Optimizing Factors Affecting Somatic Embryogenesis in Cineraria

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Abstract

We established a reproducible protocol for somatic embryogenesis and plant regeneration of cineraria. 2,4-Dichlorophenoxy acetic acid (2,4-D) had a significant effect on somatic embryo formation. Addition of cytokinins such as 2-isopentenyladenine (2-iP), 6-benzyladenine (BA), and thidiazuron (TDZ) to the 2,4-D containing medium enhanced the frequency of somatic embryo induction and average number of somatic embryos per explant. However, the nature of SE varied depending on combination of 2,4-D and cytokinins. Cotyledon and leaf explants developed somatic embryos on Murashige and Skoog (MS) medium supplemented with 3.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA. Among the two explants, leaves were found to be the most effective for somatic embryogenesis and subsequent plant regeneration. Most of the embryos developed from the cotyledon explants showed precocious germination. Furthermore, somatic embryos obtained from the cotyledon explants developed hyperhydric shoots. Thus, induction and development of SE in cineraria is also affected by the age of the explants. Globular embryos developed into normal plantlets through heart, torpedo, and cotyledonary stages, similar to zygotic embryos, when cultured on MS medium supplemented with gibberellic acid (GA_3). The in vitro-developed plantlets were successfully acclimatized in the greenhouse with 98 % survival.

Keywords

Ammonium nitrate • Auxin • Benzyladenine • Gibberellic acid • Ornamental plants • Plant regeneration • Temperature

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4.1 Introduction

Somatic embryogenesis is the process of histodifferentiation for regeneration of complete plants by haploid or diploid somatic cells which resemble zygotic embryos (Williams and Maheswaran 1986). The process of histodifferentiation of a somatic cell to an embryogenic one is mediated by abiotic stress imposed by culture medium composition and subculture interval (Feher 2008). A number of factors including type and composition of plant growth regulators (PGRs) (Sivanesan et al. 2011), light conditions, explant source (Sharma and Rajam 1995), and genotype (Kim et al. 2003) can influence the process of successful somatic embryogenesis. Several types of stress, such as wounding, heavy metal ions, osmotic stress, high salt concentrations, and high levels of PGRs, have been suggested to induce the somatic embryo (SE) in plants (Zavattieri et al. 2010). Thus, somatic embryogenesis is a process that is normally suppressed in plants, and stress treatments are derepressing the somatic embryogenesis process. Somatic embryogenesis is a multistep regeneration process in plants starting with proembryogenic masses that progress to the next stages maintaining the bipolar pattern of cell. Auxins and cytokinins are essential to maintain proembryogenic proliferation (Xu et al. 2013), whereas embryo formation is triggered by the withdrawal of PGRs (Bozhkov et al. 2002). The further development of early somatic embryos and maturation requires abscisic acid (Rai et al. 2011; Su et al. 2012). Initiation of SE is strongly controlled by genetic additive effects, and as a result plants of some families' respond well in propagation, while others are difficult to propagate by somatic embryogenesis (Salvo et al. 2014).

Cineraria are attractive pot plants belonging to the family Asteraceae. *Senecio cruentus* is an ornamental species; it originates in Canary Islands and is widely cultivated for its colorful flowers. Cineraria can be easily propagated by seeds, but seedlings are heterozygous producing flowers of different colors (Malueg et al. 1994). It is propagated vegetatively by cuttings; however, shortage of plant materials has often been affecting the large-scale commercial propagation (Sivanesan and Jeong 2012). Thus, propagation of new selections could be achieved more rapidly using in vitro propagation methods. In vitro clonal propagation of ornamental plants is an important technique that can be applied for large-scale production of select plants or cultivars. Clonal propagation through somatic embryogenesis has become an essential method for the improvement of most economically important plants. Plantlet regeneration through somatic embryogenesis is also a potential tool for the production of synthetic seeds and genetic transformation. In addition, this process is also considered as a most suitable system for physiological and morphological studies in plant during morphological development and conservation of desired genotypes through mass propagation (Lelu-Walter et al. 2013). Effective procedures for the initiation of somatic embryogenesis from mature plants permit more rapid propagation of elite plants, thus increasing genetic gains in each breeding generation (Dey et al. 2015). Direct somatic embryogenesis reduces the time required for plant propagation, which may be beneficial to minimize culture-induced genetic changes (Sivanesan et al. 2011). Somatic embryogenesis and plant regeneration have been reported in *Senecio* species (Malueg et al. 1989, 1994; Nam et al. 2005), but low frequency of embryo induction and conversion, abnormal embryos, and hyperhydricity hinder the application of these protocols for large-scale commercial propagation.

