjiang of China as explants, and obtained 48.3% of callus induction frequency. In the study of Sun & Hong (2009), they used both genotypes, WT (wild-type) and JS [a new variety, same as JIS-1 in the report of Liu et al. (2002) and Jisheng 1 in the report of Liu et al. (2004)], collected from Jilin of China, suggesting that WT had slightly higher callus induction and plant frequency frequencies than JS which had been improved to 88.3% and 70.8 %, respectively. In another study of Sun & Hong (2010a), they also used WT and JS as explants and had 75.6% and 71.0% of callus induction and plant regeneration frequencies, respectively. In this report, WT also showed higher callus induction and plant regeneration potential than JS.

Isolate	Collection origin and year	Explants	Callus induction frequency (%)	Plant regeneration frequency (%)	Reference
w	Inner Mongolia, China	Mature seeds	29.05	23.68	Cui et al. (1990)
w	Jilin, China	Young rhizoma	22.50	14.80	

Isolate	Collection origin and year	Explants	Callus induction frequency (%)	Plant regeneration frequency (%)	Reference
NM-1	Ximeng, Inner Mongolia, China	Immature stacys	88	38	Liu et al. (2002)
NM-1	Ximeng, Inner Mongolia, China	Leaf sections	60	0	
NM-1	Ximeng, Inner Mongolia, China	Mature embryos	45	0	
WZMQY	Hailaer, Inner Mongolia, China	Immature stacys	---	3	
SL	Shuangliao, Jilin, China	Immature stacys	---	11	
GLT	Gaolintun, Inner Mongolia, China	Immature stacys	---	21	
YHT-w	Yihuta, Inner Mongolia, China	Immature stacys	---	20	
CL-w	Changling, Jilin, China	Immature stacys	---	8	
HUIG	Changlin, Jilin, China	Immature stacys	---	8	
CHC-01	Changchun, Jilin, China	Immature stacys	---	10	
JIS-1	Changchun, Jilin, China	Immature stacys	---	6	
JIS-4	Changchun, Jilin, China	Immature stacys	---	12	
Nongmu 1	Inner Mongolia, China	Immature inflorescence	90.29	43.66	Liu et al. (2004)
C-2	Inner Mongolia, China	Immature inflorescence	54.23	7.69	
C-3	Inner Mongolia, China	Immature inflorescence	90.72	6.67	
C-4	Inner Mongolia, China	Immature inflorescence	87.12	5.71	
C-5	Inner Mongolia, China	Immature inflorescence	87.79	12.82	
C-6	Inner Mongolia, China	Immature inflorescence	93.21	9.46	
Jisheng 1	Jilin, China	Immature inflorescence	33.35	10.34	
W4	Inner Mongolia, China	Immature inflorescence	64.95	4.71	Qu et al. (2004)
w	Jilin, China in 2001	Mature seeds	24	26.67	
w	Anda, Heilongjiang, China in 2003	Seeds	68	36	Kim et al. (2005)
w	Anda, Heilongjiang, China in 2003	Leaves	51	36	
w	Anda, Heilongjiang, China in 2003	Embryos	39	36	

Isolate	Collection origin and year	Explants	Callus induction frequency (%)	Plant regeneration frequency (%)	Reference
	China in 2003				
A (grey-green leaf)	Daqing, Heilongjiang, China	Mature seeds	20	2	Wei et al. (2005)
B (yellow-green leaf)	Daqing, Heilongjiang, China	Mature seeds	6	4	
C (grey leaf)	Daqing, Heilongjiang, China	Mature seeds	12	2	
w	Daqing, Heilongjiang, China	Mature seeds	48.3	---	Kong et al. (2008)
WT	Siping, Jilin, China	Mature seeds	88.3	70.8	Sun & Hong (2009)
WT	Siping, Jilin, China	Root segments	71.0	70.8	
WT	Siping, Jilin, China	Leaf base segments	66.7	70.8	
JS	Changchun, Jilin, China	Mature seeds	83.3	68.1	
JS	Changchun, Jilin, China	Root segments	75.0	68.1	
JS	Changchun, Jilin, China	Leaf base segments	74.7	68.1	
WT	Siping, Jilin, China	Mature seeds	75.6	71.0	Sun & Hong (2010a)
WT	Siping, Jilin, China	Leaf base segments	30.0	71.0	
JS	Changchun, Jilin, China	Mature seeds	56.4	69.2	
JS	Changchun, Jilin, China	Leaf base segments	28.9	69.2	

Table 2. Summary of callus induction and plant regeneration frequencies in tissue culture systems using different genotypes. --- means undefined or unverified in the relevant reference. w means wild-type plants in its collection origin

2.3 Medium compositions

2.3.1 Culture media for callus induction

Except effects of explants type and genotypes, the effect of medium compositions in each stage of LC tissue culture is also important and never neglected (Table 3). Gao (1982) has conducted three culture media to induce callus using rhizoma, but could only induce 20% of explants into callus, which also had not high potential of plant regeneration. To improve callus induction conditions, Cui et al. (1990) attempted Murashige and Skoog (MS, Murashige & Skoog, 1962), B5 and 8114 containing 1 ~ 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) to induce callus, suggesting that B5 or MS with 4 mg/l 2,4-D is the most appropriate for callus induction of this grass. Despite there is no a great increase in callus induction frequencies, with about 22%, the study of Cui et al. (1990) is the first report talking about the importance of callus type for regeneration and optimization method of callus

