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DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

Interactive effect of light, temperature and TDZ on the regeneration potential of leaf discs of *Fragaria x ananassa* Duch

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Abstract This is the first report where shoot regeneration in strawberry cultivar Chandler has been achieved simultaneously through both somatic embryogenesis and shoot bud formation. Direct somatic embryogenesis was observed in leaf discs which were cultured on medium containing MS salts + B_5 vitamins + 2% glucose + 18.16 μM thidiazuron (TDZ) and given both chilling and dark treatment for 2 wk at 4±2°C followed by incubation at 25±2°C under 16-h photoperiod for third wk. After 3 wk, these explants were then subcultured on medium containing MS salts + B_5 vitamins + 2% glucose and incubated under 16-h photoperiod at 25±2°C for further growth and development. Direct regeneration via *de novo* shoot bud formation was observed in leaf disks which were given dark treatment and were cultured on medium containing MS salts + B_5 vitamins + 2% glucose supplemented with 9.08 μM TDZ. There was a synergistic effect of photoperiod, dark, and chilling treatments on somatic embryogenesis, whereas chilling treatment had an inhibitory effect on shoot organogenesis.

Keywords Strawberry · TDZ · *In vitro* · Organogenesis · Somatic embryogenesis · Regeneration

Introduction

Growth and regeneration *in vitro* is a complex phenomenon and is influenced by a number of genetic and environmental factors. As every species seems to have its own specific requirements, there are several reports about the substances and conditions which help cells to differentiate (Sen et al. 2002). Being an economically important crop, the application of plant tissue culture and plant genetic engineering in strawberry cultivation is of special value to obtain improved or desirable traits like disease resistance, insect resistance and to enhance the shelf life of this fruit crop (Husaini and Srivastava 2006). Strawberry is a suitable target for improvement through direct gene manipulations especially because of the genetic limitations associated with high heterozygosity and polyploidy which hamper the improvement through traditional breeding methods (James 1987). The clonal propagation of strawberry (Boxus et al. 1977) provides an added advantage for the stable transfer of a desired gene into a commercially important genotype without sexual recombination.

Regeneration via shoot organogenesis has been described in different cultivars of strawberry, and many workers have reported the use of thidiazuron for shoot regeneration from leaf discs in strawberry (Schaart et al. 2002; Passey et al. 2003; Zhao et al. 2004; Yonghua et al. 2005), but no one has studied its effect along with that of dark treatment, chilling treatment, and photoperiod on the morphogenetic response of strawberry leaf explants. These reports describe the use of different concentrations of thidiazuron (TDZ) for shoot regeneration in different strawberry cultivars. Wang et al. (1984) had explored somatic embryogenesis in strawberry, and their most effective medium for inducing somatic embryos contained 2,4-D (22.62 μM), BA (2.22 μM), and casein hydrolysate (500 mg/l), whereas Lis (1987) had reported the formation of adventitious buds and somatic embryos using the medium of Lee and de Fossard (1977).

TDZ, a substituted phenyl urea, was first used for the mechanized harvesting of cotton bolls and more recently

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incorporated into tissue culture media as a means of inducing regeneration, as it acts as a substitute for both the auxin and cytokinin requirements of organogenesis and somatic embryogenesis in several species (Murthy et al. 1998). TDZ is responsible for biosynthesis of cytokinin and preserves endogenous hormones in plant tissues (Capelle et al. 1983; Thomas and Katterman 1986).

In the investigation reported here, TDZ-induced regeneration of strawberry was studied with the following specific objectives: (1) to test the effect of varying TDZ concentration on the induction of shoot organogenesis and somatic embryogenesis; (2) to test the effect of chilling treatment and dark treatment on the induction of shoot organogenesis and somatic embryogenesis; (3) to test the effect of exposure to varying light photoperiods on morphogenetic response of cultured leaf explants.

Materials and Methods

Explant source and preparation. The nodal explants of 9-mo.-old plants of strawberry cultivar Chandler, procured from Division of Pomology, SKUAST-K and maintained in a glasshouse in the Herbal Garden of Jamia Hamdard University, served as a source for establishing the *in vitro* cultures. Green leaves from 20-d-old plantlets of these *in vitro* cultures, maintained under culture room conditions [temperature: 25±2°C; light intensity, 44.85 μE m^{-2} s^{-1}; photoperiod: 16/8-h (day/night)] were used as explants. The leaflets were separated, and leaf discs (0.5–1.0 cm) were prepared by cutting along the midvein and the edges.