The addition of auxin to the culture medium is required to induce somatic embryos in most plant species. However, in some plant species, the inclusion of cytokinin alone is sufficient to induce somatic embryos. In cineraria, addition of both auxin and cytokinin to the culture medium is required for the induction of SE (Malueg et al. 1994; Nam et al. 2005). The maturation, germination, and conversion of SE into plantlets are other important steps during somatic embryogenesis. Several chemical and physical factors affect the development and conversion of SE such as abscisic acid (ABA), gibberellic acid (GA_3), polyethylene glycol (PEG), sucrose, light intensity,

humidity, and temperature. We optimized the conditions for somatic embryo induction and studied the effect of ABA, PEG, and sucrose concentration on embryo maturation and germination (Nam et al. 2005). In this chapter, we describe an efficient procedure for somatic embryogenesis and plant regeneration from cotyledon and leaf explants in *S. cruentus*.

4.2 Somatic Embryogenesis

Seeds of *S. cruentus* 'Tokyo Daruma' (Sakata Seed Co., Yokohama, Japan) were sterilized with 70 % (v/v) ethanol for 30 s and 2.0 % (v/v) sodium hypochlorite for 10 min. Each treatment was followed by four rinses with sterile distilled water, and the seeds were germinated on Murashige and Skoog (MS) medium containing 3 % (w/v) sucrose and 0.8 % (w/v) agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 20 min. The cultures were maintained at 25 ± 2 °C under a16 h photoperiod with 15 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Cotyledon (0.5 cm) and leaf (0.5–1.0 cm) explants were taken from 7- to 21-day-old seedlings, respectively. For SE induction, the explants were cultured on MS supplemented with various concentrations of auxins [2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), or α-naphthaleneacetic acid (NAA)] alone or in combination with cytokinins (2-isopentenyladenine (2-iP), 6-benzyladenine (BA), or thidiazuron (TDZ)] (Table 4.1). The cultures were maintained for 2 weeks at 25 ± 2 °C in the dark and then exposed to light of 45 μmol m^{-2} s^{-1} PPFD provided by cool white fluorescent light with a light/dark cycle of 16/8 h for 4 weeks. To study the effects of ammonium nitrate (NH_4NO_3) and temperature on SE induction, cotyledon and leaf explants were cultured on optimal SE induction medium (MS + 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} BA)

Table 4.1 Effects of plant growth regulators on somatic embryogenesis from cotyledon and leaf explants of *S. cruentus* after 6 weeks of culture

PGRs (mg l^{-1})						Somatic embryo induction (%)		No. of somatic embryos/explant	
2,4-D	IBA	NAA	2iP	BA	TDZ	Cotyledon	Leaf	Cotyledon	Leaf
0						0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.0						13.3 ± 2.2	18.0 ± 3.0	14.0 ± 1.6	28.3 ± 1.7
2.0						37.7 ± 1.9	39.5 ± 2.4	26.7 ± 1.2	39.0 ± 2.2
3.0						52.8 ± 3.5	66.8 ± 3.7	32.0 ± 1.7	45.0 ± 1.6
3.0			1.0			84.7 ± 1.2	87.6 ± 2.6	63.6 ± 3.3	84.3 ± 4.6
3.0				1.0		93.6 ± 3.4	97.0 ± 1.4	87.3 ± 2.1	135.3 ± 5.7
3.0					1.0	92.0 ± 2.4	95.6 ± 1.0	77.3 ± 1.7	101.6 ± 2.8
	1.0					Root	Root	Root	Root
	2.0					Root	Root	Root	Root
	3.0					Callus	Callus	Callus	Callus
	3.0		1.0			84.7 ± 1.2	92.0 ± 2.0	5.3 ± 0.9	7.3 ± 0.5
	3.0			1.0		84.7 ± 1.2	92.0 ± 2.0	7.0 ± 1.4	11.0 ± 0.8
	3.0				1.0	84.7 ± 1.2	92.0 ± 2.0	9.0 ± 0.8	13.7 ± 1.7
		1.0				Root	Root	Root	Root
		2.0				Callus + root	Callus + root	Callus + root	Callus + root
		3.0				Callus	Callus	Callus	Callus
		3.0	1.0			84.7 ± 1.2	92.0 ± 2.0	6.0 ± 1.6	19.6 ± 1.3
		3.0		1.0		84.7 ± 1.2	92.0 ± 2.0	12.0 ± 1.6	23.7 ± 2.6
		3.0			1.0	87.2 ± 4.6	92.0 ± 2.0	15.3 ± 0.9	21.6 ± 1.2

