RESEARCH ARTICLE
J. Crop Sci. Biotech. 2008 (June) 11 (2) : 107 ~ 100

A High-Efficiency Direct Somatic Embryogenesis System for Strawberry (*Fragaria x ananassa* Duch.) Cultivar Chandler

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<Received May 10, 2008 / Accepted June 9, 2008>

Abstract

A high-efficiency, reproducible somatic embryogenesis system for strawberry cultivar Chandler was developed. Thirty-one somatic embryos per explant (max no.) were recorded in leaf discs which were cultured on medium containing MS salts + B_5 vitamins + 2% glucose + 4.0 mg l^{-1}TDZ (Thidiazuron) and incubated at 10 ± 1 ℃ under darkness for one week followed by three weeks under 16-h photoperiod. The scanning electron microscopic (SEM) ontogeny revealed the normal development of somatic embryos from globular to heart-shaped and dissection microscopy from torpedo-shaped to cotyledonary-stage embryos. The maximum germination percentage of 48% could be obtained on MS medium containing kinetin (1.0 mg l^{-1}) and the maximum survival percentage (79%) of plantlets after four weeks was found to be in the mixture of vermiculite, peatmoss, and soilrite (1:1:1).

Key words: Strawberry, TDZ, somatic embryogenesis, regeneration

Introduction

Strawberry (*Fragaria x ananassa* Duch.) is one of the most popular soft fruits and is cultivated in plains as well as in the hills up to an elevation of 3,000 m in humid or dry regions (Darrow and Walgo 1934). Strawberries are produced in 71 countries worldwide on 506,000 acres and are among the highest-yielding fruit crops (Husaini and Abdin 2008). Its popularity can be judged from the fact that in the last 20 years strawberry breeding activity has led to the commercial introduction of 463 new cultivars from 35 different countries, by 79 public agencies, and 32 private companies (Faedi et al. 2002). The fruit is in great demand for fresh market as well as in fruit processing industry market for preparing jams and other products.

Plantlet regeneration via organogenesis in leaf cultures of *Fragaria x ananassa* has been studied extensively and is well documented (Yonghua et al. 2005; Zhao et al. 2004; Passey et al. 2003; Schaart et al. 2002; Barcelo et al. 1998). Only a few studies however, have so far focused on somatic embryogenesis in strawberry (Donnoli et al. 2001; Lis 1987; Wang et al. 1984). These studies have primarily demonstrated the importance of various plant growth regulators and growth media in achieving regeneration via somatic embryogenesis. In a previous study, our group published the first report where shoot regeneration in strawberry was achieved simultaneously through both somatic embryogenesis and shoot bud formation (Husaini and Abdin 2007). The study focused on shoot induction and morphogenetic response of cultured leaf explants under complex environmental conditions of light, temperature, and TDZ, and identified some developmental constraints that affect somatic embryogenesis in strawberry. The present study was carried out for developing a reliable, reproducible, and highly efficient somatic embryogenesis system for strawberry cultivar Chandler and exploit the potential of TDZ for the induction of somatic embryogenesis in cultured leaf explants. The study also examined the effect of temperature on the induction and maintenance of somatic embryogenesis in strawberry cultivar Chandler. The rationale for developing an efficient somatic embryogenesis system for strawberry was to provide a useful system for producing artificial seeds as well as a means for

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recovering genetically modified plants. The somatic embryogenesis system reported here was tested successfully for regeneration of transgenic strawberry plants tolerant to salt stress (Husaini and Abdin 2008).

Materials and Methods

Leaf discs of strawberry cultivar Chandler were derived from fully expanded, green leaves of plantlets maintained under culture room conditions [temperature: 26 ± 2 ℃; light intensity: 2300 lux; photoperiod: 16/8-h (day/ night)]. The leaflets were separated and leaf discs (0.5-0.6 cm) were prepared by cutting along the mid vein and the edges. After cutting the leaf material into small discs these were cultured on culture medium containing MS salts (Murashige and Skoog 1962), B_5 vitamins (Gamborg et al. 1968), 2% glucose, 0.8% agar supplemented with eleven different concentrations of TDZ viz. 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 mg l^{-1}. These explants were then incubated under three different temperature regimes during the first four weeks of culture: One week under darkness followed by three weeks under 16-h photoperiod (at 2300 lux light intensity) at 5 ± 1 ℃ (regime A), 10 ± 1 ℃ (regime B), and 15 ± 1 ℃ (regime C).