types with cultured on B5 or MS with 1 mg/l 2,4-D before shoot organogenesis. Later, Liu et al. (2002) detected effect of various 2,4-D concentrations (0.5 ~ 2.5 mg/l) on callus induction frequency, suggesting that 2.0 mg/l 2,4-D could induce the highest callus induction frequency, but the callus induction frequencies depend on different plant genotypes. Qu et al. (2004) talked effects of various culture medium types (MS, B5, N6 and MSB) and 2,4-D concentrations (0 ~ 4.0 mg/l) on callus induction frequency, and showed that 2.5 mg/l 2,4-D when added into MS culture medium, induce relatively higher callus compared to other 2,4-D concentrations and there was no significant change on callus induction frequencies among MS, B5, N6 and MSB media all supplemented with 2.0 mg/l 2,4-D. Liu et al. (2004) firstly attempted N6 medium in LC, that is more appropriate for tissue culture of gramineous plants due to lower concentrations of inorganic salts, and some components such as glutamine, proline, and casein hydrolytes that might act as nitrogen supplier also helped enhancement of callus induction. Kim et al. (2005) increased the concentrations of thiamine·HCl (VB1), glycine, and inositol in MS basic salts with additional application of 1.0 mg/l, 2.0 mg/l, and 100 mg/l, respectively. With the addition of 1.5 mg/l 2,4-D, it could cause the highest callus induction frequency. Lower and higher 2,4-D concentrations did not satisfy the demands of high callus induction frequency. However, Wei et al. (2005) suggested that effect of 1.0 mg/l 2,4-D on callus induction is remarkable, and the effect on callus induction is inversely proportionate to the 2,4-D concentration. In this study, compact embryogenic callus could be translated from soft and watery callus with 1 ~ 2 times of subculture on the same medium used for callus induction. Zhang et al. (2007) continued to use 2.0 mg/l 2,4-D as the optimal 2,4-D concentration for callus induction of LC according to the report of Liu et al. (2004). The difference was that there was a process of callus status regulation with transferring callus onto MS medium supplemented with 1.0 mg/l abscisic acid (ABA), 100 mg/l casein hydrolytes, 300 ~ 500 mg/l glutamine, 500 mg/l proline and 2.0 mg/l 2,4-D from MS medium only containing 2.0 mg/l 2,4-D. Newly formed callus appeared white, translucent, watery, and nearly ropy with slow growth, that could not be used for plant regeneration (Zhang et al., 2007; Sun & Hong, 2010a). In Zhang et al. (2007) study, they also investigated N6/MS medium alternation to stimulate the formation of embryogenic callus and the proliferation, and make embryogenic callus compact. Compared to MS medium that is in favor of the embryogenesis of callus and the proliferation of embryogenic callus, N6 medium contains higher nitrate-nitrogen concentrations that results in the formation of compact structure of embryogenic callus, the maintenance of the embryogenesis. In the studies of Kong et al. (2008) and Lu et al. (2009), they all applied only 2,4-D to induce callus production, however, the former suggested 2.0 mg/l 2,4-D is the optimal concentration, while the later suggested moderate low 2,4-D concentration (0.5 mg/l) is more suitable in callus induction from rhizoma than high concentration (1.0 mg/l) and low concentration (0.1 mg/l). To optimize the tissue culture conditions further, Sun & Hong (2009) investigated effects of plant hormone 2,4-D, nitrogen supplier and high osmosis maker, glycine and proline, nitrate-nitrogen enhancer, KNO₃ on callus induction by L₉(3⁴) orthogonal test, suggesting that using mature seeds as explants demands higher 2,4-D concentration, the optimal medium compositions varies in different explants and genotypes. In our following study, Sun & Hong (2010a) added freshly 5.0 mg/l L-glutamic acid combined with 2.0 mg/l 2,4-D in MS medium, suggesting that the inclusion could significantly promote primary callus induction. Culturing on the same

medium for 1 ~ 2 months was essential for the embryogenic callus maturation and the optimization of callus status.

Isolate	Optimal components in media	plant regeneration	Reference
w	B5 or MS + 2,4-D 4 mg/l	MS + 0.5 mg/l BA	Cui et al. (1990)
NM-1	MS + 2 mg/l 2,4-D	MS + 1.0 mg/l KT, 0.5 mg/l NAA	Liu et al. (2002)
WZMQY		MS + 1.0 mg/l KT, 0.5 ~ 1.0 mg/l NAA	
SL		MS + 1.0 mg/l KT, 0.5 mg/l NAA	
GLT		MS + 1.0 mg/l KT, 0.5 mg/l NAA	
YHT-w		MS + 1.0 mg/l KT, 0.5 mg/l NAA	
CL-w		MS + 1.0 mg/l KT, 1.5 mg/l NAA	
HUIG		MS + 1.0 mg/l KT, 0.5 ~ 1.0 mg/l NAA	
CHC-01		MS + 1.0 mg/l KT, 0.5 mg/l NAA	
JIS-1		MS + 1.0 mg/l KT, 1.0 mg/l NAA	
JIS-4		MS + 1.0 mg/l KT, 0.6 mg/l NAA	
w	MS + 2.5 mg/l 2,4-D	MS + 0.5 mg/l BA	Qu et al. (2004)
Nongmu 1	N6 + 5.0 mg/l Glutamine, 500 mg/l Proline, 500 mg/l Casein hydrolytes, 2.0 mg/l 2,4-D	N6 + 1.0 mg/l KT, 1.0 mg/l BA	Liu et al. (2004)
Jisheng 1			
C-5			
C-4			
C-3			
W4			
C-2			
C-6			
w	MS + 1.5 mg/l 2,4-D, 1.0 mg/l Thiamine HCl, 2.0 mg/l Glycine, 100 mg/l Myo-inositol	MS + 2.0 mg/l KT, 0.5 mg/l NAA	Kim et al. (2005)
A (grey-green leaf)	MS + 1.0 mg/l 2,4-D	1/2 MS + 0.5 ~ 1.5 mg/l NAA	Wei et al. (2005)
B (yellow-green leaf)	MS + 2.0 mg/l 2,4-D		
C (grey leaf)	MS + 1.0 mg/l 2,4-D		
Zaipei-3	MS + 2.0 mg/l 2,4-D	MS/N6 + 2.0 mg/l 2,4-D, 1.0 mg/l ABA, 100 mg/l Casein hydrolytes, 300 ~ 500 mg/l Glutamine, 500 mg/l Proline	Zhang et al. (2007)
w	MS + 2.0 mg/l 2,4-D	---	Kong et al. (2008)
---	MS + 0.5 mg/l 2,4-D	---	Lu et al. (2009)

