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Original Paper

Highly efficient system for plant regeneration from leaf and stem explants in *Dahlia*

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Abstract Protocol of plant regeneration from leaf and stem explants was established in dahlia (*Dahlia*×*pinnata*) 'Yamatohime' in *in vitro* culture. Nodular calli were successfully induced from leaf and stem explants on 30 g l^{-1} sucrose-containing MS medium supplemented singly with one of the cytokinins, 6-benzyladenine (BA), zeatin or thidiazuron (TDZ) and the highest frequency of nodular callus induction was obtained on medium containing 10 mg l^{-1} TDZ. The nodular callus induced on this medium subsequently produced a tissue with numerous shoot primordial structures called 'mass of shoot primordia' (MSP), which was maintained at least for more than 2 years on the same medium. Shoot induction occurred after transfer the MSP onto hormone-free MS medium, in which replacement of sucrose by maltose was necessary. The shoots excised from the MSP were rooted on hormone-free MS medium and successfully grown under greenhouse conditions. The regenerated plants showed the same morphological characteristics to those of the control plants and flowered normally.

Key words: *Dahlia*×*pinnata*, mass of shoot primordia, shoot regeneration, TDZ.

The genus *Dahlia* belongs to Asteraceae family and is native to Mexico, Central America and Colombia. Numerous cultivars of dahlia (*Dahlia*×*pinnata*) with wide variations in flower color, size and shape have so far been produced by intra- and interspecific hybridizations. They are grown as a popular tuberous ornamental crop used for cut flower and as pot and garden plants. For cultivation of dahlias, viral diseases are the most serious problems since many cultivars are always facing to extinction because of the high susceptibility to some viruses such as dahlia mosaic virus (Pappu et al. 2005). To avoid the virus diseases, meristem-tip culture has been widely used to obtain virus-free plants, which was first successfully reported in dahlia cultivars (Morel and Martin 1952). The technique was then improved by Mori (1971), Mullin and Schlegel (1978) and Wang et al. (1988). However, virus-free plants remain susceptible to the problematic viruses and are usually re-infected within 2–3 years under the common cultivation conditions, if those plants are cultivated with contagious sources (Mori 1971). Unfortunately, no useful germplasms are available for conferring resistance to virus diseases in dahlia. In addition, although dahlia has large variations in flower color and morphology, there have been constant demands for novel characters such as blue flowers. To achieve these breeding objectives, it is now expected to utilize genetic transformation methods in dahlia. Although it is indispensable to establish an efficient protocol for *in vitro* plant regeneration for conducting genetic transformation studies in dahlia, only few studies on the tissue culture of this ornamental plant have been reported using shoot tip and axillary buds (Kongthong et al. 1999) and cotyledon and hypocotyls (Fatima et al. 2007) as the explants. In the present study, we describe an efficient and long-term sustainable protocol for plant regeneration from leaf and stem explants in dahlia.

Materials and methods

Plant material

Dahlia (*Dahlia*×*pinnata*), 'Yamatohime', which has pink, single (8 petals) and medium-sized flowers, was mainly used in this study. Other 8 cultivars, 'Amy K', 'Black Cat', 'Hakusan', 'Kokucho', 'L'Ancresse', 'Summer Firework', 'Kamakura' and 'Sakurajyouou', were also used for testing the applicability of the plant regeneration system established using 'Yamatohime'. *In vitro* plants of 'Yamatohime' were initially produced by inoculating 0.3 mm width shoot apex onto MS medium (Murashige and Skoog 1962) containing 30 g l^{-1} sucrose, 0.2% gellan gum (Gelrite; Wako Pure Chemical Industries, Osaka, Japan), 0.01 mg l^{-1} α-naphthaleneacetic acid (NAA) (Sigma-Aldrich, St. Louis, MO, USA) and 0.01 mg l^{-1} 6-benzyladenine