Culture conditions. After cutting the leaf material into small discs, these were cultured on culture medium contain-

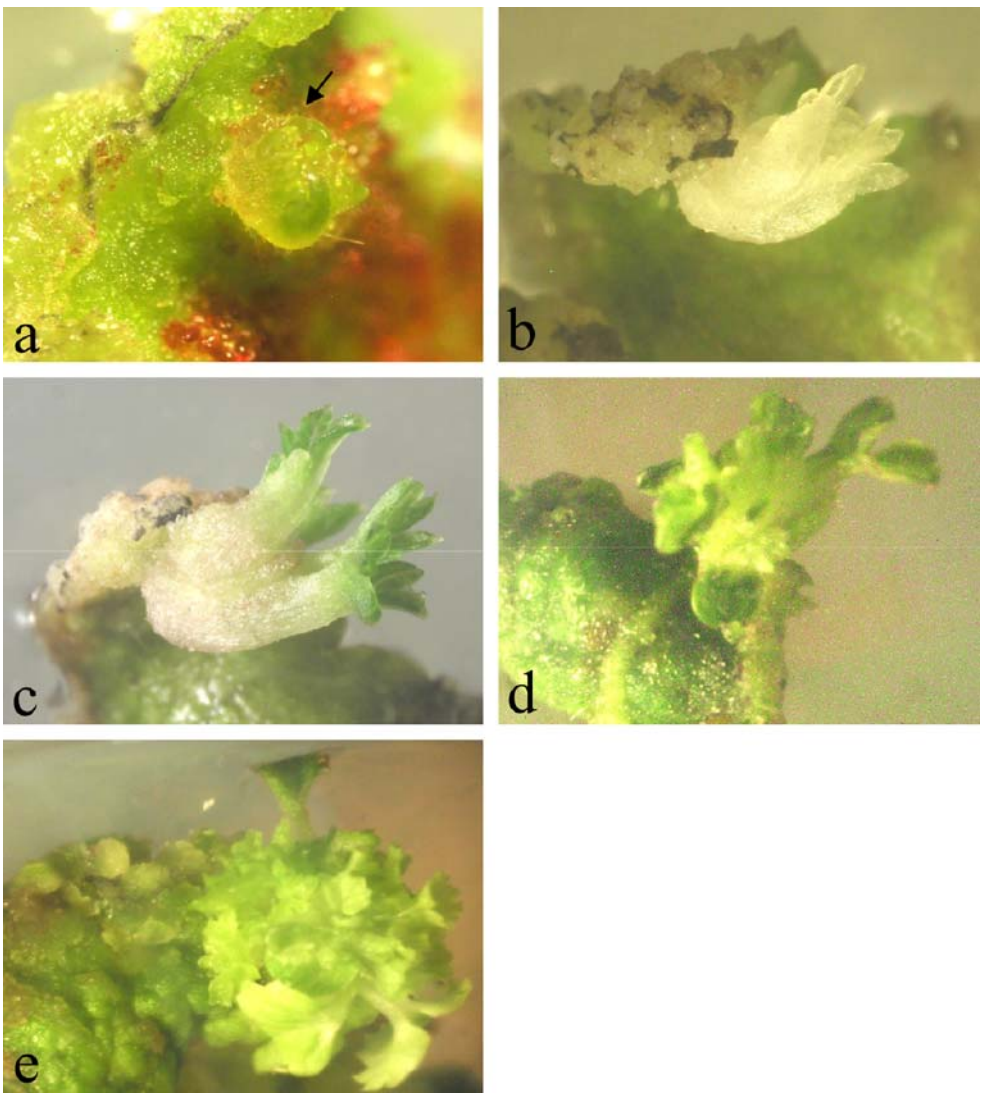

Figure 1. Direct shoot regeneration in *Fragaria x ananassa*. (*a*) Shoot bud initiation on the leaf margin (*arrow*); (*b*, *c*) Differentiation of shoot bud; (*d*, *e*) Multiple shoot formation.

ing MS salts (Murashige and Skoog 1962), B_5 vitamins (Gamborg et al. 1968), 2% glucose, 0.8% agar supplemented with seven different concentrations of TDZ viz 4.54, 6.81, 9.08, 11.35, 13.62, 15.89, and 18.16 μM, respectively.

These explants were then subjected to three different treatments, i.e., incubated:

1. directly under three different photoperiod conditions (24-, 16-, 12-h light) at 44.85 μE m^{-2} s^{-1} light intensity provided by cool-white fluorescent tube lights at 25±2°C. After 3 wk, these explants were then subcultured on culture medium containing MS salts, B_5 vitamins, 2% glucose, 0.8% agar and kept under same cultural conditions,
2. for 2 wk under complete darkness and then at 44.85 μE m^{-2} s^{-1} light intensity at 24-, 16- and 12-h photoperiods for third wk, temperature remaining constant at 25±2°C. After 3 wk, these explants were then subcultured as mentioned above for further growth and development,
3. for 2 wk under complete darkness at chilling temperature 4±2°C and then transferred to 44.85 μE m^{-2} s^{-1} light intensity at 24-, 16- and 12-h photoperiods for third wk at 25±2°C. After 3 wk, these explants were then subcultured as mentioned above.