Isolate	Optimal components in media	plant regeneration	Reference
WT	MS + 1.0 mg/l 2,4-D, 4.0 mg/l Glycine, 0.3 g/l Proline, 1.0 g/l KNO ₃	MS + 0.2 mg/l NAA, 2.0 mg/l KT, 2.0 g/l casamino acid	Sun & Hong (2009)
	MS + 1.0 mg/l 2,4-D, 4.0 mg/l Glycine, 0.3 g/l Proline, 1.0 g/l KNO ₃		
	MS + 0.5 mg/l 2,4-D, 2.0 mg/l Glycine, 0.5 g/l Proline, 1.0 g/l KNO ₃		
JS	MS + 2.0 mg/l 2,4-D, 1.0 mg/l Glycine, 1.0 g/l Proline, 1.0 g/l KNO ₃		
	MS + 0.5 mg/l 2,4-D, 2.0 mg/l Glycine, 0.5 g/l Proline, 1.0 g/l KNO ₃		
	MS + 1.0 mg/l 2,4-D, 1.0 mg/l Glycine, 0.5 g/l Proline, 2.0 g/l KNO ₃		
WT	MS + 2.0 mg/l 2,4-D, 5.0 mg/l L-glutamic acid	MS + 0.2 mg/l NAA, 2.0 mg/l KT, 2.0 g/l casamino acid	Sun & Hong (2010a)
JS		MS + 0.5 mg/l NAA, 2.0 mg/l KT, 2.0 g/l casamino acid	

Table 3. Summary of the optimal medium compositions in callus induction and plant regeneration stages. --- means undefined or unverified in the relevant reference. w means wild-type plants in its collection origin

2.3.2 Culture media for plant regeneration

The final aim of plant tissue culture is still plant regeneration, of which the appropriate concentration combination of medium compositions in plant regeneration media plays an important role (Table 3). From 1982, Gao (1982) has been able to regenerate whole plants from rhizoma, just with low plant regeneration frequency (24.2%). Later in 1990, Cui et al. (1990) performed plant regeneration on MS medium containing 0.5 mg/l 6-benzyladenine (BA), but this still did not largely enhance plant regeneration frequency (14.8 ~ 23.68%). Qu et al. (2004) also investigated plant regeneration on MS medium containing 0.5 mg/l BA, and similar results were obtained, with 26.67% of the plant regeneration frequency. Liu et al. (2002) attempted kinetin (KT) and α -naphthalene acetic acid (NAA) to induce shoot organogenesis in plant regeneration stage, and obtained 38% of the highest plant regeneration frequency using NM-1 genotype. In 2004, Liu et al. (2004) further optimized plant regeneration medium compositions using N6 medium supplemented with 1.0 mg/l KT and 1.0 mg/l BA, and increased plant regeneration frequency (43.66%) once again using Nongmu 1 genotype. In this study, plant regeneration efficiencies varying according to different genotypes are obvious that C-4 and W4 showed only 5.71 ~ 4.71% of plant regeneration frequency. In following study of Liu et al. (2006), it was reported that N6 medium supplemented with 0.3 ~ 2.5 mg/l BA and 0.3 ~ 2.5 mg/l KT could efficiently regulate callus status and thus induce high plant regeneration frequency. Kim et al. (2005) also detected effects of NAA and KT on plant regeneration frequency and found 0.5 mg/l

NAA the most suitable for plant regeneration of wild-type plants collected from Heilongjiang, China when combined with 2.0 mg/l KT in MS medium. Wei et al. (2005) reported that embryogenic callus induction and shoot organogenesis could be accomplished one-step on consistent culture media. Zhang et al. (2007) found ABA, casein hydrolytes, glutamine and proline combined with alternately culture on MS/N6 medium could efficiently improve callus status, and stimulate plant regeneration. Summarized previous studies, Sun & Hong (2009, 2010a) freshly added 2.0 g/l casamino acid combined with 2.0 mg/l KT and low concentrations of NAA (0.2 ~ 0.5 mg/l) in MS medium to increase plant regeneration efficiency, and resulted in relatively high frequencies (54.0 ~ 71.0%).

2.4 Other effects on callus induction

2.4.1 Temperature

Optimal temperature is mainly considered as the requirement of plant growth, however, temperature as one influence factor in plant tissue culture is rarely reported. For LC, most tissue culture systems have been performed under 22 ~ 28°C without special explanation (Table 4). Until in 2008, Kong et al. (2008) firstly brought forward that variable temperature results in high callus induction and proliferation frequencies through improving the callus status. Callus induction frequency under variable 16°C/26°C was twice higher compared to that under invariable 26°C, that was explained that alternating temperature could break seed dormancy and thus induce callus induction.

Isolate	Explants	Temperature (°C)	Objection and Remarks	Reference
---	Rhizoma	---	Plant regeneration	Gao 1982
Wild-type collected from Jilin, China	Young rhizoma	---	Regulation of callus status and plant regeneration	Cui et al. (1990)
Wild-type collected from Nei Mongolia, China	Mature seeds			
NM-1	Immature stacys	25	Plant regeneration	Liu et al. (2002)
	Mature embryos		Non regenerated plants	
	Leaf sections		Non regenerated plants	
Wild-type collected from Jilin, China in 2001	Mature seeds	26	Plant regeneration	Qu et al. (2004)
Nongmu 1	Immature inflorescence	25	Plant regeneration	Liu et al. (2004)
Jisheng 1				
C-5				
C-4				
C-3				
W4				
C-2				
C-6				
Wild-type collected from Jilin, China in 2002	Mature seeds	---	Research on salt-tolerance of callus	Qu et al. (2005)
Wild-type collected from Anda, Heilongjiang, China in 2003	Embryos	24 ± 2	Plant regeneration	Kim et al. (2005)
	Seeds			
	Leaves			