Values are mean ± standard deviation (SD) of results

Table 4.2 Effects of ammonium nitrate concentration and temperature on somatic embryogenesis from cotyledon and leaf explants of *S. cruentus*

NH_4NO_3		Somatic embryo induction (%)		No. of somatic embryos/explant	
(mg l^{-1})	Temperature (°C)	Cotyledon	Leaf	Cotyledon	Leaf
0		18.2 ± 1.8	27.7 ± 3.3	2.3 ± 0.9	4.6 ± 1.7
412.5	15	29.6 ± 1.7	36.7 ± 1.7	5.6 ± 0.9	7.7 ± 0.5
825.0		52.7 ± 3.7	58.0 ± 1.6	6.3 ± 1.7	9.3 ± 0.9
1650		62.3 ± 3.7	67.0 ± 1.6	9.7 ± 0.5	14.0 ± 0.8
0		38.7 ± 2.1	40.0 ± 1.6	5.6 ± 1.2	6.3 ± 1.2
412.5	20	62.3 ± 2.5	71.6 ± 2.1	13.7 ± 1.2	18.0 ± 1.0
825.0		76.3 ± 1.7	84.7 ± 1.2	17.0 ± 0.8	23.3 ± 2.5
1650		87.0 ± 2.2	91.3 ± 1.6	21.7 ± 3.3	29.7 ± 1.3
0		44.0 ± 1.6	39.3 ± 2.7	12.3 ± 1.7	16.0 ± 2.2
412.5	25	63.0 ± 2.2	75.3 ± 1.2	34.0 ± 1.6	55.6 ± 2.6
825.0		86.3 ± 3.8	90.6 ± 2.5	53.0 ± 3.7	76.6 ± 2.5
1650		93.6 ± 3.4	97.0 ± 1.4	87.3 ± 2.1	135.3 ± 5.7

Values are mean ± standard deviation (SD) of results

with 0, 412.5, 825.0, or 1650 mg l^{-1} NH_4NO_3, and the cultures were maintained for 2 weeks at 15, 20, or 25 ± 2 °C in the dark and then exposed to light of 45 µmol m^{-2} s^{-1} PPFD. Globular stage embryos were transferred to the MS containing various concentrations of GA_3 for SE maturation and germination (Table 4.2).

4.2.1 Role of Plant Growth Regulators (PGRs) on Somatic Embryo Induction

PGRs play crucial roles in the induction and development of SEs. Cotyledon and leaf explants failed to develop SE on MS medium devoid of PGRs, and their color gradually turned from green to brown exhibiting necrosis. Somatic embryogenesis was also induced on auxin-free media in several plants. Thus, the presence of PGRs in the culture medium was essential for the induction of somatic embryos in cineraria. Auxin is often required for the induction of callus, root, or SE from various explants, and the process of somatic embryogenesis is generally initiated on the culture medium supplemented with high concentrations of auxins. In alfalfa, supplementation of low concentration of 2,4-D induced callus from leaf protoplast, while at ten times higher levels of 2,4-D provoked in forming embryo-like structures (Feher et al. 2002). However, somatic embryogenesis was induced in several plants on auxin-free media (Jimenez 2001). Among the auxins, only 2,4-D promoted direct somatic embryogenesis in both explants of cineraria, whereas IBA and NAA induced callus and roots from the cut ends and surface of the explants. 2,4-D is one of the most widely used herbicides in the world, and Feher et al. (2003) suggested that it functions as a stress chemical as well as an auxin. Besides, auxinic herbicides have been shown to interact with ethylene and ABA, thereby increasing the cellular levels of these stress hormones (Feher et al. 2003). The seedling explants of carrot formed SEs when the culture medium was supplemented with ABA (Nishiwaki et al. 2000). Increasing concentration of sucrose or addition of NaCl or CdCl to the PGR-free medium induces SEs in carrot (Zavattieri et al. 2010). The stress caused by in vitro culture conditions and media composition can also induce SE formation. 2,4-D has been proven to be the most potent auxin and effective for SE induction in several ornamental plants (Tanaka et al. 2000; Sivanesan et al. 2011, 2012).

