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Plant Regeneration via Direct Somatic Embryogenesis in Three Strawberry (*Fragaria ananassa* Duch.) Cultivars

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ABSTRACT

Cultivated strawberry (*Fragaria×ananassa* Duch.) is one of the most important fruit plants for both fresh consumption and food processing. The aim of this study was plant regeneration via direct somatic embryogenesis in three strawberry cultivars of Kurdistan, Paros and Camarosa. For this purpose, leaf blade of cultivars were cultured on Murashige and Skoog (MS) medium supplemented with Thidiazuron (TDZ) at 1, 2, 3, and 4 mg/L alone or in combinations with the different concentrations (0, 0.25, 0.5, and 1 mg/L) of auxin 2,4-dichlorophenoxy acetic acid (2,4-D). All data were analyzed by one-way analysis of variance (ANOVA) using factorial experimental based on completely randomized design with four replication and means were compared with Duncan's tests at $p< 0.05$. The highest induction frequency of embryogenesis and number of somatic embryos per explants was obtained on MS medium containing 3 mg/L TDZ supplemented with 0.25 mg/L 2,4-D. In this medium, the maximum number of globular embryos per explants obtained for the cultivars of Camarosa (25.75), Paros (22.00) and Kurdistan (14.75). The globular embryos of the leaf explants developing into cotyledonary embryos were cultured on MS medium supplemented with sucrose at a concentration of 1.5, 3, 6, 9, and 12%. Among the tested concentrations, 6% sucrose was found superior for uniform embryo developmental stages.

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1. Introduction

Cultivated strawberry (*Fragaria* × *ananassa* Duch.) is fruit plants for fresh consumption and food processing. It is a high value product that is growing all over the world. This plant is clonally propagated by runner (Hancock *et al*., 2008).

The process of somatic embryogenesis consists in cultivating somatic cells under suitable conditions for the formation of embryonic cells, which undergo a morphophysiological process that produces somatic embryos and later complete plants (Wójcikowska and Gaj, 2017). In zygomatic embryogenesis, the embryo is inside the seed, making it difficult to study, but somatic embryogenesis allows easy manipulation and control of the culture conditions for the study of morphophysiological, biochemical, and molecular processes that occur during the development in higher plants (Kumar and Van Staden, 2017).

Somatic embryogenesis has made it possible to propagate a large number of plants of different species. It has two different pathways called direct and indirect somatic embryogenesis (Loyola-Vargas *et al.*, 2016; Horstman *et al.*, 2017). Cultivation of explants on media containing the appropriate balance of plant growth regulators can induce formation of embryos directly from the explanted tissue (direct somatic embryogenesis) or induce proliferation of embryogenic callus (indirect somatic embryogenesis) (Pulianmackal *et al.*, 2014). The direct embryogenesis occurs when a plant cell produces embryos without callus formation

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while indirect embryogenesis requires one additional step for callus formation prior to embryo development (Horstman *et al.*, 2017).

One of the most important factors in inducing somatic embryogenesis in plants is growth regulator, which has been reported in approximately 80% of somatic embryogenesis induction protocols (Nic-Can and Loyola-Vargas, 2016). Most of the plant growth regulators used are auxins, alone or in combination with other regulators (Wójcikowska and Gaj, 2017). Different components are involved in the mechanism of regulation of the auxins' response genes (Sghaier *et al.*, 2018). Plants growth regulators of auxin play an important role in somatic embryogenesis in many plant species, and the use of 2,4-D is very common for this purpose. However, this auxin can produce epigenetic and genetic changes in the cells (Fehér, 2015). Auxin (2,4-D) in maturation medium can actually inhibit the development of embryos and somatic embryo maturation occurs usually on medium without any auxins (Vondráková *et al.*, 2011).

Somatic embryogenesis occurs due to changes in the level of existing growth regulators, especially auxins and cytokinins in the plant tissue culture medium (Moradi *et al.*, 2017).