Each treatment consisted of three replicate petriplates (each containing 12 explants), and the experiment was conducted thrice.

Cultures were scored on day 36 for the percentage of explants which either had embryos or bud primordia. The regenerants that emerged with true leaves from the cut surface

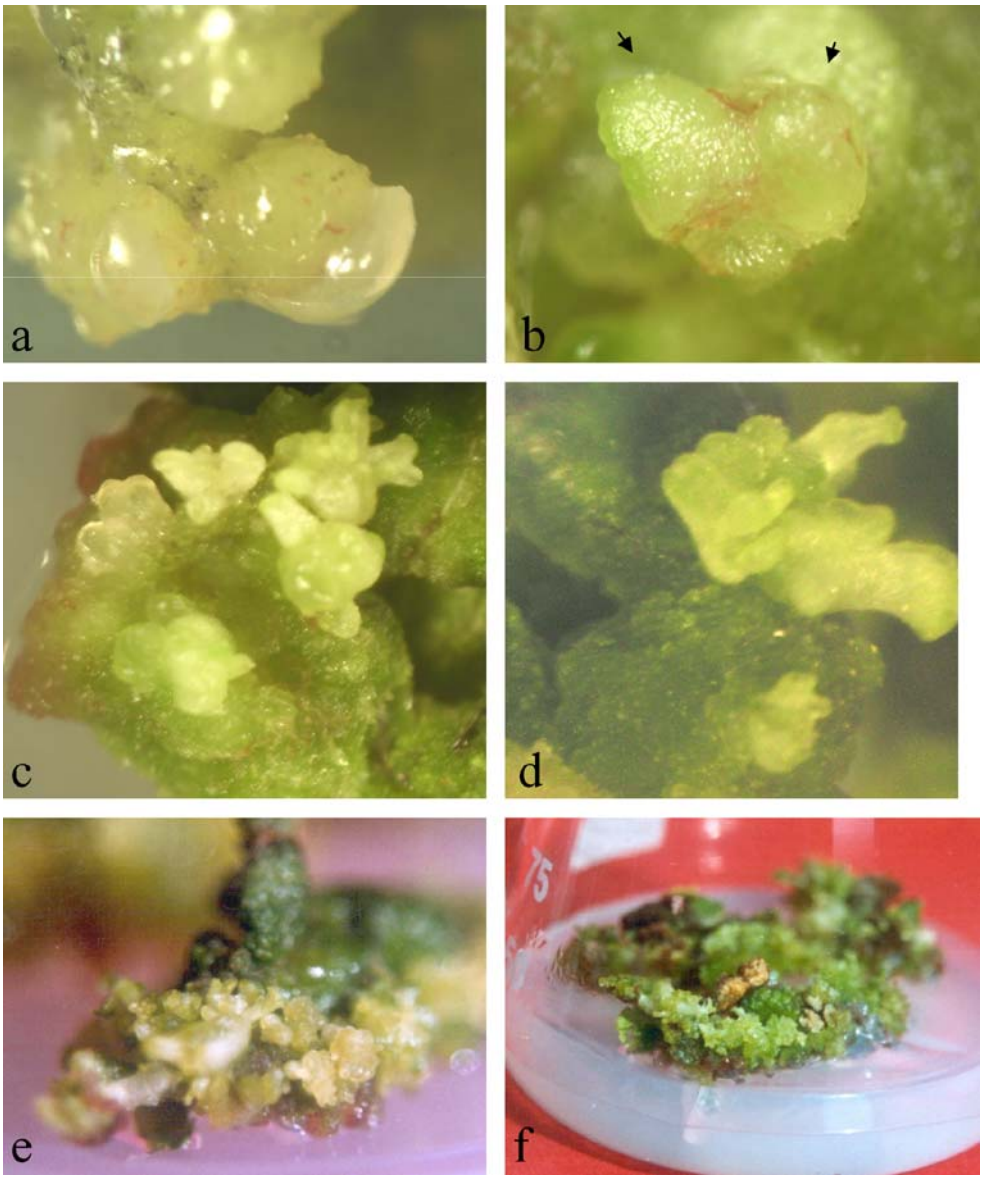

Figure 2. Direct somatic embryogenesis in *Fragaria x ananassa*. (*a*) Advanced globular embryos; (*b*) Heart-shaped embryo with cotyledonary primordia (*arrows*); (*c*) Advanced cotyledonary embryos; (*d*) Embryos germinating; (*e*,*f*) Multiple shoot regeneration.

of the explant were recorded as shoots, whereas the structures that appeared globular and loosely attached on the surface of the explant were scored as somatic embryos. The number of total regenerants was estimated by adding the number of shoots and somatic embryos.