Incubating explants under a short period of darkness may be beneficial for in vitro morphogenesis by reducing blackening and chlorosis and

preventing the formation of some growth inhibitors. Dennis (2000) reported that culture in the darkness suppressed unwanted tissue differentiation in explants tissues, for example, by limiting the development of plastids into chloroplasts. SEs were induced under light conditions, but culturing the explants 14 days in the dark followed by 4 weeks under light conditions resulted in high frequency of SE induction. This is in agreement with our previous study (Nam et al. 2005). Similar result was also found in other ornamental species such as *Campanula punctata* (Sivanesan et al. 2011), *Crocus vernus* (Sivanesan et al. 2012), and *Dendranthema grandiflorum* (Tanaka et al. 2000). The tissue sensitivity of the explants to the PGRs may have been altered by the dark treatment, thereby resulting in a higher frequency of embryo formation (Zobayed and Saxena 2003). In geranium continuous exposure of light treatment significantly reduced the level of endogenous plant growth hormones (Hutchinson et al. 2000). Globular embryos induced directly on the surface of the explants after weeks of culture, without a callus phase, and the embryos were yellow, compact, and pale. Of the various concentrations of 2,4-D tested, the highest frequency of somatic embryo induction (66.8 %) and mean number of SE (45.0) per leaf explant were obtained on MS supplemented with 3.0 mg l^{-1} 2,4-D (Table 4.1). In contrast, SE did not induce from the cotyledon explants of cineraria 'Jester Pink' on MS ammended with 2,4-D, while 'Early Blue' formed SE on the medium containing 2,4-D (Nam et al. 2005). Thus, somatic embryogenesis in cineraria is cultivar dependent. Somatic embryogenesis has been described as being genetically determined. Cineraria seeds are heterozygous in nature; therefore, further studies on genetic analysis in various cultivars of cineraria may help to find out the genes involved in somatic embryogenesis in cineraria. However, SEs were initiated from all cultivars of cineraria on MS containing auxin plus cytokinins. In several plants, a combination of auxin and cytokinin stimulates the formation of SEs.

The frequency of SE induction and average number of SEs per explant significantly increased when the optimal concentration of 2,4-D (3.0 mg l^{-1}) was combined with cytokinins. However, the nature of SEs varied depending on combination of 2,4-D and cytokinins. The explants developed fused SEs on MS supplemented with 2,4-D and 2iP after 6 weeks of culture (Fig. 4.1a). Auxin polar transport was reported to be essential for bilateral symmetry during early plant embryogenesis. Liu et al. (1993) reported that TIBA, trans-cinnamic acid, and 9-hydroxyfluorene-9-carboxylic acid induced the formation of fused cotyledons in Indian mustard. Cytokinin influences cell-to-cell auxin transport by modification of expression of several auxin transport compounds. The addition of ABA to the induction medium significantly decreased the proportion of abnormal embryos in several plants. In soybean, the supplementation of ABA to the culture medium containing 2,4-D, adenine, and kinetin increased the number of SEs which grew from the globular to heart stage (Phillips and Collins 1981). SEs with abnormal cotyledons failed to convert into normal plantlets (Todd and Yeung 1993). Normal SE formed two meristematic centers and then organized normal two cotyledons. The addition of ABA induces dormancy of SEs, which makes embryos to form two suitable meristematic centers (Ammirato 1985). Thus, incorporation of ABA to the SE induction medium may reduce the formation of abnormal embryos. When the medium was supplemented with 2,4-D and BA, a few embryos germinated precociously (Fig. 4.1b). The inclusion of high molecular weight PEG to the SE induction medium significantly improves the synchrony of development and the quality of mature SEs, particularly in terms of storage reserves, and prevents precocious germination (Attree et al. 1992). Nam et al. (2005) reported that addition of 7.8 μM ABA or 3.0 % PEG or 6.0 % sucrose to the culture medium prevented precocious germination in cineraria. On medium containing 2,4-D and TDZ, the explants formed SE and also callus on the surface after 6 weeks of culture (Fig. 4.1c). The callus formation may be due to the high activity of TDZ. Thidiazuron, a cotton defoliant, has been shown both auxin and cytokinin effects (Guo et al. 2011). Ferreira et al. (2006) reported that treatment of TDZ significantly increases the

