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Effect of genotype, explant size, position, and culture medium on shoot generation of *Gerbera jamesonii* by receptacle transverse thin cell layer culture

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Abstract

An unique procedure for the mass shoot propagation of *Gerbera* using receptacle transverse thin cell layer (tTCL) culture procedure was developed. Genotype, flower bud age, explant size, position of receptacle tTCLs and culture media were found to affect the success of culture. Ten interspecific crosses of *Gerbera* showed different shoot regeneration rates and callus induction via receptacle tTCL culture, all of which had shoot regeneration rates higher than 57%. Flower buds collected on the 10th day resulted in 91% shoot regeneration after 6 weeks of culture on basal MS medium [Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15, 475–497] supplemented with 0.02 mg l^{-1} thidiazuron (TDZ), 0.8 mg l^{-1} adenine and 10% (v/v) coconut water (CW). This was significantly higher than those from flower buds on the 7th and 14th days (22% and 54%), respectively. Shoot regeneration rate was the highest (94–100%) in the middle layers of the receptacle. For mass shoot propagation, shoot clusters were subcultured on half-strength MS medium supplemented with 0.5 mg l^{-1} indole-3-butyric acid (IBA), 0.5 mg l^{-1} 6-benzyladenine (BA) and 2.0 mg l^{-1} kinetin after every 4 weeks. Plantlets formed when single shoots were cultured on half-strength MS medium containing 1 mg l^{-1} IBA. All plantlets acclimatized well in the greenhouse.

Keywords: *Gerbera jamesonii*; Genotype; Receptacle; TDZ; TCL

1. Introduction

Gerbera jamesonii (Asteraceae) is an important commercial flower native to South Africa and Asia and commercially grown all over the world. Although it can be propagated by seed, cultivated gerbera are extremely heterozygous and need more time to flower. Conventional propagation and breeding are facing with problems that require the application of modern methods of biotechnology. In the case of asexual propagation multiplication rate is too slow. Among the various methods, multiplication through division of clumps is most common. It can also be multiplied by cuttings. Nevertheless, tissue culture multiplication through which a million-fold increase per year of a desired plant may be obtained is an ideal method since no abnormalities have been reported so far.

Different methods of *in vitro* multiplication and regeneration of *Gerbera* have been previously described, including adventitious root formation and callus induction from young leaves (Pierik and Segers, 1972), direct adventitious shoot formation from excised capitulum explants (Pierik et al., 1973, 1975) and from isolated shoot tips (Murashige et al., 1974). Further studies on *Gerbera* micropropagation were also reported including callus induction from ovules (Meynet and Sibi, 1984), direct adventitious shoot formation from leaves (Hedtrich, 1979), adventitious shoot regeneration from petiole-derived callus (Orlikowska et al., 1999). Nhut et al. (2003) also found that the addition of TDZ induced callus more effectively than either BA or kinetin. However, the use of TDZ for direct shoot formation from receptacle tTCLs has never been described.

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The objective of this study was to develop an efficient and practical method for receptacle tTCL culture. TDZ was used to induce direct shoots in order to reduce culture time and increase the multiplication rate. Specific objectives were to identify responsive genotypes, to determine the optimum flower bud age and appropriate medium composition for successful *Gerbera* receptacle tTCL culture.

2. Materials and methods

2.1. Plant materials

Receptacles (1.5–2.0 cm in diameter) of 10 interspecific crosses of young *Gerbera* flowers (7–14 days old) were washed thoroughly under tap water for 20 min, soaked in My Hao detergent (Viet Nam) for 15 min, then washed thoroughly under tap water again for 2 h, rinsed six times with distilled water, and then submerged in a 7% (w/v) solution of $Ca(ClO)_2$ for 25 min and rinsed six times in sterile distilled water. Sterilized receptacles were cut into thin layers (0.2–0.5 mm thick).

2.2. Experimental designs

2.2.1. Effect of different culture media on shoot formation of different positions of explants

In this experiment, the effect of different TDZ concentrations (0.01–1.0 mg l^{-1}) on shoot formation was examined first (data not shown). TDZ was then combined with other components such as adenine and coconut water (CW) to increase the *Gerbera* receptacle tTCL shoot regeneration ability. tTCLs of 10 crosses were placed on modified MS media containing 30 mg l^{-1} sucrose, 10 mg l^{-1} agar, and different plant growth regulators (PGRs) (Table 1). Twenty healthy layers were cultured in each treatment with three replications per treatment.