Abbreviations: BA, 6-benzyladenine; HF, hormone-free; MSP, mass of shoot primordia; NAA, α-naphthaleneacetic acid; TDZ, thidiazuron.
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(BA) (Wako Pure Chemical Industries, Osaka, Japan). Then the plants were propagated by inoculating the shoots with 2 leaves excised from the original *in vitro* plants on MS medium containing 30 g l^{-1} sucrose and 0.8% agar. The pH was adjusted to 5.8 with KOH or HCl prior to the addition of agar and autoclaving at 121°C for 15 min. These cultures were incubated at 25±1°C under a 16 h photoperiod at 35 μmol m^{-2} s^{-1} with cool white fluorescent light. The shoots were subcultured at one month intervals to obtain enough mother stock cultures for experiments. The explants were excised from the donor cultures 1 month after transfer onto fresh medium.

Culture media and callus induction

Two kinds of explants, stem and leaf segments from *in vitro*-cultured shoots, were cut into small segments (stems: 0.5–1 cm length; leaves: 0.5 cm×0.5 cm) and placed on media for callus induction. MS medium containing 30 g l^{-1} sucrose and 0.25% gellan gum was used as the basal medium, which was supplemented with either NAA, 4-amino-3,5,6-trichloropicolinic acid (picloram ; Sigma-Aldrich, St. Louis, MO, USA), BA or thidiazuron (TDZ) (Wako Pure Chemical Industries, Osaka, Japan) at the concentrations of 1 and 10 mg l^{-1}. The medium was adjusted to pH 5.8 with KOH or HCl before autoclaving. The callus and the mass of shoot primordia (MSP) formation was evaluated after 8 weeks and 3 months of culture, respectively, and the type of callus was evaluated after 3 months of culture. For each hormonal treatment, 15 leaf and 15 stem explants were used, and the experiment was repeated 3 times.

For the experiments to study the effect of the type of cytokinin on callus induction, leaf explants (0.5 cm×0.5 cm) were excised from the youngest fully expanded leaves of *in vitro*-cultured plantlets and placed on MS medium containing 30 g l^{-1} sucrose, 0.25% gellan gum and 10 mg l^{-1} cytokinin (BA, kinetin, TDZ or zeatin) with abaxial side in contact with the medium. The excised leaf explants were also cultured on medium containing different concentrations of TDZ (1, 5, 10, 15, 20 mg l^{-1}). In these experiments, 20 leaf explants were used and after 8 weeks of culture, each of the enlarged explants with nodular calli were divided into four pieces (ca. 10–15 mm in diameter) and the experiment was repeated 3 times. The cultured tissues were subcultured at 2 week-intervals and the browned parts were removed at each subculture. The number of MSP totally formed from initial explants was scored 3 months after initiation of culture.

Plant regeneration

MSP obtained from leaf explants on MS medium supplemented with 10 mg l^{-1} TDZ were subcultured on the same medium at 2 week-intervals after dividing into small pieces (ca. 5 mm in diameter) at each subculture for proliferation. About 2 months after induction of the MSP, they were subcultured on the same medium for 1 month after cutting into small pieces, and then transferred onto hormone-free (HF) MS medium containing 30 g l^{-1} maltose and 0.8% agar. Subsequently, these MSP were subcultured every 1 month on the same HF medium and shoot regeneration was observed at the end of each subculture. In addition, 2 year-old MSP, which had been subcultured on MS medium supplemented with 10 mg l^{-1} TDZ at 1 month-intervals, were used to examine the ability of shoot regeneration after dividing into small pieces (ca. 5 mm in diameter) and subcultured on MS medium supplemented with 10 mg l^{-1} TDZ for one month. The experiment was performed 3 times, each with 30 MSP. The regenerated shoots were excised and cultured on HF MS medium containing 30 g l^{-1} sucrose and 0.8% agar for rooting. The rooted plantlets were transferred to pots filled with fine-grained soil and grown in a growth chamber at 22°C under a 14 h photoperiod for acclimatization. After 2 to 3 weeks, the well-established plantlets were transferred to greenhouse and grown under there conditions.