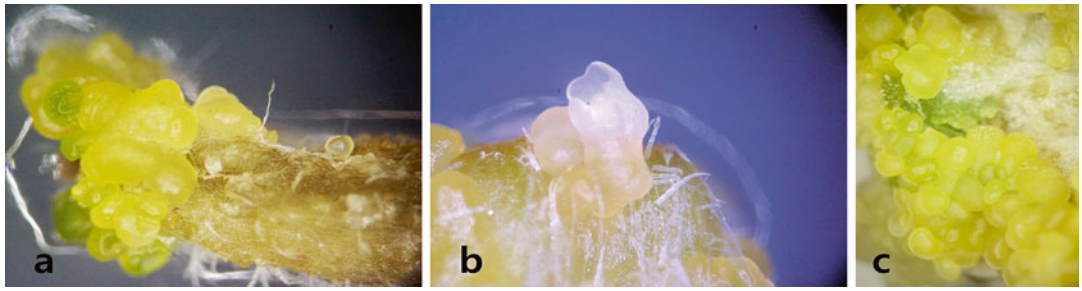

Fig. 4.1 Effects of combination of 2,4-D and cytokinins on somatic embryogenesis from explants of *S. cruentus* after 6 weeks of culture. (**a**) 2,4-D + 2iP. (**b**) 2,4-D + BA. (**c**) 2,4-D + TDZ

endogenous cytokinins and IAA levels of the explants of *Dendrobium*. Thus, reducing the concentration of TDZ in the induction medium can minimize the callus formation and the number of SEs. Both explants formed SE and callus on MS medium containing IBA or NAA in combination with cytokinins after 6 weeks of culture. Of the various combinations of auxins and cytokinins studied, the highest frequency of SE induction (97.0 %) and mean number of SE (135.3) per leaf explant were obtained on MS supplemented with 3.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA. Similar result has also been reported in cineraria 'Hansa' (Malueg et al. 1994) and 'Jester Pink' (Nam et al. 2005). The use of 2,4-D and BA for SE induction has been reported for other members of Asteraceae (Filho et al. 1993; Correa et al. 2009).

4.2.2 Influence of Explants on Somatic Embryo Induction

Somatic embryogenesis in cineraria has been achieved using various explants such as cotyledons (Malueg et al. 1994; Nam et al. 2005), hypocotyls (Nam et al. 2005), and leaves (Malueg et al. 1994). The ability of cineraria cultivars to develop SEs is influenced by the type of explant. Nam et al. (2005) reported that cotyledon explants were found to be the best for SE induction than hypocotyls. In this study, cotyledon and leaf explants produced SEs on MS supplemented with 3.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA (Table 4.1). However, leaf explants were more efficient than cotyledon in SE induction. This may be due to variation in the endogenous levels of PGRs in the explants. SE began to appear mostly on the cut end of cotyledon within 2 weeks of culture (dark condition), while SEs were formed on the cut ends and surface of the leaf explants after 3 weeks of culture. Most of the embryos developed from the cotyledon explants showed precocious germination after 6 weeks of culture (Fig. 4.2a). Addition of ABA, PEG or sucrose to the culture medium prevents precocious germination, but most of the SE-derived plantlets exhibited hyperhydricity (Nam et al. 2005). Therefore, the normal process of SE development is closely coupled with the osmotic environment surrounding the SEs (Yeung and Claudio 2000). On the other hand, compact yellow globular embryos were formed on the leaf explants after 6 weeks of culture. Thus, induction and development of SE in cineraria is also affected by the age of the explants. In most plant species, younger explants were more responsive than the older explants for SE induction. However, mature leaf explants found to be the best for SE induction and plant regeneration in cineraria.