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°C 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).

Statistical analysis. The effects of temperature, light or dark treatment were tested by the *F* test for the number of explants that had embryos or buds and by analysis of variance using MSTATC software. Ten explants from each medium × treatment combination were assayed each time.

Results

Shoot regeneration in strawberry leaf explants occurred simultaneously through both somatic embryogenesis as well as shoot bud formation. Small shoots were visible on the explants after 3 to 4 wk of culturing (Fig. 1*a–e*). There was a significantly greater number of shoots on explants (22±0.47 shoots per explant) cultured on the medium supplemented with 9.08 μM TDZ and exposed to dark treatment coupled with incubation at 16-h photoperiod.

Somatic embryos were observed in explants after 4 wk of culture (Figs. 2*a–f*, 3*a–d*). In general, embryos originated on the surface of the leaves and near leaf margins. Greater number of somatic embryos (26±0.82 somatic embryos per explant) developed on explants cultured on the medium supplemented with 18.16 μM TDZ and exposed to both chilling and dark treatment coupled with incubation at 16-h photoperiod. The embryos were loosely attached to the surface of the source tissue and could be easily detached. The explants cultured on medium containing no TDZ failed to produce somatic embryos (data not shown).

Effect of TDZ on regeneration. Maximum shoot organogenesis (22±0.47 shoots per explant) was observed on 9.08 μM TDZ where the cultures were exposed to dark treatment and incubated at 16-h photoperiod (Table 1; Fig. 4). The somatic embryos per explant (26±0.82 per explant) were maximum on 18.16 μM TDZ where the cultures were exposed to dark as well as chilling treatments and incubated at 16-h photoperiod (Table 1; Fig. 4). Further, the maximum number of regenerants (24 embryos + 6.67 buds per explant) was recorded in explants cultured on the medium supplemented with 18.16 μM TDZ (Table 2; Fig. 5).

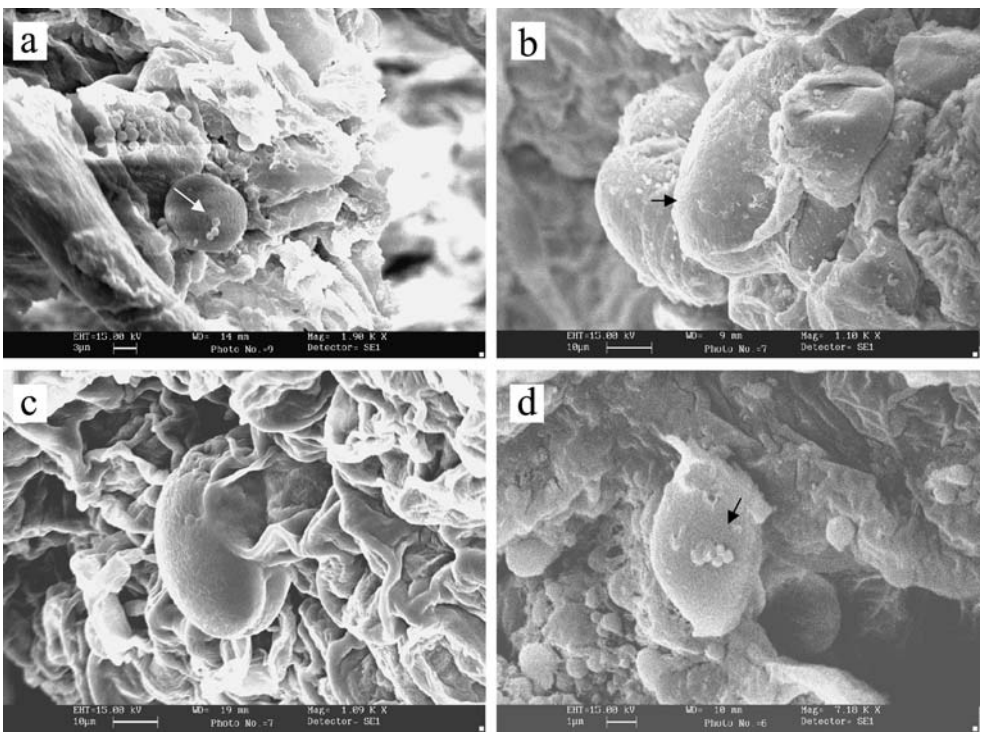

Figure 3. SEM images of somatic embryos in *Fragaria x ananassa*. (*a*) Globular embryo with secondary embryo (*arrow*) on surface; (*b*) Differentiation of advanced globular towards heart stage; (*c*) Early heart shaped embryo with two incipient cotyledons; (*d*) Torpedo stage embryo with secondary embryos (*arrow*).