Data analysis

Data obtained for the nodular calli and the MSP induction were subjected to the analysis of variance (ANOVA) using the SPSS statistical package. Tukey's HSD test was performed to identify significant differences among the treatments, with significance level of $p<0.05$. The arcsine transformation was performed on all percentage data before statistical analysis.

Results

Effects of cytokinins on induction of nodular callus and MSP

On media containing only auxin (1 or 10 mg l^{-1} NAA and 1 mg l^{-1} picloram), some leaf and stem explants formed yellow nodular calli (Table 1), which occasionally produced adventitious roots but no shoots during the subculture on the same medium. When these yellow nodular calli were transferred onto medium containing only cytokinin (1 or 10 mg l^{-1} TDZ) or both auxin and cytokinin (1 mg l^{-1} picloram and 1 or 10 mg l^{-1} TDZ), yellow green calli were partially formed on the surface of yellow nodular calli. However, these two types of calli failed to proliferate during the subcultures on media containing only cytokinin or both auxin and cytokinin without producing shoot. On the other hand, both leaf and stem explants cultured on medium containing only cytokinin at 1 or 10 mg l^{-1} (either BA or TDZ) formed green nodular calli (Table 1). Among the 4 cytokinins (BA, kinetin, TDZ and zeatin) further tested at 10 mg l^{-1} using leaf explants, kinetin failed to induce nodular calli, whereas other 3 cytokinins, successfully produced green nodular calli at the frequencies of 33% with BA, 95% with zeatin and 100% with TDZ, respectively (Table 2). In the presence of TDZ, cultured leaf explants initiated to swell after several days of culture and enlarged into the size of about 4 times after 4 weeks of culture. Then green nodular calli formation was observed at the cut surfaces and wounded parts of the explants. After 8 weeks of culture, enlarged explants with nodular calli

Table 1. The effect of kind and concentration of plant growth regulators on callus and mass of shoot primordia (MSP) induction from leaf and stem explants of dahlia 'Yamatohime'.

Explant	Plant growth regulator	Concentration ($mg\,l^{-1}$)	% of explants forming calli	Callus color	Total No. of MSP formed from initial explants (Average No. per explant)
Leaf	NAA	1	2.2 c	Yellow	0.0±0.0 c (0)
		10	4.4 bc	Yellow	0.0±0.0 c (0)
	picloram	1	0.0 c		
		10	0.0 c		
	BA	1	0.0 c		
		10	35.6 b	Green	0.7±0.3 c (0.05)
	TDZ	1	100.0 a	Green	2.7±0.3 b (0.18)
		10	100.0 a	Green	12.3±0.9 a (0.82)
Stem	NAA	1	11.1 bc	Yellow	0.0±0.0 c (0)
		10	4.4 bc	Yellow	0.0±0.0 c (0)
	picloram	1	13.3 bc	Yellow	0.0±0.0 c (0)
		10	0.0 c		
	BA	1	22.2 bc	Green	0.0±0.0 c (0)
		10	0.0 c		
	TDZ	1	95.6 a	Green	1.0±0.0 bc (0.05)
		10	100.0 a	Green	1.0±1.0 bc (0.05)

All of the calli obtained in this study were nodular. The explants were subcultured at 2 week-intervals and nodular callus formation and mass of shoot primordia formation were evaluated after 8 weeks and 3 months of culture, respectively. Each value represents a mean±SE of the three independent experiments each with 15 explants. Tukey's HSD test was performed to identify significant differences among the 16 treatments. The arcsine transformation was performed on all percentage data before statistical analysis. Values with different letters are significantly different at $p<0.05$.

Table 2. The effect of different kind of cytokinin on callus and mass of shoot primordia (MSP) induction from leaf segments of dahlia 'Yamatohime'.