TDZ is a suitable regulator for in vitro propagation in a wide range of plants. It is generally used to induce organogenesis and direct somatic embryogenesis, alone or in combination with other growth regulators (Guo *et al.*, 2011; Mahendran and Bai, 2016). Many studies indicated that the use of TDZ alone could induced direct somatic embryogenesis and it is better efficacy over other purine-type cytokinins even at low concentrations (Feng and Chen, 2014). TDZ has cytokinin-like activity and can be used as an alternative for both auxin and cytokinin (Kou *et al.*, 2016).

Although, the formation of somatic embryos in strawberry have been reported recently by the current authors. Somatic embryogenesis of strawberries is still in a preliminary stage and some more research is needed to develop it (Biswas *et al.*, 2007). During past decades, TDZ gained a lot of attention due to its prominent role on in vitro culture, with both auxin and cytokinin like effects, in different plant species (Debnath, 2018). Although limited reports on somatic embryogenesis are available in few small fruit crops such as strawberry (Biswas *et al.*, 2009). The aim of this study was plant regeneration via direct somatic embryogenesis in leaf explants from three strawberry cultivars response to TDZ and 2,4-D or combinations.

2. Materials and methods

Runner tips were prepared from three strawberry cultivars of ‘Kurdistan’, ‘Paros’ and ‘Camarosa’ and washed under running tap water for 30 minutes to remove surface contaminants. Surface sterilization was done inside the laminar air flow cabinet by 30 seconds dipping the explants in 70% (v/v) ethanol and for 15 minutes in the aqueous solution of 0.5% (v/v) sodium hypochlorite. After three washes in sterile double distilled water, sterilized runner tips (3–4 mm) were cultured on MS medium supplemented with 0.5 mg/L BA, 0.1 mg/L GA_3 and 0.1 mg/L IBA, as described by Boxus (1999). The pH of the medium was adjusted to 5.8 using 0.1n NaOH or HCl and medium were solidified with 0.8% agar before autoclaving. The explants were cultured in a growth chamber under 16/8 h light/dark cycle at 25±2°c.

2.1. Effect of growth regulator on somatic embryogenesis

Pieces from fully expanded young leaves of 4-5 week-old plantlets from auxiliary shoots of in vitro cultured runner tips were used as explants. The discs (each approximately 5×5 mm) were cultured at the abaxial surface in contact with the direct somatic embryogenesis media. Leaf explants were cultured on MS medium supplemented with TDZ at 1, 2, 3, and 4 mg/L alone as well as the combination with different concentrations (0, 0.25, 0.5, and 1 mg/L) of 2,4-D for direct somatic embryogenesis induction. 6 explants were cultured in each Petri dish (100 x 20 mm). During direct somatic embryogenesis period, all the cultures were incubated at under 16/8 h light/dark cycle in 25 ± 1°c.

2.2. Effect of sucrose on somatic embryo development

In order to develop somatic embryos, globular stage embryos were transferred to MS media with 1.5%, 3%, 6%, 9%, and 12% concentrations of sucrose without growth regulator. The results include the percentage of explants exhibiting somatic embryogenesis, the percentages of globular stage embryos developing into cotyledonary ones as well as the number of somatic embryos per responding explants 4-6 weeks after subculture.

2.3. Conversion of embryos

To regenerate whole plants, cotyledonary somatic embryos were transferred to Ms basal medium containing 3% sucrose and 1 mg/L GA3 and incubated under a 16 h light and 8 h dark photoperiod at 25 ± 1°c. Plantlets developed from somatic embryos were subjected to acclimation, transplanted into plastic bags containing sterile peat moss and perlite (1:2), and maintained in a growth chamber under 16/8 h light/dark photoperiod at 25 ± 1°ᶜ and relative humidity of 90-95% for four weeks and then transferred to greenhouse.

2.4. Statistical analysis

Thirty-six explants were used in each treatment and the experiments were conducted and all data were analyzed by one-way analysis of variance (ANOVA) using factorial experimental based on completely randomized design with four replication and means were compared with Duncan's tests at $p < 0.05$ whit SAS program.

3. Results

For purpose of direct somatic embryogenesis, leaf blade of Kurdistan, Paros, and Camarosa cultivars were cultured on MS medium supplemented with TDZ alone as well as in combination with different concentrations of 2,4-D. Initiation of somatic embryos was begun on explants within 3–4 weeks of inoculation on media and somatic embryos were observed on the margins of leaf explants. It is observed that direct Somatic embryogenesis was not affected by the callus formation because they grew fast and produced proglobular embryos (Fig. 1a) and after two weeks the proglobular embryos developed into globular embryos (Fig. 1b) and after 3–4 weeks, these matured into cotyledonary-stage embryos (Fig. 1c).