Table 1. Interactive effect of TDZ and photoperiods coupled with dark and chilling treatments on the number of somatic embryos and number of shoots developing per explant

Medium: MS salts + B_5 Vit. + Glucose (2%)

TDZ concentration (μM)	Number of somatic embryos per explant			Number of shoots per explant		
	Photoperiod			Photoperiod		
	24/0	16/8	12/12	24/0	16/8	12/12
4.54	1.00±0.00	2.00±0.00	1.00±0.00	0.00±0.00	3.67±0.47	0.67±0.47
6.81	0.00±0.00	1.00±0.00	0.00±0.00	0.67±0.47	5.00±0.82	0.00±0.00
9.08	0.00±0.00	4.67±0.47	1.00±0.82	1.33±0.47	18.67±0.82	3.33±0.47
11.35	0.00±0.00	4.00±0.82	1.00±0.82	0.67±0.47	16.00±0.82	0.00±0.00
13.62	0.00±0.00	4.00±0.82	1.33±0.47	0.67±0.47	5.00±0.82	3.00±0.82
15.89	3.10±0.70	19.00±0.82	8.00±0.82	1.00±0.00	5.00±0.82	1.33±0.47
18.16	2.67±0.47	26.00±0.82	13.33±1.25	2.00±0.82	4.00±0.82	2.67±0.47
		CD at 5%			CD at 5%	
Photoperiod		0.682			0.694	
TDZ concentration		0.356			0.362	
Photoperiod × TDZ concentration		1.181			1.202	

Each value is the mean±standard error.

Effect of dark treatment on regeneration. The leaf explants that were inoculated directly under light turned the medium brownish black, whereas the explants that were inoculated under dark for first 2 wk showed a significant decrease in tissue browning. Furthermore, dark treatment increased shoot organogenesis considerably in the explants that were cultured on 9.08 μM TDZ and kept under 16-h photoperiod (22±0.47 shoots per explant; Table 2; Fig. 5), whereas when accompanied by chilling, a decrease in number of shoots (18.67±0.82 shoots per explant; Table 1; Fig. 4) was observed.

Similarly, dark treatment increased the number of somatic embryos considerably in the explants that were cultured on 18.16 μM TDZ and kept under 16-h photoperiod (24±0.82 somatic embryos per explant; Table 2; Fig. 5), and when accompanied by chilling, further increase in number (26±0.82 per explant; Table 1; Fig. 4) was observed.

Effect of chilling treatment on regeneration. There was an overall decrease in the number of shoots per explant when chilling treatment accompanied dark treatment (Tables 1, 2). However, it moderately increased the number of somatic embryos per explant especially when explants were cultured on 18.16 μM TDZ and were given dark treatment (Tables 1, 2; Figs. 4, 5). There was no significant effect of chilling on the maximum number of regenerants (≈30) obtained at 18.16 μM TDZ (Figs. 4, 5).

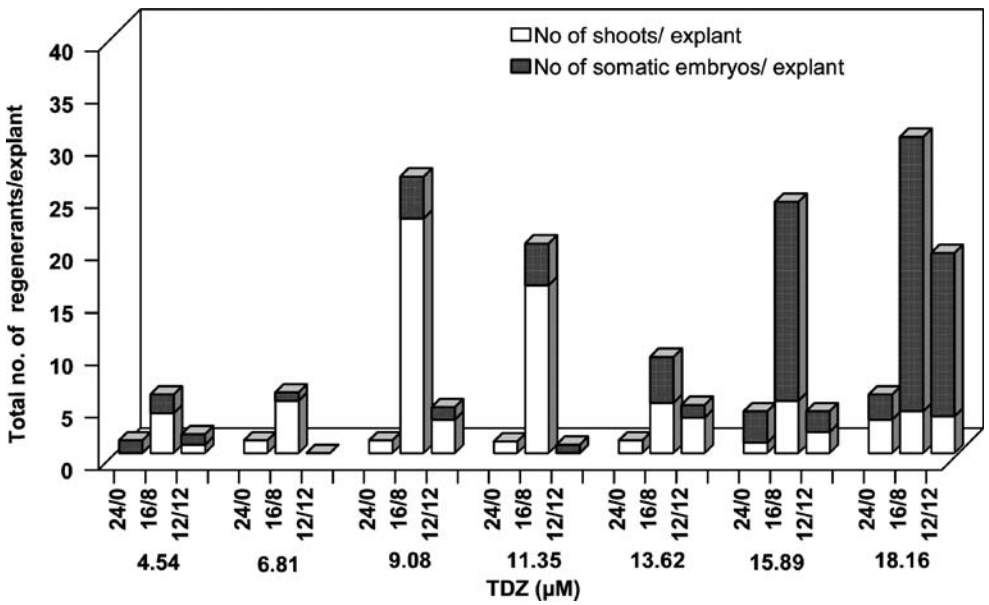

Figure 4. Interactive effect of TDZ, photoperiods, dark and chilling treatments on the total number of regenerants developing from leaf discs.