Isolate	Explants	Temperature (°C)	Objection and Remarks	Reference
A (grey-green leaf) collected from Daqing, Heilongjiang, China	Mature seeds	25 ± 2	<i>In vitro</i> 12 plants regenerated from this system	Wei et al. (2005)
B (yellow-green leaf) collected from Daqing, Heilongjiang, China				
C (grey leaf) collected from Daqing, Heilongjiang, China				
Zaipei-3	Young spikes	22-26	Regulation of callus status	Zhang et al. (2007)
Wild-type collected from Daan, China in July, 2004	Mature seeds	16/26	Research on the relationship between variable cultivating temperature and seed dormancy and callus induction frequency	Kong et al. (2008)
---	Roots	---	Optimization of callus induction	Lu et al. (2009)
	Rhizoma			
	Leaves			
WT, wild-type collected from Siping, Jilin, China	Mature seeds	28 ± 2	Plant regeneration and optimization of callus induction medium by four-factor-three-level [L ₉ (3 ⁴)] orthogonal test	Sun & Hong (2009)
	Leaf base segments			
	Root segments			
JS, a new variety collected from Jisheng Wildrye Excellent Seed Station, Changchun, Jilin, China	Mature seeds	28 ± 2	Optimization of callus induction and plant regeneration media by the addition of growth regulators and plant regeneration	Sun & Hong (2010a)
	Leaf base segments			
	Root segments			
WT, wild-type collected from Siping, Jilin, China	Mature seeds	28 ± 2	Optimization of callus induction and plant regeneration media by the addition of growth regulators and plant regeneration	Sun & Hong (2010a)
	Leaf base segments			
JS, a new variety collected from Jisheng Wildrye Excellent Seed Station, Changchun, Jilin, China	Mature seeds	28 ± 2	Optimization of callus induction and plant regeneration media by the addition of growth regulators and plant regeneration	Sun & Hong (2010a)
	Leaf base segments			

Table 4. Summary of temperature used for callus induction and plant regeneration of *L. chinensis*. --- means undefined or unverified in the relevant reference

2.4.2 Seed dormancy

Seed dormancy of LC is the key factor in the inhibition of germination rate that is considered as the main connection with callus induction frequency (Cui et al., 1990), thus, breaking dormancy becomes the sticking point of increasing germination rate and subsequent enhancing callus induction frequency. Ma et al. (2005) has reported that dormancy style of LC belongs to inhibitor-induced physiological dormancy, and one of the key inhibitors is ABA. However, Zhang et al. (2007) reported that low concentration of ABA helps the callus embryogenesis and maintenance of callus compact structure. Zhang et al.

(2007) also suggested that low concentration of ABA creates high-osmotic and dry conditions to stimulate cell growth, while high concentration inhibits the callus embryogenesis regulation and even cause callus browning. Except the variable temperature applied by Kong et al. (2008), many methods including polyethylene glycol (PEG) treatment, exogenous hormone addition and saturation in flowing cold water have also been investigated by scientists (Ma et al., 2005).

2.4.3 Nitrogen source

High ammonium-nitrogen MS medium is reported to be able to stimulate the callus embryogenesis, while low ammonium-nitrogen B5 medium is more suitable for tissue and suspension cell culture of some plant species than MS medium (Table 5). Early in 1990, Cui et al. (1990) have investigated callus induction of LC on B5 medium, but have not obtained significant results compared to that on MS medium. Later, the results of Qu et al. (2004) suggested further B5 did not cause significantly high callus induction frequency in tissue culture of LC. Except B5 medium, high nitrate-nitrogen N6 medium is reported to favor in the formation of callus compact structure and maintenance of callus embryogenesis (Table 5). From the year of 2004, Qu et al. (2004) and Liu et al. (2004) have chosen N6 as basic salts in optimal tissue culture media. However, Qu et al. (2004) found that using N6 and even MSB medium for callus induction did not have remarkable changes compared to using MS medium. Until in 2007, Zhang et al. (2007) further investigated MS/N6 alternating medium to meet needs of callus induction, embryogenesis and maintenance. To improve the callus status and maintain the embryogenesis, many scientists also added some organic nitrogen

Component	MS	N6	B5	MSB
	Concentration (mg/l)	Concentration (mg/l)	Concentration (mg/l)	Concentration (mg/l)
KNO ₃	1900	2830	2500	1900
NH ₄ NO ₃	1650	463		1650
KH ₂ PO ₄	170	400		170
MgSO ₄ ·7H ₂ O	370	185	250	370
CaCl ₂ ·2H ₂ O	440	165	150	440
KI	0.83	0.80	0.75	0.83
H ₃ BO ₃	6.2	1.6	3.0	6.2
MnSO ₄ ·4H ₂ O	22.3	4.4	10	22.3
(NH ₄) ₂ SO ₄			134	
ZnSO ₄ ·7H ₂ O	8.6	1.5	2.0	8.6
Na ₂ MoO ₄ ·2H ₂ O	0.25		0.025	0.25
CuSO ₄ ·5H ₂ O	0.025		0.025	0.025
CoCl ₂ ·6H ₂ O	0.025		0.025	0.025
Na ₂ -EDTA	37.3	37.3	37.3	37.3
FeSO ₄ ·7H ₂ O	27.8	27.8	27.8	27.8
Inositol	100		100	100
Glycine	2.0	2.0		2.0
Nicotinic acid	0.5	0.5	1.0	1.0
VB1	0.1	1.0	10	10
VB6	0.5	0.5	1.0	1.0

Table 5. Components of common culture media including MS, N6, B5 and MSB

sources, such as glutamine, proline, casein hydrolytes, glycine and casamino acids (Liu et al., 2004; Zhang et al., 2007; Sun & Hong 2009, 2010a), and many evidence suggested that these accessions of organic nitrogen sources, or even additional nitrate-nitrogen sources like KNO_3 (Sun & Hong 2009), have greatly improved the callus status and enhanced the callus embryogenesis and maintenance.