3.1. Effect of growth regulator and cultivar on somatic embryogenesis

Percentage of embryogenic explants and number of globular embryos per explants for leaf blade in different cultivars were significantly different ($p<0.01$). The data obtained in this study indicated that the response of embryogenic percentage and number of globular embryos per explants were greatly dependent on the concentration of TDZ combination with different concentrations of 2,4-D. TDZ combination with the low concentration of 2,4-D improves percentage embryogenic and number of globular embryos per explants. The highest response of percentage embryogenic and number of globular embryos per explants was obtained in MS containing 3 mg/L TDZ + 0.25 mg/L 2,4-D in all tested cultivars. This combination yielded the highest percentage embryogenic explant and number of globular embryos per explants from Camarosa (83.10% embryogeny and 25.75 embryones per explants), Kurdistan (78.05% embryogeny and 14.75 embryones per explants) and Parose (74.10% embryogeny and 22 embryo per explants) (Table 1).

The lowest value of direct somatic embryogenic (0%) was for all concentration of TDZ combination with the high concentration of 2,4-D combination (2 mg/L). Because callus induction was observed within two weeks after inoculation leaf explants on MS medium containing different concentrations of TDZ combination with the high concentration of 2,4-D (Table 1).

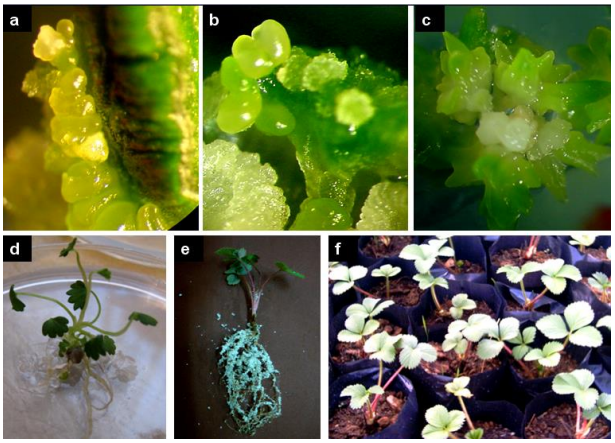

Figure 1. Direct somatic embryogenesis system developed for strawberry; a) Somatic embryo induction on culture MS medium containing 3 mg/L TDZ supplemented with 0.25 mg/L 2,4-D; b) Globular-stage embryos; c) Cotyledonary-stage embryos on culture MS medium containing 6% sucrose; d) Conversion of somatic embryos to plantlet on MS medium containing 1 mg/L GA_3; e) Converted plantlet with well-developed roots in pots in the growth chamber; f) Potted plants in the greenhouse.

3.2. Effect of sucrose on somatic embryo development

Results indicated that the development of somatic embryos from the globular to the cotyledonary stage was strictly dependent on the concentration of sucrose in the medium. The highest percentage of stimulated embryos and normal development occurred on media with 6% sucrose (Table 2). Ms medium supplemented with 6% sucrose were found superior not only for giving optimum growth rate of embryonic culture but

also a uniform embryo developmental stage (Fig 1c). The frequency of developing embryos decreased on media containing lower (1.5 and 3%) or higher (9 and 12%) concentrations of sugar. The percentage of cotyledonary-stage embryos was also different among the cultivars. Paros cultivar gave the highest frequency and Kurdistan the lowest (Table 2).