After four weeks, the explants were incubated at 26 ± 2 ℃ on MS basal medium or MS medium supplemented with either Kn or GA_3 (1.0-2.0 mg l^{-1}). Cultures were scored on day 36 for the number of somatic embryos per explant and day 43 for percentage of somatic embryos germinating. Data on somatic embryos was recorded using a dissection microscope. Each treatment consisted of three replicates (each containing 12 explants) and the experiment was repeated twice. Statistical analyses were carried out by two-way classification of ANOVA (Cochram and Cox 1957), to evaluate whether the means were significantly different, taking $P < 0.05$ as significance level.

For scanning electron microscopy, the viewing surface of the tissues was cleaned with 0.1 M phosphate buffer (pH 7.4) after fixation. Fixation was for 18-h at 4 ℃, in modified Karnovsky's fluid made in 0.1 M Phosphate buffer (pH 7.4). The specimens were dehydrated in graded acetone solution. Critical Point Drying was done with liquid CO_2 using Polaron Jumbo Critical Point Dryer and Gold Sputter Coating was carried out under reduced pressure in an inert argon gas atmosphere (Agar Sputer Coater P 7340). After sputter coating, the tissues were examined under Scanning Electron Microscope (Leo 435VP) operated at 15 KV (David et al. 1973).

Results and Discussion

The somatic embryogenesis system developed for strawberry comprises of four culture steps, viz i) Somatic embryo induction on culture medium containing MS salts, B_5 vitamins, 2% glucose, 0.8% agar supplemented with 4.0 mg l^{-1} TDZ; ii) Embryo germination and shoot development on MS medium with 1.0 mg l^{-1} Kn (Kinetin); iii) Plantlet development on MS basal medium; and iv) Acclimatization on soil free substrate containing Vermiculite+ Peat moss+ Soilrite (1:1:1).

Somatic embryos were observed on the margins of leaf explants after four weeks of culture (Figures 1 a,b). Upon one week of incubation on MS basal medium, the globular embryos developed into heart-stage embryos (Figures 1c,d) and upon two weeks, these matured into cotyledonary-stage embryos (Figure 1e). The embryos were loosely attached to the surface of the source tissue and could be easily detached. Incubation in dark condition during first week of culturing successfully prevented darkening of culture medium, caused due to exudation of phenolic compounds from explants followed by oxidation to quinines (George 1993; Taji and Williams 1996).

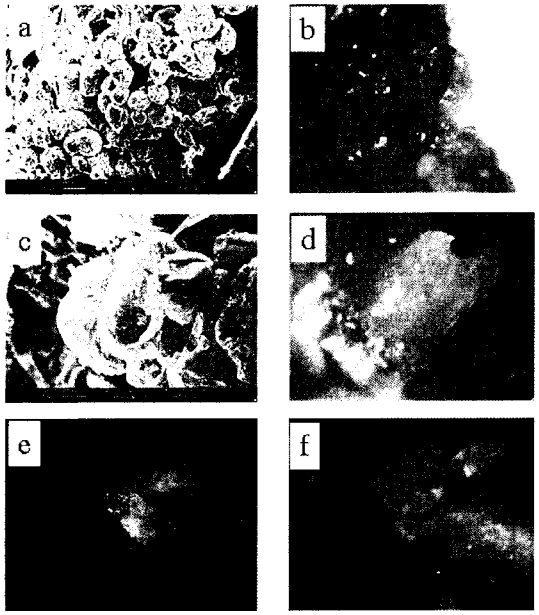

Fig. 1. Direct somatic embryogenesis in *Fragaria x ananassa* Duch. (a) Cluster of proglobular embryos (SEM image), (b) Globular embryos on leaf epidermis appearing individually or in clusters, (c) Differentiation of advanced globular towards heart stage (SEM image), (d) Heart-shaped embryo, (e) Advanced cotyledonary embryos, and (f) Embryos germinating.