2.4.4 Others

Pre-culture on medium with lower 2,4-D concentrations than medium used for primary callus induction before transferring onto plant regeneration medium results in efficient and rapid plant regeneration (Liu et al., 2002b). Cui et al. (1990) firstly attempted this method in improvement of callus status, suggesting that removing 2,4-D and adding inositol and casein hydrolytes could regulate callus status to increase plant regeneration frequency. Later, this method continued to be used in many studies (Liu et al., 2002b; Qu et al., 2004; Kim et al., 2005; Wang et al., 2006; Kong et al., 2008), as well as suggesting that lower 2,4-D concentration could help the following plant regeneration. In addition, to quicken the production of embryogenic callus and *in vitro* regenerated plants of LC, a novel plant regeneration system from suspension-derived callus has been established by Sun & Hong (2010). This cell suspension culture system makes significantly greater increment of callus biomass and more stable culture conditions than the conventional tissue culture system.

3. *In vitro* embryogenesis of dicotyledon

The medicinal plant, ES was used as the model dicotyledonous plant for understanding the embryogenic callus induction and plant regeneration. The factors affecting embryogenic callus induction efficiency and plant regeneration potential would be presented here.

3.1 General introduction of ES

ES is a woody medicinal plant that grows only in cold regions of Asia (Lee, 1979). Due to the over-exploitation of ES, combined with poor seed setting and/or failure to set seed (Yu et al., 2003), this plant has become an endangered species in several countries, and even classified as rare, protected plants by the Environmental Ministry in Korea (Jung et al., 2004). To develop the farm cultivation of ES, many investigations are involved in the natural growth conditions of habitats. As known that the region, Hokkaida in Japan is the location adapting to the natural growth and seed production of ES, Park et al. (1995) therefore compared the natural condition factors such as local temperature and sunshine duration of Hokkaida in Japan with several locations in Korea to select a proper seed production site in Korea. This investigation suggested that Daegwanryeong in Korea is the most suitable for ES cultivation from seed propagation, because its climate characteristics are mostly similar to those of Hokkaido. In the further investigation, Park et al. (1996) mentioned Mountain Deokyu situated at 127°45'E, 35°52'N, is one of main habitats of ES in Korea. To understand more habitat information to instruct farm cultivation of ES in Korea, Park et al. (1996) surveyed the local climate, soil components, and symbiotic plant species as information inferences. To optimize the cultivation conditions of ES, Han et al. (2001) investigated the effect of shading treatments on the growth of ES, and suggested that 50% shading net treatment was most effective for yield. Kim et al. (2003) deemed that shading treatments could increase not only apparent quantum yield, but carboxylation efficiency and re-phosphorylation.

Since long-term stratification during afterripening period is required to induce maturation of the zygotic embryos in mature seeds (Isoda & Shoji, 1994), Park et al. (1997) studied the characteristics of embryo elongation after stratification and the dehiscence rate during afterripening period, which would help improve seed propagation of this species.

In addition, seed dormancy also entangles germination and propagation of this species. ES is known to have double dormancy: morphological rudimentary dormancy influenced by surrounding endosperm, and physiological dormancy after post-maturation of zygotic embryos (You et al., 2005). To date, several studies have been attempted to break seed dormancy in order to promote the seed germination, but most studies only focus on its physiological dormancy. For example, Li et al. (2003a) investigated a method for breaking the physiological dormancy of dehisced ES seeds, and suggested that storage at 5°C for 85 d could most effectively increase germination rates up to more than 90%. In the report by Li et al. (2003b), they performed cold stratification before sowing, combined with gibberellic acid (GA₃) soak. This result showed 10 d-cold stratification at 4°C following afterripening, and soaking in 500 ppm GA₃ for 3 d could also effectively promote germination. As the effective influence of GA₃ soak on germination, Lim et al. (2008) also applied this method as pretreatment of ES seeds, however, due to the different experimental materials and specific sensitivity to GA₃ soak, they elucidated the optimal GA₃ concentration was 300 mg/l for promoting the seed afterripening. And Toros sterilization was synchronously performed in ES seeds, showing positive effect on reducing dehiscent rates and suppressing fungi actions. To break another dormancy of ES, You et al. (2005) applied endosperm removal during *in vitro* culture of excised seeds and plant regeneration, and the removal of endosperm tissue not only broke the morphological rudimentary dormancy but markedly stimulated the growth of rudimentary zygotic embryos. To improve the efficiency of dormancy breaking, GA₃ treatment in 2.0 mg/l was together used in the *in vitro* culture of excised seeds.

Except of seed propagation, stem cutting propagation is widely used for propagation of ES, however, difficult rooting is a major problem to resolve. Park et al. (1994) suggested that rooting could be successfully induced from cut of stems after 3 ~ 12 d-culture, and the season for cutting propagation is also important, the late September being the best cutting season in Korea. Han et al. (2001) indicated that up-ground 30 cm-length cutting was the most effective for branching stem length, plant height and yield.

Despite a great process has been achieved on conventional propagation, this propagation pattern is known limited by some disadvantages, such as requiring enormous time and labor, and particularly long-term stratification for ES (Choi & Jeong, 2002). Thus, the establishment of more efficient propagation methods is urgently needed. Decently, *in vitro* callus induction and plant regeneration through embryogenesis has become rapid, efficient propagation means.

3.2 *In vitro* plant regeneration

Based on the plasticity and totipotency of plants, tissue culture technology has now become a remarkably useful tool in experimental studies, such as rapidly achievement of plant breeding and mass propagation. Based on one hundred years' investigation, plant tissue culture technologies have achieved a great progress in many aspects including the effects of plant growth regulators, auxins, and cytokinins, genotype-dependence, and callus type-dependence.