Table 2. Interactive effect of TDZ and photoperiods coupled with dark treatment on the number of somatic embryos and number of shoots developing per explant

Medium: MS salts + B_5 Vit. + Glucose (2%)						
TDZ concentration (μM)	Number of somatic embryos per explant			Number of shoots per explant		
	Photoperiod			Photoperiod		
	24/0	16/8	12/12	24/0	16/8	12/12
4.54	1.00±0.00	1.33±0.47	0.67±0.47	0.00.00	1.67±0.47	2.33±0.47
6.81	0.00±0.00	0.67±0.47	0.00±0.00	1.67±0.47	4.67±0.47	0.00±0.00
9.08	1.33±0.47	4.33±0.47	4.33±0.47	5.00±0.82	22.00±0.47	2.33±0.47
11.35	0.00±0.00	4.00±0.82	1.33±0.47	1.33±0.47	16.67±0.47	3.67±0.47
13.62	0.00±0.00	4.67±0.47	0.67±0.47	1.33±0.47	4.67±0.47	3.67±0.47
15.89	2.33±0.47	15.67±0.47	1.67±0.47	1.00±0.00	6.33±0.47	1.67±0.47
18.16	6.67±0.47	24.00±0.82	5.00±0.82	4.67±0.47	6.67±0.47	5.33±0.47
		CD at 5%			CD at 5%	
Photoperiod		0.544			0.569	
TDZ concentration		0.284			0.297	
Photoperiod × TDZ concentration		0.943			0.906	

Each value is the mean±standard error.

Moreover, it was interesting to note that the explants cultured on 18.16 μM TDZ and incubated at 12-h photoperiod developed significantly larger number of somatic embryos (13.33±1.25 per explant) when dark treatment was accompanied by chilling treatment in comparison to those that were given dark treatment alone (5±0.82 per explant; Tables 1, 2).

Effect of photoperiod on regeneration. The 16-h photoperiod is the overall best for shoot regeneration (via both organogenesis as well as somatic embryogenesis) from leaf explants on all treatments (Tables 1, 2, 3; Figs. 4, 5, 6). An interesting observation, however, was that when explants were cultured on 18.16 μM TDZ and were given chilling treatment, 12-h photoperiod proved to be better than many other treatments of 16-h incubation (Table 1; Fig. 4).

Discussion

In vitro plant organization might involve a two-step process where first, a cell or a tissue acquires developmental competency (totipotency) and then subsequently is determined for one structure or another by environmental factors (Decout et al. 1994). In wild carrot, two clearly separated regenerative processes, rhizogenesis and embryogenesis,