2.2.2. Effect of genotype on in vitro response of receptacle transverse TCL culture

Ten interspecific crosses were used for this study. At least 260 to over 400 healthy receptacle tTCLs were cultured in 250 ml flasks containing 30 ml medium at two tTCLs per flask. MS basal medium supplemented with 0.02 mg l^{-1} TDZ, 0.8 mg l^{-1} adenine and 10% (v/v) CW was used for this experiment. Twenty healthy layers were cultured in each treatment with three replications per treatment. The callus induction and shoot regeneration rate were recorded after 6 weeks of culture.

2.2.3. Effect of flower bud age on the in vitro response of cross II receptacle tTCL culture

To determine the optimum flower bud age for receptacle TCL culture, young flower buds from the ''responsive'' cross II were collected on the 7th, 10th, and 14th day (flower bud age). Receptacle tTCLs of different flower buds were cultured in 250 ml flasks containing 30 ml MS medium supplemented with 0.02 mg l^{-1} TDZ, 0.8 mg l^{-1} adenine and 10% (v/v) CW. Survival rate and tTCL morphogenesis were recorded after 4 and 6 weeks of culture. Procedures for surface disinfection and culture medium were the same as described above. Twenty healthy layers were cultured in each treatment with three replications per treatment.

2.2.4. Effect of cross II receptacle tTCL position on callus and shoot formation

Different layers (0.2–0.5 mm thick) from different positions on the receptacle were used for testing the callus and shoot induction capacity. Explants were cultured on basal MS medium supplemented with 0.02 mg l^{-1} TDZ, 0.8 mg l^{-1} adenine and 10% (v/v) CW. Data was recorded after 6 weeks of culture. Thirty layers were cultured in each treatment with three replications per treatment.

2.2.5. Effect of culture medium on mass shoot propagation of cross II shoot clusters

Sections of receptacles containing adventitious shoots were placed on half-strength MS media containing 0.5 mg l^{-1} IBA, 0.5 mg l^{-1} BA in combination with kinetin at different concentrations: 0.5, 1.0, 2.0 and 3.0 mg l^{-1}. Data was recorded after 6 weeks of culture. Thirty shoot clusters were cultured in each treatment with three replications per treatment.

2.2.6. Effect of IBA on root formation

Single shoots derived from cross II shoot clusters were rooted by placing on half-strength MS medium containing 0.2, 0.5, 1.0 or 2.0 mg l^{-1} IBA. Data was recorded after 6 weeks of culture. Forty shoots were cultured in each treatment with three replications per treatment.

Table 1
Effect of different culture media on the *in vitro* response of *Gerbera* receptacle tTCLs

Culture medium[a]	Survival rate (%)		Receptacle tTCL morphogenesis after 6 weeks culture (%)			No. of shoots/explant
	4 weeks	6 weeks	Callus	Shoot	Callus and shoot	
(1)	76 c	51 c	38 a	45 d	17 b	1.0 c
(2)	80 b	68 b	14 b	56 c	20 a	1.2 c
(3)	74 c	41 d	2 c	87 b	11 c	2.1 b
(4)	95 a	87 a	0	92 a	8 d	4.4 a

Different letters within a column indicate significant differences at $p = 0.05$ by Duncan's multiple range test.

[a] Media were used in this experiment: (1) MS medium supplemented with 0.02 mg l^{-1} TDZ only; (2) MS medium supplemented 0.02 mg l^{-1} TDZ, 10% (v/v) coconut water (CW); (3) MS medium supplemented 0.02 mg l^{-1} TDZ, 0.8 mg l^{-1} adenine; (4) MS medium supplemented 0.02 mg l^{-1} TDZ, 0.8 mg l^{-1} adenine, 10% (v/v) coconut water.