3.2.1 Effect of 2,4-D

In general, 2,4-D is an important inducer for somatic embryogenesis, and this inducer has also been used for the induction of somatic embryos of ES in many investigations (Gui et al., 1991; Han & Choi, 2003). And the importance of 2,4-D has been early affirmed by Gui et al. (1991) that 0.5 mg/l 2,4-D could produce mature embryos developed somatic embryos directly from swollen cotyledon and embryo axes, but most embryos only germinated on the above medium without 2,4-D. In the following investigations, the most optimal callus induction media were mainly composed with 2,4-D alone or combined with the addition of another growth inducer, thidiazuron (TDZ). For instance, Choi & Soh (1993) suggested that 1.0 mg/l 2,4-D could induce more calli from various explants of ES, and successfully achieve the transference from callus induction to plant regeneration on the same medium. Yu et al. (1997b) suggested that treatment with 2,4-D had better efficiency in callus induction than treatment of TDZ, however the plant regeneration was reversed. Yu et al. (1997a) have investigated the effects of 2,4-D and TDZ on callus formation and plant regeneration, suggesting that treatment of 2,4-D induced more calli than treatment of TDZ alone, and treatment of 2,4-D combined with TDZ had higher callus formation than treatment of 2,4-D alone. In addition, Yu et al. (1997a) also attempted various basic salts as main medium, such as WPM and SH, among which SH medium containing 1.0 mg/l 2,4-D showed 83% of callus induction frequency. Yu et al. (1997a) suggested that plant regeneration differed depending on the mature degree of immature embryo. Choi et al. (1999a) induced directly embryogenic callus without intervening callus formation on MS medium containing 1.0 mg/l 2,4-D, but the embryogenic callus formation frequency was not very high. However, embryogenic calli were transferred to MS medium lacking 2,4-D to induce somatic embryo development, and amounts of somatic embryos were produced. Li & Yu (2002) attempted to induce callus from various explants, different genotypes, and both 2,4-D concentrations (2.0 mg/l and 4.0 mg/l), and suggested that MS medium containing 2.0 mg/l 2,4-D combined with 2.0 mg/l TDZ, or 4.0 mg/l 2,4-D and 1.0 mg/l TDZ had the highest efficiency in callus formation. According to callus induction of ES, the most optimal 2,4-D concentrations were reported to be arranged between 0.5 mg/l and 4.0 mg/l (Table 6). Generally, the concentrations of alone 2,4-D were relatively low, arranging between 0.5 mg/l and 1.0 mg/l, while the concentrations of 2,4-D combined with TDZ showed higher than those with alone 2,4-D addition, arranging between 1.0 mg/l and 4.0 mg/l.

From our summary, it was obvious that 2,4-D is critical for callus initiation and embryogenic callus formation in ES, particularly when combined with the supplement of TDZ.

3.2.2 Effects of other callus inducers

Except the important growth inducer, 2,4-D, other many growth inducers also played important roles in callus induction, somatic embryo maturity, and even plant regeneration (Table 7). In the early report of Gui et al. (1991), BA and NAA were also used for callus induction and embryogenesis. Medium supplemented BA combined with NAA or 2,4-D only caused embryos enlarge, swell, callus, but did not produce somatic embryos or adventitious buds or shoots. This suggested that BA or NAA was not more efficient than 2,4-D in embryogenesis of ES. However, 0.5 mg/l 2,4-D or 1.0 ~ 3.0 mg/l indole-3-acetic acid (IAA) or 0.5 mg/l zeatin or 0.2 mg/l NAA was suggested to be favored in somatic embryo development and maturity by Gui et al. (1991). Later, TDZ that has both auxin- and cytokinin-

like activity and can be substituted for auxins or combinations of auxins and cytokinins (Shen et al., 2007; Singh et al., 2003), was used to improve the callus induction conditions of ES (Yu et al., 1997a). It was suggested that alone TDZ, IAA, NAA, or BA with different concentrations was investigated to induce callus induction (Yu et al., 1997a), only alone BA could induce callus successfully. The callus induction frequency caused by alone BA was very low, reaching only 20%. However, if optimal concentration of TDZ (0.7 mg/l) mixed with the addition of 2,4-D, the callus induction frequency was largely increased, having 4-5-fold increase (Yu et al., 1997a). For plant regeneration, Yu et al. (1997a) suggested that the combination of growth inducers did not had better regeneration efficiency than single addition of growth inducer, and lower concentration of TDZ showed the highest plant regeneration frequency in MS, MSB5, and B5 medium. In the following study by Yu et al. (1997b), the optimal concentration of TDZ showed closed relationships with the basic salts in callus induction medium, suggesting that alone 2,4-D without the addition of TDZ produced high callus induction frequency using SH medium as the basic salts, while higher concentration of TDZ was required to be combined with 2,4-D when using WPM medium as the basic salts (Table 6). In addition, Li & Yu (2002) firstly attempted to use indole-3-butyric acid (IBA), combined with TDZ to induce callus, however, the callus induction frequencies of different ES genotypes were generally lower than those with the treatment of 2,4-D combined with TDZ.

Growth inducers	Concentration (mg/l)	Remarks	References
2,4-D	0.5		Gui et al. (1991)
	1		Choi & Soh (1993)
	2	Combined with the addition of 0.7 mg/l TDZ	Yu et al. (1997a)
	1	Using SH medium as the basic salts	Yu et al. (1997b)
	1	Combined with the addition of 3.0 mg/l TDZ, using WPM medium as the basic salts	
	1		Choi et al. (1999a)
	1		Choi et al. (1999b)
	2	Combined with the addition of 2.0 mg/l TDZ	Li & Yu (2002)
	4	Combined with the addition of 1.0 mg/l TDZ	

Table 6. The most optimal concentrations of 2,4-D in callus induction

Growth inducers	Concentration (mg/l)	Treatment for somatic embryo development	References
BA	0.5 ~ 2.0	2,4-D 0.5 mg/l, or IAA 1.0 ~ 3.0 mg/l, or zeatin 0.5 mg/l, or NAA 0.2 mg/l	Gui et al. (1991)
NAA	0.5		
TDZ	0.7	2,4-D 1.0 ~ 4.0 mg/l, or TDZ 0.02 ~ 2.2 mg/l, or IAA 1.0 ~ 2.0 mg/l, or NAA 1.0 ~ 2.0 mg/l, or BA 1.0 mg/l	Yu et al. (1997a)
TDZ	3.0	Combination of 2,4-D 0.1 ~ 2.0 mg/l and TDZ 0.1~3.0 mg/l	Yu et al. (1997b)
TDZ	0.07 ~ 10	Combination of the addition of TDZ with 2,4-D or IBA, and culture in MS free liquid medium	Li & Yu (2002)
IBA	10		

Table 7. Other growth inducers during callus induction stage and treatment for somatic embryo development

3.2.3 Explant type

Explant type is another important factor affecting callus initiation efficiency. Generally, younger, more rapidly growing tissue is most effective. There have been many evidences indicating that plant regeneration potentials in many plant species have a direct correlation with the developmental stage of the explants tissue, such as in rice (Sahrawat & Chand, 2001), wheat (Wernicke & Milkovits, 1984), oat (Chen et al., 1995). For the monocotyledonon LC, mature seed has been reported to be the optimal explant for callus initiation according to the description of Part 2.1. For the dicotyledon ES, the investigations and attempts about appropriate explants have also been much studied (Table 8).