4.2.3 Effects of Different Concentrations of NH_4NO_3 and Temperature on Somatic Embryo Induction

Growth and morphogenesis in tissue cultures are greatly influenced by the availability of nitrogen

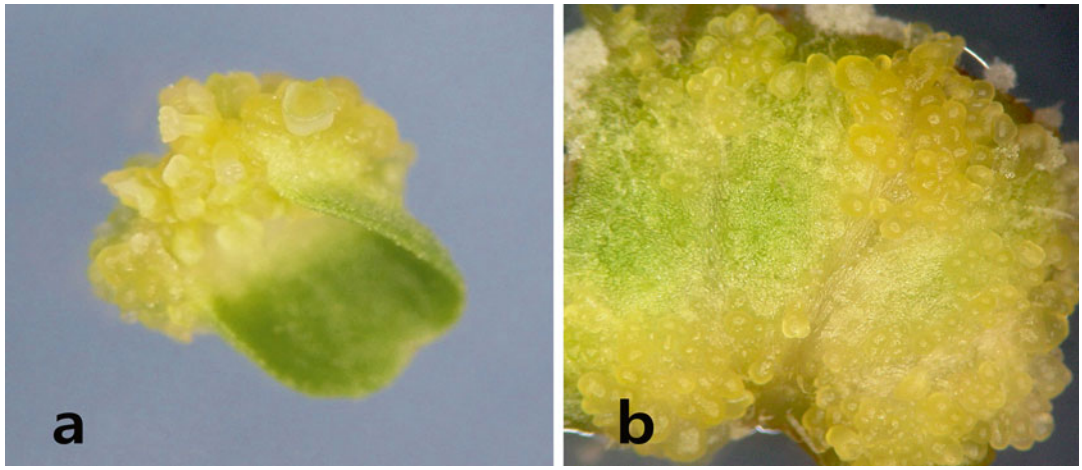

Fig. 4.2 Effects of combination of 2,4-D and BA on somatic embryogenesis from explants of *S. cruentus* after 6 weeks of culture. (**a**) Cotyledonary explant. (**b**) Leaf explant

and the form in which it is used (Sivanesan and Jeong 2012). Ammonium nitrate concentrations have been shown to be important for the induction of somatic embryogenesis in many plants (Smith and Krikorian 1989; Choi et al. 1998; Greer et al. 2009). Gertsson (1988) reported that a low number of adventitious shoots were obtained from petiole explants of *Senecio hybridus* when the total nitrogen in MS was increased to 75 mM and that more shoots were produced when the total nitrogen was reduced to 30 mM. In contrast, decreasing the levels of NH_4NO_3 had a negative effect on adventitious shoot induction in *S. cruentus* (Sivanesan and Jeong 2012). In this study, the frequency of SE induction and mean number of SEs per explant gradually decreased with decrease in the level of NH_4NO_3 concentration from 1650 to 0 mg l^{-1} (Table 4.2). The quality of the globular stage embryos did not differ markedly in used treatments. Morphogenesis including embryogenesis in culture is also influenced by temperature. The explants were maintained at three different temperatures in order to study the effect of temperature on somatic embryogenesis. Decreasing the culture temperature from 25 to 15 °C decreased the frequency of SE induction. The explants maintained under 25 °C developed maximum number of SEs, while those maintained under 15 °C produced less number of SEs. The results suggest that NH_4NO_3 concentration and temperature had a significant effect on somatic embryogenesis in cineraria. The morphology of globular embryos was affected by NH_4NO_3 concentration and temperature. The globular embryos developed on SE induction medium containing low levels of NH_4NO_3 (412.5, 825.0 mg l^{-1}) at 20 °C were purple in color (Fig. 4.3a). Thus, anthocyanin synthesis could be stimulated in cineraria in vitro cultures by reducing NH_4NO_3 levels and temperature. Variegated plantlets were produced from the globular embryos when the embryos were transferred to the germination medium devoid of NH_4NO_3 and at 15 °C (Fig. 4.3b). Somaclonal variation has also been observed in cineraria (Sivanesan and Jeong 2012) when the shoot cultures are maintained at 15 °C. Anthocyanins are flavonoid metabolites that contribute to the coloration of various plant tissues and in vitro cultured cells and tissues. The application of ammonium has been found to decrease the level of anthocyanins in petals of *Gerbera hybrida* (Huang et al. 2008), while nitrogen deficiency increased the levels of anthocyanins in *Arabidopsis thaliana* (Scheible et al. 2004). Temperature also affects anthocyanin synthesis in various plants. Low temperature enhances anthocyanin synthesis in *A. thaliana* (Leyva et al. 1995) and *Zea mays* (Christie et al. 1994).