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Table 2
Effect of *Gerbera* genotypes on callus induction and shoot regeneration by receptacle transverse thin cell layer after 6 weeks of culture

Genotype	No. of explants	Callus induction (%)	Shoot regeneration (%)	No. of shoots/explant
I	260	15 d	85 c	3.0 c
II	480	5 f	95 a	5.1 a
III	430	30 b	70 e	2.2 d
IV	350	10 e	90 b	3.6 b
V	400	43 a	57 f	1.1 e
VI	450	12 d	88 b	2.9 c
VII	350	24 c	76 d	2.2 d
VIII	280	31 b	69 e	2.1 d
IX	340	21 c	79 d	2.3 d
X	300	14 d	86 c	3.4 b

Different letters within a column indicate significant differences at $p = 0.05$ by Duncan's multiple range test.

2.3. Basic media

Basic media are MS basal medium (Murashige and Skoog, 1962) supplemented with different PGRs at different concentrations.

2.4. Culture conditions

Experiments on shoot regeneration, and mass shoot propagation and rooting stages were conducted in 250 and 500 ml Erlenmeyer flasks, respectively. Media were supplemented with 30 g l^{-1} sucrose and 10 g l^{-1} agar with pH adjusted to 5.8 before autoclaved at 121 °C 1 atm for 15 min. All cultures were incubated at 25 °C under a 12-h light photoperiod under cool white fluorescent light at 3000 lux.

2.5. Acclimatization

Four thousand plantlets with well-developed roots were transferred to autoclaved soil held in 50 cm × 50 cm styrofoam trays, maintained in the greenhouse at 25 °C, 80–85% relatively humidity and under natural light. Plantlets were watered every day for 1 month. Plantlets were maintained in the shade outside the greenhouse for 2 weeks before being transferred to a nursery garden.

2.6. Statistical analysis

Each experimental treatment was carried out with at least 20 glass vessels each of which contains five TCL explants. Data was analyzed for significance by analysis of variance with mean separation by Duncan's multiple range test (Duncan, 1995).

3. Results

3.1. Effect of different culture media on the in vitro response of Gerbera cross II receptacle tTCL

In this experiment, it was found that culture media affected survival rate and morphogenetic ability of receptacle tTCLs. TDZ concentration of 0.02 mg l^{-1} TDZ was shown to be optimal for shoot formation. In combination with different PGRs for enhancing shoot regeneration, after 6 weeks of culture, receptacle tTCLs were able to regenerate most vigorously on medium supplemented with 0.02 mg l^{-1} TDZ, 0.8 mg l^{-1} adenine, and 10% (v/v) CW. On this medium survival rate was the highest (87%), while shoot regeneration rate was 92% with four or five shoots per explant and without callus formation (Table 1). The survival rate was lowest in medium containing 0.02 mg l^{-1} TDZ only, in which the callus regeneration rate was highest (Table 1).

3.2. Effect of Gerbera genotype on callus induction and shoot regeneration by receptacle transverse thin cell layer after 6 weeks of culture

It was shown that interspecific cross II had the highest shoot regeneration rate (95%) with four to six shoots per explant and the lowest callus induction rate (5%) (Table 2). In addition, cross IV also had a high shoot generation rate (90%) but not as high as cross II. Cross V had the lowest shoot regeneration rate (57%), but the highest callus induction rate (43%).

3.3. Effect of flower bud age on the in vitro response of Gerbera cross II receptacle tTCL after 6 weeks of culture

Flower bud age affected survival rate and receptacle tTCL morphogenesis. Ten-day-old flower buds were demonstrated to be optimal for receptacle culture (Table 3). After 4 weeks of culture, the survival rate was 100%, decreasing to 98% after 6 weeks of culture, but always higher than that of other treatments; shoot regeneration rate was 91%, 5% of explants induced callus, and callus and shoot morphogenesis rate was 4%.

Table 3
Effect of flower bud age on the *in vitro* response of *Gerbera* cross II receptacle tTCL after 6 weeks of culture

Flower bud age (days)	Survival rate (%)		Receptacle tTCL morphogenesis (%)			No. of shoots/explant
	4 weeks	6 weeks	Callus	Shoots	Callus and shoots	
7	16 c	11 c	66 a	22 c	12 b	1.3 c
10	100 a	98 a	5 c	91 a	4 c	4.3 a
14	54 b	41 b	12 b	54 b	34 a	2.1 b

Different letters within a column indicate significant differences at $p = 0.05$ by Duncan's multiple range test.