First, swollen cotyledon and embryo axes were used to induce callus, and they develop somatic embryos from the epidermal or subepidermal layer of the cotyledons or embryonic axes (Gui et al., 1991). The precedence of both explants, though, was not discussed, the somatic embryos rapidly developed into globular or heart-shaped structures, and germinated normally. Choi & Soh (1993) attempted various explants to initiate callus induction, including cotyledon, hypocotyl, radicle, and intact embryo and wounded embryo, of which wounded embryo produced the highest somatic embryo formation (77.8%). With 6 week-culture, wounded embryo-derived callus appeared torpedo shape and even cotyledonary embryo, while other calli derived from intact embryo, cotyledon, hypocotyl, or radicle appeared globular, heart shape, few torpedo shape, but no cotyledonary embryo. Later, immature embryo was firstly used as explant by Yu et al. (1997a, b), and produced high somatic embryo formation.

Embryogenic cells treated as artificial seeds for obtaining plants directly have been reported for several crops of agricultural interest (Kitto & Janick, 1985; Redenbaugh et al., 1986; Choi & Jeong, 2002). To achieve ES embryogenic cells as artificial seeds, Choi et al. (1999a, b) investigated different explants to attempt to initiate callus induction in a simple and efficient way. Among cotyledon, hypocotyl, and root of zygotic embryos, hypocotyl was considered as the most optimal explant of ES, and had the highest frequency of somatic embryo formation. Using hypocotyl-derived embryogenic cells from this system, mass production through large-scale tank culture were successfully obtained, with approximately 27-fold increment of fresh weight of somatic embryo after 4 week-culture (Choi et al., 2002). In addition, mass production through cell suspension culture was also done by comparing the somatic embryo formation from cotyledon, hypocotyl, and radicle-derived embryogenic cells (Han & Choi, 2003). In this study, embryogenic cells derived from hypocotyl of zygotic embryos showed the highest growth rate and somatic embryo formation of 89%. Based on the above suggestions, it is indicated that even mass production of plant cell through large-scale suspension culture has been successfully obtained, ES plantlets produced from this system could be more convenient to be used as a source of medicinal raw materials. However, due to direct sowing of artificial seeds in the field for practical use, low soil survival becomes a major problem (Redenbaugh et al., 1986). Herein, Choi & Jeong (2002) overcame the problem of low soil survival, and reported firstly encapsulated somatic embryos as ES artificial seeds to achieve all development status from artificial seeds to whole plants. Later, Jung et al. (2004) further improved this system with the addition of carbon sources to the encapsulation matrix, and obtained that 96% of the encapsulated embryos converted to plantlets with well-elongated epicotyls.

Explants used in the study	The most optimal explant	Somatic embryo formation (%)	References
Swollen cotyledon	---	83.3	Gui et al. (1991)
Embryo axes			
Hypocotyl	Wounded embryo	77.8±9.6	Choi & Soh (1993)
Cotyledon			
Radicle			
Embryo (intact and wounded)			
Immature embryos	Immature embryos	86.7	Yu et al. (1997a)
Immature embryos	Immature embryos	83.3	Yu et al. (1997b)
Hypocotyl	Hypocotyl	---	Choi et al. (1999a)
Cotyledon	Hypocotyl	75	Choi et al. (1999b)
Hypocotyl			
Root			
Young leaf	Petiole	3.0	Li & Yu (2002)
Stem			
Node			
Petiole			
Penduncle			
Flower			
Root			
Cotyledon from zygotic embryo, seedling, and plant	Hypocotyl from zygotic embryo	89±7.2	Han & Choi (2003)
Hypocotyl from zygotic embryo, seedling, and plant			
Radicle from zygotic embryo, seedling, and plant			

Table 8. Explants of callus initiation and somatic embryo formation. --- means undefined in the relevant reference

3.2.4 Other effects on embryogenesis and plant regeneration

As known that seed dehydration accompanied by the maturation of zygotic embryos results in the dormancy of zygotic embryos (Gray et al., 1987), thus, the desiccation of somatic embryos not detrimental to survival is very efficient in the long-term conservation of somatic embryos. In light of these theories, Choi & Jeong (2002) investigated the dormancy characteristics of ES somatic embryos and induced encapsulated somatic embryos maintain in the dormancy status by a high sucrose treatment. Moreover, maintaining ES somatic

embryos from cell suspension cultures under low temperature (4°C) was also considered to be able to achieve long-term animatingly conservation (Li et al., 2004). These treatments help a long-term conservation of artificial seeds and an enhanced resistance to dehydration of somatic embryos. You et al. (2005) carried out that removal of endosperm from seeds could markedly stimulate the growth of rudimentary zygotic embryos to induce more rapid germination of rudimentary zygotic embryos by *in vitro* culture of excised seeds. And in their later investigation (You et al., 2006), the roles of plasmolyzing pretreatment for zygotic embryos were evaluated on the induction of somatic embryos, suggesting that this pretreatment could result in sharply increased callose concentration in ES zygotic embryos, and callose accumulation could then stimulate the reprogramming of epidermal cells into embryogenically competent cells and finally induce somatic-embryo development from single cells. The further and detailed mechanism of enhanced somatic embryo formation through plasmolysis treatment was revealed that the expression level of callose synthase gene increased with response to 2,4-D, sucrose, and mannitol, and the callose played an important role in separating cell in epidermis from neighboring cells and consequently developing into embryogenic potential cells (Xilin et al., 2010).