Auxin and cytokinin balance has long been recognized as a key factor in regeneration in most dicot plants (Murashige 1977). TDZ, a substituted phenyl urea used in the present study, acts as a substitute for both the auxin and cytokinin requirements of organogenesis and somatic embryogenesis in several species (Murthy et al. 1998; Visser

Table 1. Effect of treatment regimes A, B, and C* on the number of somatic embryos developing on different concentrations of TDZ.

TDZ concentration (mg l^{-1})	Number of somatic embryos per explant		
	Regime		
	A (5 ± 1 ℃)	B (10 ± 1 ℃)	C (15 ± 1 ℃)
1.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1.5	0.00 ± 0.00	0.67 ± 0.47	1.00 ± 0.00
2.0	1.67 ± 0.47	4.67 ± 0.47	2.80 ± 0.47
2.5	1.91 ± 0.52	5.00 ± 0.82	3.30 ± 0.82
3.0	2.98 ± 0.40	5.67 ± 0.47	3.80 ± 0.82
3.5	17.00 ± 0.82	19.67 ± 0.47	14.00 ± 0.82
4.0	23.00 ± 0.82	31.00 ± 0.95	19.00 ± 0.82
4.5	11.00 ± 0.82	8.67 ± 0.47	9.67 ± 0.47
5.0	4.30 ± 0.47	8.00 ± 0.82	7.00 ± 0.82
5.5	4.00 ± 0.82	6.67 ± 0.47	5.67 ± 0.47
6.0	3.30 ± 0.47	1.67 ± 0.47	3.00 ± 0.82

*Regimes A, B, and C correspond to incubation of explants for one week under darkness followed by three weeks under 16-h photoperiod (at 2300 lux light intensity), at 5 ± 1 ℃, 10 ± 1 ℃ ,and 15 ± 1 ℃, respectively.
Each value is the mean ± SE with at least 3 replicates.

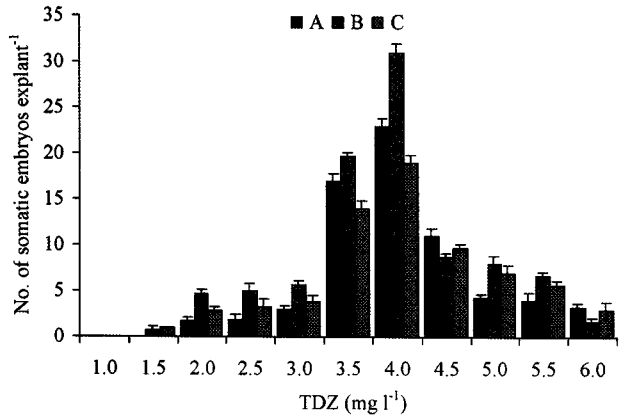

Fig. 2. Effect of treatment regimes A, B, and C* on the number of somatic embryos developing from leaf discs.
*Regimes A, B, and C correspond to incubation of explants for one week under darkness followed by three weeks under 16-h photoperiod (at 2300 lux light intensity), at 5 ± 1 °C, 10 ± 1 °C ,and 15 ± 1 °C, respectively.
The *bars* represent mean ± standard error.

et al. 1992;). The number of somatic embryos increased with increase in the concentration of TDZ in all the three temperature regimes until it reached the maximum (31 ± 0.95 somatic embryos per explant) on 4.0 mg l^{-1} TDZ, where the cultures were incubated at 10 ± 1 °C (regime B) (Table 1; Figure 2). However, at supra-optimal concentrations the number of normal somatic embryos decreased while the number of shriveled and deformed embryos increased (data not shown). The temperature regime B recorded the highest number of somatic embryos per explant (31 ± 0.95) followed by regime A (23 ± 0.82). These results clearly demonstrate that the concentration of TDZ is the primary factor responsible for induction of somatic embryogenesis, and incubation on temperature regime B (10 ± 1 °C) has a complementary effect on increasing the number of somatic embryos per explant. Incubation on optimum temperature and concentration of TDZ caused redirection of the developmental program in cells and acquisition of competence in the embryogenic process. This competence to take up the embryogenic pathway might be due to some temperature-regulated proteins, as shown by a temperature-sensitive line (ts11) of carrot (Giuliano et al. 1984) that produces somatic embryos at 24 °C, but at 32 °C embryos are arrested at the globular stage. However, when the proteins secreted into the cultured medium by the cells grown at 24 °C are added to the culture growing at 32 °C, normal development is restored and the embryos are able to pass beyond the globular stage and complete their development (Baldan et al. 1997; De Jong et al. 1992; Lo Schiavo et al. 1990). One of those proteins has been identified as a glycosylated acid endochitinase, EP3, with a molecular mass of 32 kD (De Jong et al. 1992).