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Table 4
Effect of *Gerbera* cross II receptacle tTCL positions on callus and shoot formation after 6 weeks of culture

Explant position (receptacle)	Receptacle tTCL morphogenesis (%)			No. of shoots/explant
	Callus	Shoot	Callus and shoot	
Position 1*	8 b	75 e	17 a	1.2 d
Position 2*	10 a	80 d	10 b	2.2 c
Position 3*	3 c	87 c	10 b	2.0 c
Position 1**	0	94 b	6 c	3.1 b
Position 2**	0	96 b	4 c	4.2 a
Position 3**	0	100 a	0	4.1 a

1* → 3*: exterior layers of receptacle; 1** → 3**: middle layers of receptacle. Different letters within a column indicate significant differences at $p = 0.05$ by Duncan's multiple range test.

3.4. Effect of Gerbera cross II receptacle tTCL positions on callus and shoot formation after 6 weeks of culture

The position of receptacle tTCL affected the receptacle TCL morphogenic ability. Middle receptacle layers had greater morphogenic ability than exterior layers. The percentage of shoot formation from receptacle tTCL middle layers was more than 94%, with average number of shoot per explant of five or six, especially in the layer from position 3, which had the highest percentage (100%), without callus formation (Table 4). Position 1 from exterior layers had the lowest shoot morphogenic percentage (75%). Efficiency decreased from the middle to exterior layers. Larger middle layers were easier to regenerate than smaller ones (Pierik, 1987). Larger explants are sometimes easier to regenerate than smaller ones, and this may be due to the presence of more nutrient reserves. Larger explants have a larger surface area, hence absorbing nutrients more easily. Both size of explant and degree of wounding in explant preparation can affect regeneration capacity, small explants being easily wounded, resulting in decreased regeneration rate.

3.5. Effect of culture media on mass shoot propagation of Gerbera cross II shoot clusters after 4 weeks of culture

In this experiment, mass shoot propagation of *Gerbera* cross II shoot clusters on half-strength MS medium containing 0.5 mg l^{-1} IBA, 1 mg l^{-1} BA in combination with kinetin at the concentration 2.0 mg l^{-1} was the most optimal (Table 5). The shoot number from shoot clusters cultured on medium supplemented with 0.5 mg l^{-1} IBA, 1 mg l^{-1} BA and 0.5 mg l^{-1} kinetin was lowest (7.9), but shoots were highest (Table 5).

3.6. Effect of culture media on root formation of Gerbera cross II single shoots after 4 weeks of culture

Gerbera cross II root formation after 4 weeks of culture on half-strength MS medium containing 1.0 mg l^{-1} IBA was the most effective (Table 6). Longer roots support better plantlet growth, resulting in taller plants than from short-root plants. There was no root formation on MS medium containing 2 mg l^{-1} IBA. Most plants require the presence of auxins for efficient root regeneration. This need is not constant since after root initiation (high auxin requirement), outgrowth of the root primordium requires a low concentration, and a continuous high auxin concentration will inhibit root elongation. The optimum IBA concentration was 1.0 mg l^{-1} (Table 6).

4. Discussion

It was demonstrated that regeneration efficiency depends significantly on medium components such as minerals, nutrients, sugar, vitamins and PGRs. Shoot formation is often enhanced by the combination of auxins and cytokinins. TDZ has been shown to promote differentiation of organized centers of growth in cultured tissues at much lower concentrations, and shoot regeneration occurs with an efficiency comparable to or greater than that of other cytokinins (Kerns and Meyer, 1986; Fellman et al., 1987; Fiola et al., 1990). TDZ, a non-purine, cytokinin-like compound has been shown to exhibit stronger effects than conventional cytokinins over a wide range of species, being effective for shoot proliferation and adventitious shoot organogenesis (Huetteman and Preece, 1993). Its mode of action may be attributed to its action to induce cytokinin accumulation (Victor et al., 1999) and enhance the accumulation and translocation of auxin within TDZ-exposed tissue (Murch and Saxena, 2001). Due to many optimal characteristics, TDZ promotes the induction of shoot regeneration. TDZ when supplemented to the medium at an appropriate concentration, enhanced callus formation. Furthermore, other, separate experiments with BA showed that no shoot was formed on tTCLs (Nhut et al., unpublished). However, shoot regeneration and the survival rate were higher (92%) when the medium was also supplemented with other components

Table 5
Effect of culture media containing 0.5 mg l^{-1} IBA, 0.5 mg l^{-1} BA in combination with different kinetin concentrations on mass shoot propagation of *Gerbera* cross II shoot clusters after 4 weeks of culture

Kinetin (mg l^{-1})	No. of shoots/explant	Shoot height (cm)
0.5	7.9 d	3.7 a
1.0	11.4 c	3.3 b
2.0	15.7 b	2.8 c
3.0	22.2 a	1.6 d

Different letters within a column indicate significant differences at $p = 0.05$ by Duncan's multiple range test.