Table1. Mean comparison of the effect of different TDZ and 2,4-D concentrations on direct somatic embryogenesis of leaf explants of strawberry cultivars

TDZ (mg/L)	2,4-D (mg/L)	Percentage of explant embryogenesis			Number of globular embryos per explant		
		Cultivar			Cultivar		
		Kurdistan	Paros	Camarosa	Kurdistan	Paros	Camarosa
1	0.0	22.32^{u}	25.05^{st}	30.07^{qr}	1.97^{qr}	2.80^{pq}	3.42^{nop}
2	0.0	46.45^{m}	46.15^{mn}	51.15^{k}	5.52^{kl}	6.57^{ij}	8.00^{gh}
3	0.0	61.15^{h}	65.02^{f}	69.20^{e}	12.27^{e}	14.30^{d}	15.50^{c}
4	0.0	56.05^{i}	53.20^{j}	64.15^{h}	7.62^{gh}	7.37^{hi}	8.40^{fgh}
1	0.25	21.10^{v}	29.20^{r}	19.17^{vw}	3.02^{opq}	$2.65p^{q}$	4.00^{mno}
2	0.25	56.02^{i}	63.17^{g}	65.02^{f}	8.62^{fg}	9.32^{f}	8.15^{gh}
3	0.25	78.05^{b}	74.10^{c}	83.10^{a}	14.75^{cd}	22.00^{b}	25.75^{a}
4	0.25	63.17^{g}	71.05^{d}	68.12^{e}	14.52^{cd}	12.74^{e}	14.00^{d}
1	0.5	7.20^{z}	12.12^{y}	17.20^{wx}	0.30^{st}	0.90^{st}	1.00^{rst}
2	0.5	18.15^{w}	24.22^{t}	29.17^{r}	3.00^{opq}	2.90^{pq}	3.37^{nop}
3	0.5	33.17^{p}	31.27^{q}	42.22^{o}	5.42^{kl}	4.57^{lm}	6.00^{jk}
4	0.5	41.22^{o}	45.15^{n}	48.17^{l}	4.32^{mn}	5.50^{kl}	5.52^{kl}
1	1	0.00	0.00	0.00	0.00	0.00	0.00
2	1	0.00	0.00	0.00	0.00	0.00	0.00
3	1	11.10^{yz}	15.10^{x}	12.85^{y}	0.62^{st}	1.15^{rst}	0.50^{st}
4	1	19.07^{vw}	26.05^{s}	25.12^{st}	1.30^{rs}	2.62^{pq}	2.37^{pq}
1	2	0.00	0.00	0.00	0.00	0.00	0.00
2	2	0.00	0.00	0.00	0.00	0.00	0.00
3	2	0.00	0.00	0.00	0.00	0.00	0.00
4	2	0.00	0.00	0.00	0.00	0.00	0.00

Different letters in each column indicate a significant difference ($p<0.05$)

Table2. Mean comparison of the effect of sucrose concentration on the development of somatic embryos in the leaf explants of strawberry cultivars

Sucrose (%)	Number of globular embryos per explant			Number of cotyledonary embryos per explant		
	Cultivar			Cultivar		
	Kurdistan	Paros	Camarosa	Kurdistan	Paros	Camarosa
1.5	2.85^{g}	3.37^{g}	3.05^{g}	0.70^{j}	1.10^{i}	0.00^{k}
3	5.77^{f}	7.35^{d}	7.20^{de}	2.07^{h}	2.75^{g}	2.07^{h}
6	11.68^{b}	12.68^{ab}	13.35^{a}	5.75^{b}	6.50^{a}	5.95^{b}
9	7.97^{cd}	8.62^{c}	8.55^{c}	3.97^{e}	4.60^{d}	5.15^{c}
12	5.55^{f}	7.27^{de}	6.17^{ef}	1.97^{h}	3.05^{g}	3.37^{f}

Different letters in each column indicate a significant difference ($p<0.05$)

3.3. Plant regeneration

The average germination rate of somatic embryos on MS basal medium containing 1 mg/L GA_3 was about 65–67% for all cultivars. Plantlets developed from somatic embryos (Fig. 1d) were subjected to acclimation, transplanted into plastic bags and maintained in a growth chamber for four weeks (Fig. 1e). The rooted plants were hardened and transferred to soil with 75% to 80% survival rate (Fig. 1f).

4. Discussion

The effects of different concentrations of TDZ and 2,4-D on the percentage of responding explants and number of globular embryos per explants are shown in Table 1. Somatic embryo induction and somatic embryos were induced on media supplemented with TDZ alone as well as the combination with low concentrations of 2,4-D. However, the process of somatic embryogenesis is affected by many factors.