The cotyledonary embryos germinated after one week of subculturing (Figure 1f) and the maximum germination percentage of 48% could be obtained on MS medium containing kinetin (1.0 mg l^{-1}) (Table 2). Clumps with small shoots and roots (up to 1.5 cm long) developing from the explants (after seven weeks) were then transferred to MS medium without growth regulators for further growth and development. These clumps developed into complete plantlets on MS medium and attained a shoot length of 6 cm in four weeks (Fig. 3). Plantlets were then separated and planted into a soil-less substrate containing sterilized peatmoss or soilrite or vermiculite or

Table 2. Percentage germination of somatic embryos on MS medium.

Medium	Germination % (2 weeks)
MS	26 ± 3.1
MS+ GA3 (1.0 mg l^{-1})	16 ± 2.8
MS+ GA3 (2.0 mg l^{-1})	12 ± 2.3
MS+ Kn (1.0 mg l^{-1})	48 ± 3.0
MS+ Kn (2.0 mg l^{-1})	36 ± 2.9

All values are expressed as percentage mean ± SE with at least 3 replicates.

Table 3. Percentage survival of *in vitro* regenerated plantlets in different potting mixtures.

Potting mixture	Percentage survival (4 weeks)
Peat moss	65 ± 4.1
Soilrite	45 ± 2.6
Vermiculite	68 ± 3.1
Vermiculite+ Peat moss+ Soilrite (1:1:1)	79 ± 3.8

All values are expressed as percentage mean ± SE with at least 3 replicates.

mixture of vermiculite, peatmoss and soilrite (1:1:1). The percentage survival of these potted plants grown in growth chamber at 25 ± 2 °C and relative humidity of 70-95% was recorded after four weeks of transfer. The maximum survival percentage (79%) of plantlets after four weeks was found to be in the mixture of vermiculite, peatmoss, and soilrite (1:1:1), followed by 68% in vermiculite (Table 3). The most interesting observations of our study are: (1) Incubation on optimum temperature and concentration of TDZ causes redirection of the developmental program and acquisition of competence in the embryogenic process; (2) appropriate concentration of TDZ is the primary factor responsible for induction of somatic embryogenesis; (3) optimum concentration of TDZ improves the 'quality' of mature embryos i.e., produces normal structure and facilitates higher levels of germination or the conversion of embryos to plantlets; (4) incubation on temperature regime B (10 ± 1 °C) has a complementary effect on increasing the number of somatic embryos per explant; and (5) some temperature-regulated proteins might be involved in the induction of somatic embryogenesis in strawberry.

Fig. 3. Development of somatic embryos into plantlets. (a) Small shoots developing on MS medium with Kn (1.0 mg l^{-1}), (b) Plantlets developed on MS basal medium, (c) Plantlet showing healthy roots, and (d) Acclimatization on soil-free substrate in pots.

These results are particularly encouraging as somatic embryos were regenerated at high frequency (31 ± 0.95 per explant) as compared to previous report of 26 ± 0.82 per explant (Husaini and Abdin 2007) with a percent increase of 19.2%. Moreover the efficiency of germination into plantlets is as high as 48%. This system can therefore accelerate the introduction of improved clones into commercial production, since somatic embryos can be encapsulated and handled as artificial seeds (Torne et al. 2001). Moreover, this system has a marked advantage over the use of callus culture system for transformation, which may not be completely free from the risk of somaclonal variation (Larkin and Scowcroft 1981). The variability in embryogenic cultures is relatively less than that in organogenic cultures (Ozias-Akins and Vasil 1988) and hence it can serve as a very promising system for the regeneration of genetically transformed cells, and avoiding chimeras (Trigiano et al. 1989). It will therefore provide an appropriate regeneration system for the recovery of transgenic strawberry plants after genetic transformation (Husaini and Abdin 2008).

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