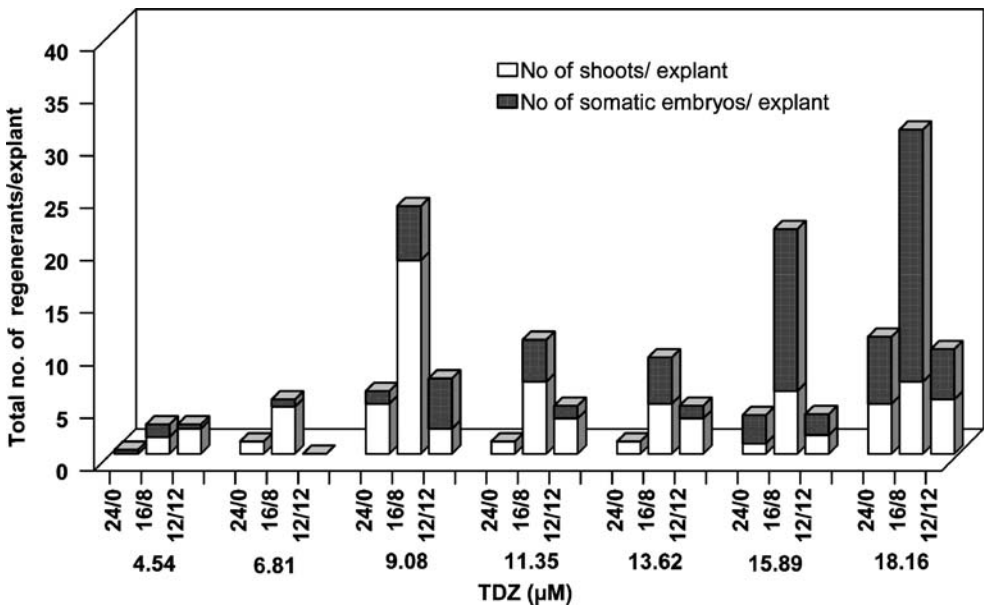

Figure 5. Interactive effect of TDZ, photoperiods and dark treatment on the total number of regenerants developing from leaf discs.

Table 3. Effect of TDZ and photoperiods on the number of somatic embryos and number of shoots per explant

Medium: MS salts + B_5 Vit. + Glucose (2%)

TDZ concentration (μM)	Number of somatic embryos per explant			Number of shoots per explant		
	Photoperiod			Photoperiod		
	24/0	16/8	12/12	24/0	16/8	12/12
4.54	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.67±0.47	2.33±0.47
6.81	0.00±0.00	0.00±0.00	0.00±0.00	2.00±0.00	4.00±0.82	0.00±0.00
9.08	0.00±0.00	1.67±0.47	0.00±0.00	0.00±0.00	8.67±0.47	1.67±0.47
11.35	0.00±0.00	0.00±0.00	1.33±0.47	0.00±0.00	5.33±0.47	0.33±0.47
13.62	0.00±0.00	0.00±0.00	1.67±0.47	0.33±0.47	2.67±0.47	1.00±0.82
15.89	0.00±0.00	7.00±0.82	1.33±0.47	0.00±0.00	1.00±0.00	1.67±0.47
18.16	1.67±0.47	10.00±0.82	1.67±0.47	0.67±0.47	4.67±0.47	1.67±0.47
		CD at 5%			CD at 5%	
Photoperiod		0.441			0.509	
TDZ concentration		0.230			0.266	
Photoperiod × TDZ concentration		0.764			0.882	

Each value is the mean±standard error.

are inducible as alternative morphogenetic events: media containing ammonium promote embryogenesis (Halperin 1966). Shift of hormone balance in the medium promotes shoots or embryoids in *Solanum carolinense* (Reynolds 1986), *Glycine max* (Barwale et al. 1986), and *Stylosanthes scabra* (Dornelas et al. 1992). In our study, we have been able to induce both somatic embryogenesis and shoot organogenesis (He et al. 1990) with identical photoperiods and dark treatment, but using different concentrations of TDZ. Shoot organogenesis was maximum when leaf explants were cultured on lower concentration of TDZ (9.08 μM), whereas somatic embryogenesis was best when leaf explants were cultured on relatively higher concentration of TDZ (18.16 μM), which is supported by the results obtained in African violet (Mithila et al. 2003). As no auxin was used in combination with TDZ, there is a conformation of some earlier findings that the most normal histodifferentiation into somatic embryos may even be achieved through the complete removal of exogenous auxins from the medium, and the presence of a cytokinin (in this case TDZ) enhances the number of somatic embryos ultimately recovered (Merkle et al. 1995). The increase in induction of somatic embryogenesis in comparison to that of organogenesis at higher TDZ doses may have occurred due to an optimum phytohormone balance within the tissue (Hutchinson et al. 1996; Murthy et al. 1996, 1998) combined with increased stress