3.3 Application for biochemical and biological events

Somatic embryogenesis has been studied as a model system for understanding the physiological, biochemical, and molecular biological events occurring during plant embryo development (Zimmerman, 1993). Among them, production of secondary metabolites through cell culture, particularly in medical plant, has long been used for commercial purposes (Roberts, 2007). To improve the culture conditions and then increase the production efficiency, many scientists have been investigated many factors affecting growth of culture materials and *in vivo* accumulation of active compounds. Ahn et al. (2003) investigated the effect of inorganic nitrogen sources such as KNO₃ and NH₄NO₃ on cell growth and production of chlorogenic acid and eleutheroside E derivative. In another investigation by Ahn et al. (2007), the effect of NO₃⁻ and NH₄⁺ on the adventitious root growth of ES and production of eleutheroside derivatives were investigated, and eleutheroside B (249 µg/g), E (788 µg/g), and E1 (43 µg/g) were increased at the highest levels by 40, 120, and 40 mM total nitrogen source, respectively. These results suggested that production of secondary metabolites through *in vitro* cultured cells could be manipulated by controlling the total concentration of nitrogen sources and the concentration ratio of NO₃⁻ and NH₄⁺ in the culture medium.

Except of nitrogen sources, light is another important factor affecting growth and organogenesis, but a factor stressing plants to consequently regulate the secretion mechanism of secondary products (Shohael et al., 2006a). Higher H₂O₂ content, malondialdehyde content and lipoxygenase activities were observed in cultured embryos under red light compared dark grown embryos, as well as activities of some antioxidant enzymes such as catalase, superoxide dismutase, glutathione S transferase, and ascorbate peroxidase were also stimulated in red light irradiated embryos. Of course, the contents of eleutheroside E and E1 were synchronously accumulated 51% and 21% higher than control under red light irradiation. Jeong et al. (2009) compared the effects of red, blue, and far-red light by irradiation of light emitting diodes (LEDs) with white fluorescent lamp, on growth,

morphogenesis and eleutheroside contents of *in vitro* cultured ES. The results indicated that *in vitro* cultured plantlets under the red/blue LEDs were taller than control, and those under blue LED showed greater leaf area, root length, and fresh weight than other light sources. Contents of eleuthroside B and E in plantlets were higher under blue LED, while content of eleuthroside E1 was the highest under fluorescent lamps.

Ahn et al. (2007, 2010) investigated the impacts of jasmonic acid (JA) on adventitious root culture of ES and eleutherosides accumulation, suggesting that JA inhibited the root growth but increased eleutherosides accumulation, as well as total phenolic contents and antioxidant activity. The highest levels of accumulation of eleutheroside B (359.9 $\mu\text{g/g}$), E (798.1 $\mu\text{g/g}$), and E1 (197 $\mu\text{g/g}$) were found under 40, 10, and 10 μM of methyl jasmonate addition, respectively.

Effects of temperature on secondary metabolite production such as eleutheroside B, E, E1, total phenolics, flavonoids, and chlorogenic acid and antioxidant enzyme activities were investigated by Shohael et al. (2006b), suggesting that culture at 24°C caused the highest production efficiency of secondary metabolites, and either lower or higher temperature could cause severe oxidative stress to form a cellular damage. Based on above results, the production of secondary metabolites, one side, was considered as the consequent result of cultured cell metabolism, the other side, as the outcome stimulated by some stress treatments. Therefore, to control the balance between reactive oxygen species (ROS) formation derived by stress treatments and consumption correlated with an array of antioxidant enzymes and redox metabolites becomes required and important. Shohael et al. (2007) further examined the ascorbate-glutathione cycle enzymes and other enzymes metabolism during somatic embryogenesis of ES, and suggested that the alterations of the glutathione redox systems play a significant role in somatic embryo development.

Genetic improvement of another application of plant tissue culture, and a good approach to improve plant physiological traits and augment the drug-yielding capacity of medicinal plants (Tejavathi & Shailaja, 1999). To authors' knowledge, only two transformation events through *Agrobacterium*-mediated transformation occurred in ES. Jo et al. (2005) transformed the human lactoferrin (*hLf*) gene into ES cells, and these transgenic ES cultured cells could produce *hLf* protein as cell growth increasing proportionally. As lactoferrin is an iron-binding glycoprotein with many biological roles, including the protection against microbial and virus infection and stimulation of the immune system, *hLf* transgenic ES plants could be used as a medicinal raw material for production of secondary metabolites. Another successful transformation event of ES was obtained in the report by Seo et al. (2005) that a squalene synthase-encoding gene derived from *Panax ginseng* (*PgSS1*) was successfully introduced into ES plants through *Agrobacterium*-mediated transformation. The transgenic plants showed up to 3-fold of squalene synthase enzyme activity higher than that of wild-type plants. Moreover, the introduced *PgSS1* gene in transgenic plants enhanced the metabolisms of phytosterol and triterpenoides, with 2.0 ~ 2.5-fold increments of their levels. These results indicated that transgenic ES cultured cells would be biotechnologically useful for the commercial production of medicinal plant cell cultures.

4. Conclusions

All biotechnological approaches like genetic engineering, haploid induction, or somaclonal variation to improve traits strongly depend on an efficient *in vitro* plant

regeneration system. Since LC as a monocotyledonous grass species and also a halophyte and ES as a dicotyledonous medicinal plant species, have increasingly great ecological and economic significant, this review would help efficiently improve traits through genetically modification.

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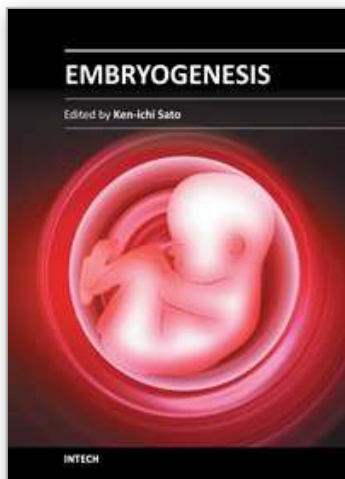
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Phone: +86-21-62489820
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