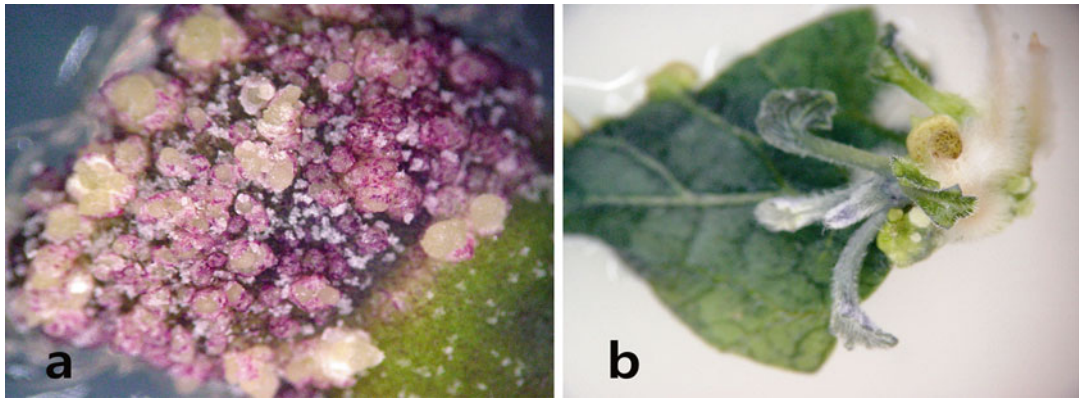

Fig. 4.3 Effects of different concentrations of NH_4NO_3 and temperature on somatic embryogenesis from explants of *S. cruentus*. (**a**) Pigmented somatic embryos developed on MS medium containing 412.5 mg l^{-1} NH_4NO_3 at 20 °C. (**b**) Variegated shoot developed from the SE

4.3 Maturation and Germination of Somatic Embryos

Embryo maturation and simultaneous conversion to plantlets is one of the steps in somatic embryogenesis, which partially depends on embryo quality (Sivanesan et al. 2012). Somatic embryogenesis is often initiated on the culture medium supplemented with PGRs; however, development beyond the globular stage was inhibited by maintaining the same induction medium. Thus, for promoting further growth and development of SEs, it is necessary to subculture the embryos to the PGR-free medium. In cineraria, globular embryos initiated on MS containing 2,4-D and BA matured and converted into plantlets on MS with activated charcoal (Malueg et al. 1994). Activated charcoal is composed of carbon arranged in a quasi-graphitic form in small particle size. It is often used in medium to improve growth and development of cell, tissue, and organ (Thomas 2008). Activated charcoal has the potential to absorb some inorganic ions, auxins, cytokinins, and phenolics. The positive effect of activated charcoal on embryo maturation and conversion was probably by adsorption of PGRs (von Aderkas et al. 2002), ethylene (Johansson 1983), and growth inhibitory substances like 5-hdroxymetylfurfural in the culture medium (Weatherhead et al. 1978), alteration of culture medium pH to an optimum level for development (Owen et al. 1991), and release of substances naturally present in or adsorbed by activated charcoal (Pan and van Staden 1998). The addition of activated charcoal to the maturation medium has also been proven beneficial for development and conversion of SEs in *Campanula punctata* (Sivanesan et al. 2011) and *Crocus vernus* (Sivanesan et al. 2012). On the other hand, ABA and osmotic stress (PEG, sucrose) have been reported beneficial for embryo maturation in cineraria (Nam et al. 2005). The combination of ABA and PEG is also used to stimulate SE maturation in *Corydalis yanhusuo* (Sagare et al. 2000) and *Panax ginseng* (Langhansova et al. 2004). Treatment of 3.8 μM ABA or 6.0 % sucrose was best for embryo maturation. ABA and sucrose are known to be important factors for seed maturation in higher plants. The authors reported that treatment of ABA was the best in promoting the conversion ratio than PEG or sucrose treatment. ABA is often used in in vitro culture to promote the maturation of SEs and to store carbohydrate reserves in embryos during maturation. The influence of ABA on somatic embryo maturation and germination has been reported in many plants (Rai et al. 2011). The conversion ratio from SEs to plantlets in cineraria was 30 % (Nam et al. 2005). Thus, it is necessary to improve the conversion ratio of somatic