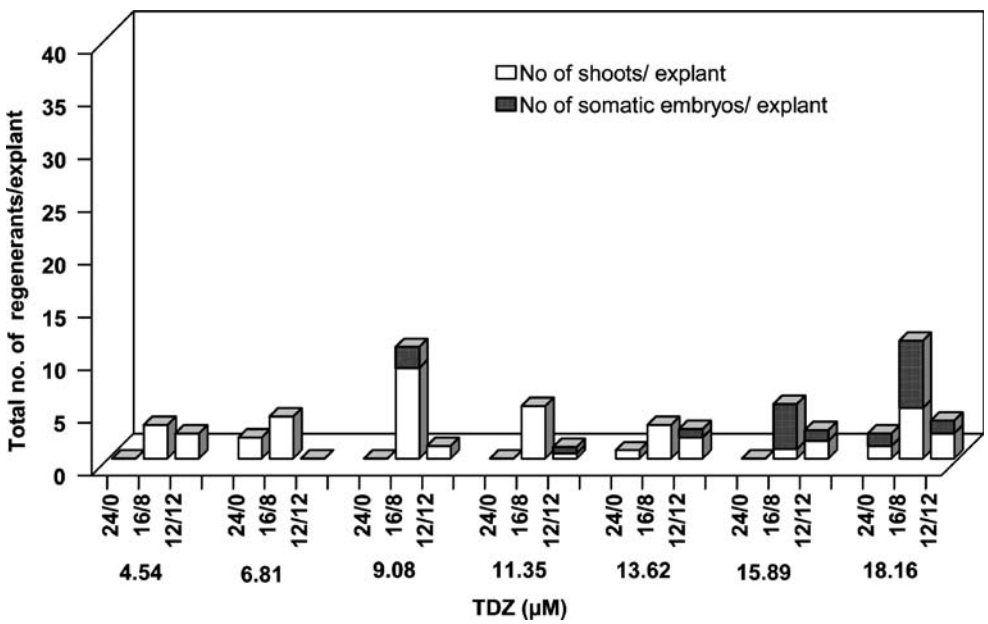

Figure 6. Effect of TDZ and photoperiods on the total number of regenerants developing from leaf discs.

imposed by chilling treatment (Decout et al. 1994; Mithila et al. 2003).

The problem of darkening of culture medium of *in vitro* cultured strawberry explants in the present study can be attributed to phenolic compounds exuding from these tissues. This process is initiated by browning of the surface of plant tissues due to the oxidation of phenolic compounds, resulting in the formation of quinines which are highly reactive and toxic to plant tissue (Taji and Williams 1996). In the present investigation, incubation in dark condition of leaf explants decreased tissue browning (George 1993) probably by arresting the enzymatic activity responsible for tissue oxidation (Titov et al. 2006). Besides this, it enhanced organogenesis in leaf explants as has been reported earlier in strawberry (Liu and Sanford 1988; Barcelo et al. 1998) as well as in several other genera, e.g., *Cucumis* (Punja et al. 1990), *Nicotiana* (Chandler et al. 1987), *Malus* (Fasolo et al. 1989). The explant cultivation in darkness during the first experimental step could also be a key factor influencing somatic embryogenesis (Fiore et al. 1997), especially when the negative effect of light on somatic embryo induction in strawberry has been reported in the cultivar Clea (Donnoli et al. 2001).

A stress treatment with the ability to down-regulate gene expression can stimulate somatic embryogenesis. Heat stress is effective for the induction of pollen embryos in canola (Pechan et al. 1991), whereas cold stress increases the embryogenic potential of strawberry (present study) and spruce (Hakman and von Arnold 1985).

Light is known to affect somatic embryogenesis through its effect on induction (Verhagen and Wann 1989) and on some morphological characteristics of differentiated somatic embryos (Halperin 1966; Ammirato and Steward 1971). Despite these powerful effects of light, little attention has been devoted to its role in *in vitro* culture (Torne et al. 2001). The *in vitro* photoperiod requirements vary from one plant species to other. Shoot bud regeneration is highest under 24-h illumination in ginger (Rout and Das 1998) and *Brassica* sp. (Jain et al. 1988), whereas it is best under 16-h photoperiod in lettuce cultures (Kadkade and Seibert 1977). However, the effect of long and short days as well as the effect of end-of-day red and far red light treatments on the somatic embryogenesis of *Araujia sericifera* petals (Torne et al. 1996) has revealed that there is a strong increase in the number and size of somatic embryos under long day. In the present study, a photoperiod of 16 h was found to be the optimum photoperiod for shoot bud regeneration as well as somatic embryogenesis. Photoperiod has been implicated in the regulation of cytokinin levels (Forsline and Langille 1975) as well as in photoconversion of phytochromes (Torne et al. 1996).

In the present study, long photoperiod along with high TDZ concentration may have provided the trigger that enabled cells to undergo changes in the developmental process and caused them to become competent for somatic embryogenesis (Kiyosue et al. 1990), as has been reported earlier by treatments like excision, low or high temperature treatments, and gamma radiation (Yeung 1995).

The most interesting aspects of our study on the regeneration of strawberry are: (1) TDZ induces regeneration via both organogenesis and somatic embryogenesis in leaf explant; (2) the number of shoots and somatic embryos induced is primarily dependent on concentration of TDZ. Shoot organogenesis is best when leaf explants are cultured on lower concentration of TDZ (9.08 μM), whereas somatic embryogenesis is best when leaf explants are cultured on relatively higher concentration of TDZ (18.16 μM); (3) the total number of regenerants increases significantly by giving dark treatment to leaf explants; (4) the 16-h photoperiod is the optimum photoperiod for shoot regeneration from leaf explants; (5) chilling treatment has an inhibitory effect on shoot organogenesis; and (5) there is a synergistic effect of photoperiod, dark, and chilling treatments on somatic embryogenesis.

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