Table 6
Effect of culture media containing different IBA concentrations on root formation of *Gerbera* cross II single shoots after 4 weeks of culture

IBA (mg l^{-1})	No. of roots/explant	Plantlet height (cm)	Root length (cm)
0.2	7.9 a	4.7 c	2.4 c
0.5	6.4 b	5.3 b	3.7 b
1.0	4.7 c	5.8 a	6.2 a
2.0	0	3.6 d	0

Different letters within a column indicate significant differences at $p = 0.05$ by Duncan's multiple range test.

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(Table 1), such as adenine (Nitsch et al., 1967). In this study, adenine and CW were used to increase shoot morphogenesis. Adenine was first used by Skoog and Tsui (1948) when they cultured tobacco stem explants, during which adventitious shoot formation occurred. CW is one of the factors promoting shoot regeneration because it contains 9-β-D-ribofuranosylzeatin, a cytokinin (Letham, 1974). As in this case, results showed that shoot formation rate on media supplemented with CW was higher than in media without it (Table 1).

Flower bud ages also play crucial role in morphogenesis based on TCL culture system. Culture of receptacles from 7-day-old flower buds was least effective. As a plant becomes older, its regenerative capacity often decreases, and parts of juvenile plants are preferred to those from adults, especially in the case of trees and shrubs. Differences in cell division and regenerative ability between juvenile and adult plants *in vitro* were found in *Hedera helix* (Stoutemeyer and Britt, 1965), *Lunaria annua* (Pierik, 1967) and *Anthurium andreanum* (Pierik et al., 1974), in which plant regeneration from juvenile plant parts occurs more readily than those from adult plants. In *Gerbera*, 7-day old as compared to 10-day-old flower buds have not yet reached a sufficiently physiologically mature state for shoot regeneration. Therefore, 10-day-old flower buds are optimal for shoot regeneration.

In our experiment, the cytokinin:auxin ratio was 2.5, suitable for mass shoot propagation. A high cytokinin concentration and low auxin concentration promote further outgrowth and development (Pierik, 1987). Murashige et al. (1974) increased *Gerbera* shoot multiplication rate on MS medium containing 0.5 mg l^{-1} IAA and 10 mg l^{-1} kinetin. This experiment used high PGR concentrations, but the number of shoots obtained was lower than in this study (the average number of shoots per cluster was 15.7). Since high PGR concentrations and high humidity result in hyperhydricity of cultures, we used half-strength MS medium to reduce hyperhydricity to improve shoot quality. These results may reduce the cost and culture time in comparison with previous experiments.

The obtained results in root formation were different from some other studies on *Gerbera* rooting which showed that the best root growth (15.2 cm) was obtained in the presence of 10 μM IBA with an 85% survival rate (Meyer and van Staden, 1987). BA was proven to be highly effective for rooting *in vitro*. Half-strength MS medium was used to reduce hyperhydricity, eliminate expensive organic supplements and reduce the concentration of others without a loss in multiplication rate. During the experiment, it was found that individually separated divisions rooted better than undivided clusters. It was also very clear that genotype affects the regeneration of explants in *Gerbera*, concurrent with results by Jerzy and Lubomski (1990), and Reynoird et al. (1993), in which each genotype has its own *in vitro* response. Similar results were also found in *Anthurium* (Geier, 1986).

5. Conclusion

The innovative and effective *in vitro* regeneration of *Gerbera* through the application of transverse thin cell layer culture has been reported. In this study, we found the appropriate media for direct shoot regeneration and investigated the effect of genotype, position and explant size on the *in vitro* response of receptacle transverse TCL culture. Moreover, an effective method utilizing TDZ for successfully programming of shoot, root and callus morphogenesis was achieved. These results are supposed to have significant contribution for more understanding about TCL-system-based morphogenesis and its variation in various varieties.

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