Plant growth regulators, which are important factors in plant development, play a crucial role in somatic embryogenesis (Gerdakaneh and Zohori, 2013). Auxin and cytokinin balance has long been recognized as a key factor in regeneration in most dicot plants (George *et al.*, 2008; Lim *et al.*, 2009). TDZ alone or in combination with other growth regulators is used for somatic embryogenesis (Debnath, 2018).

The results indicated that increasing TDZ concentration in the medium improved the frequency of globular somatic embryos. According to our results, a medium supplemented with 3 mg/L TDZ proved to be the best for a high frequency of globular embryo induction in all tested cultivars. Both higher and lower TDZ concentrations reduced the frequency of embryogenesis. Somatic embryogenesis occurs due to changes in the level of existing growth regulators, especially auxins and cytokinins in the plant tissue culture medium (Moradi *et al.*, 2017). TDZ is a suitable regulator for in vitro propagation in a wide range of plants. It is generally used to induce organogenesis and direct somatic embryogenesis, alone or in combination with other growth regulators (Guo *et al.*, 2011; Mahendran and Bai, 2016). The application of TDZ could induce somatic embryogenesis of many plant species (Lee and Chen, 2014; Tsai *et al.*, 2016). TDZ affected plant physiology, such as cellular, nutrient, transport and alters the endogenous levels of plant growth regulators (Ouyang *et al.*, 2016). TDZ has been generally used to induce organogenesis and high frequency of direct somatic embryogenesis, either alone or in combination with other growth regulators (Mahendran and Bai, 2016). Many studies indicated that the use of TDZ alone could induced direct somatic embryogenesis even at low concentrations (Feng and Chen, 2014). TDZ has cytokinin-like activity and can be used as an alternative for both auxin and cytokinin (Kou *et al.*, 2016). The data obtained in this study indicate that the response of percentage embryogenic and number of globular embryos per explants was greatly dependent on the concentration of TDZ combination with different concentrations of 2,4-D. It has been known that auxins mainly 2,4-D is required for somatic embryogenesis induction and embryo multiplication to scale-up the number of embryos which can be potentially produced by indirect somatic embryogenesis (Vondráková *et al.*, 2011) but it is necessary to remove this plant growth regulator in the later stages of expression, development and maturation because it prevents the embryo development and conversion into plant (Zavattieri *et al.*, 2010). Plant growth regulators (especially 2,4-D) in high concentration or high exposure of the explant to auxins can block normal embryo development and auxin (2,4-D) in maturation medium can actually inhibit the development of embryos and somatic embryo maturation occurs usually on medium without any auxins (Vondráková *et al.*, 2011).

The percentage of embryo formation per explant depended to cultivars. Similar results were observed during SE of four blueberry cultivars, where percent and average number of somatic embryo formation varied with genotypes (Ghosh *et al.*, 2018). The results obtained in this study indicate that somatic embryogenesis pathway was affected by the interaction between the type and concentration of plant growth regulator, and the cultivar. In fact, this kind of interaction is generally considered to play a key role in the success of plant regeneration through direct somatic embryogenesis. The highest the number of globular embryos per explant was recorded for Camarosa followed by Paros and Kurdistan (Table 1). There is a strong genetic component in the regeneration capacity of different strawberry cultivars and different responses were also observed in the induction of somatic embryos in the different genotype of strawberry by Gerdakaneh et al. (2011). Such results may indicat differences in the ability to activate key elements of the embryogenic pathway. In the present study, significant genotypic differences were observed for somatic embryos in the different genotype of strawberry.

In three cultivars, the number of embryos per explant enhanced as the concentration of sucrose increased from 1.5% to 6% and the number of embryos per explant were decreased as the concentration of sucrose increased from 6% to 12% (Table 2). Sucrose concentration play important roles in different stages of the somatic embryogenesis process and this positive effect could mimic the changes in osmolarity that occur in the environment surrounding the zygote embryo within the seed (Widoretno *et al.*, 2017). In other plant species, increasing sucrose concentrations in the medium improved the development of globular somatic embryos (Ghobeishavi *et al.*, 2015). Similar results have been reported in Lilium (Bakhshaie *et al.*, 2010).

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