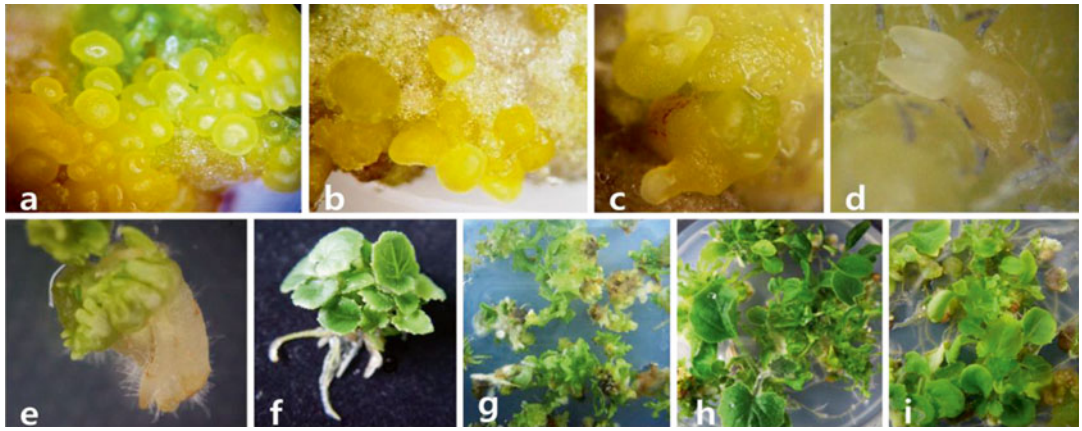

Fig. 4.4 Somatic embryogenesis and plant regeneration from cotyledon and leaf explants of *S. cruentus*. (**a**) Globular stage. (**b**) Heart stage. (**c**) Torpedo stage. (**d**) Cotyledonary stage. (**e**) Germination and root formation. (**f**) SE-derived plantlets. (**g**) and (**h**) SE germination and conversion. (**i**) Hyperhydric shoots developed from the SE obtained from cotyledon explants

Table 4.3 Effects of different concentrations of GA_3 on germination of somatic embryos after 5 weeks of culture

GA_3 (mg l^{-1})	Germination (%)
0	29.7 ± 5.9
0.5	42.7 ± 5.0
1.0	65.0 ± 5.1
2.0	84.3 ± 3.1
4.0	71.3 ± 3.3

Values are mean ± SD of results

embryos to be of practical use. In this study, globular embryos completed germination through heart, torpedo, and cotyledonary stages when the embryos were cultured on MS basal medium (Fig. 4.4a–d). However, the frequency of conversion was very low. Furthermore, SE having malformed cotyledons occurred during the embryo development. The low frequency of SE conversion to plantlets is due to the carryover effects of PGRs. GA_3 had a significant effect on SE maturation and conversion (Table 4.3). Gibberellins are known as growth-promoting hormones, being involved in several processes during plant development, like shoot elongation, flower development, breaking dormancy, and seed germination (Linkies and Leubner-Metzger 2012). The frequency of SE maturation and conversion were 29.7, 42.7, 65.0, 84.3, or 71.3 % when MS was supplemented with 0, 0.5, 1.0, 2.0, or 4.0 mg l^{-1} GA_3, respectively. The fact that GA_3 stimulates the maturation and conversion of SE is well known. However, SE obtained from the cotyledon explants developed hyperhydric shoots (Fig. 4.4). This is in agreement with our previous report (Nam et al. 2005). The results suggest that somatic embryogenesis and plant regeneration in cineraria largely depends on the type of explant. Further, understanding physiological and molecular nature of the explants will be useful for accessing the quality of the SE. Plantlets developed from the SE with shoots and roots were separated and grew further on hormone-free MS medium for 4 weeks. The in vitro-developed plantlets were successfully acclimatized in the greenhouse with 98 % survival.

4.4 Conclusion

We developed a simple and reproducible procedure for somatic embryogenesis and plant regeneration of cineraria. 2,4-D and BA had a positive effect on SE induction in both explants. Among the two explants, leaf was found to be the most

effective for somatic embryogenesis and subsequent plant regeneration. GA_3 had a positive effect on SE maturation and conversion. This protocol could be utilized for genetic transformation and large-scale commercial propagation of cineraria. Most of the SEs were attached to the explants; these must be separated from the explants and embryo clusters for artificial seed production. Further studies are needed to confirm the genetic homogeneity of the in vitro-regenerated plantlets.

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