Cytokinin ($10\,mg\,l^{-1}$)	% of explants forming nodular calli	Total No. of MSP formed from initial explants (Average No. per explant)
BA	33.3 b	2.7±1.8 b (0.14)
Kinetin	0.0 b	0.0±0.0 b (0)
TDZ	100.0 a	22.7±2.2 a (1.14)
Zeatin	95.0 a	0.0±0.0 b (0)

The explants were subcultured at 2 week-intervals and nodular callus formation was recorded after 8 weeks of culture. Then each explant with nodular calli was divided into four pieces and total number of shoot primordia formed from the explants were scored 3 months after initiation of leaf culture. Each value represents a mean±SE of the three independent experiments each with 20 explants. Tukey's HSD test was performed to identify significant differences among the 4 treatments. The arcsine transformation was performed on all percentage data before statistical analysis. Values with different letters are significantly different at $p<0.05$.

were divided each into four pieces (ca. 10–15 mm in diameter). Although the nodular calli continued to grow on the same medium, they turned brown easily (Figure 1A), which caused melting of the surrounding medium. Stem explants cultured with TDZ also swelled and formed nodular calli as in the case with leaf explants. When these nodular calli were cultured for a several weeks with subcultures at every 2 weeks on the same medium after removing browned parts and dividing into small pieces (ca. 10–15 mm in diameter), another type of

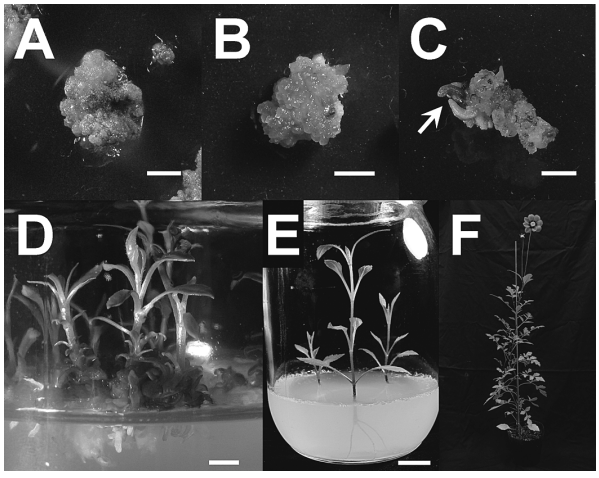

Figure 1. Plant regeneration from leaf explants in dahlia (*Dahlia*×*pinnata*) 'Yamatohime'. (A) Green nodular callus showing partial necrosis after 2 months of culture on MS medium supplemented with $10\,mg\,l^{-1}$ TDZ. At each 2 week-intervals of subculture, the browned parts were eliminated. bar=5 mm (B) Mass of shoot primordia (MSP) obtained after subculture of nodular callus for 3 months on MS medium supplemented with $10\,mg\,l^{-1}$ TDZ at 2 week-intervals. bar=5 mm (C) Malformed adventitious buds regenerated from MSP one month after subculture on MS medium supplemented with $10\,mg\,l^{-1}$ TDZ without applying regular 2 week-interval subculture. Totally 5 months after initiation of leaf culture. bar=5 mm (D) Normal shoots regeneration from the buds primordia on MSP after transfer onto maltose-containing HF MS medium with subcultures at 1 month-intervals. bar=5 mm (E) Rooting of regenerated shoot 1 month after the transfer onto sucrose-containing HF MS medium. bar=15 mm (F) Flowering of potted plant 3 months after acclimatization.

Table 3. The effect of concentration of TDZ on callus and mass of shoot primordia (MSP) induction from leaf segments of dahlia 'Yamatohime'.

Concentration of TDZ ($mg l^{-1}$)	% of explants forming nodular calli	Total No. of MSP formed from initial explants (Average No. per explant)
1	100	2.3±0.7 b (0.12)
5	100	4.3±0.3 b (0.22)
10	100	24.0±1.2 a (1.20)
15	100	11.0±4.0 b (0.55)
20	100	2.0±1.5 b (0.10)

The explants were subcultured at 2 week-intervals and nodular callus formation was recorded after 8 weeks of culture. Then each explant with nodular calli was divided into four pieces and total number of shoot primordia formed from the explants were scored 3 months after initiation of leaf culture. Each value represents a mean±SE of the three independent experiments each with 20 explants. Tukey's HSD test was performed to identify significant differences among the 5 treatments. The arcsine transformation was performed on all percentage data before statistical analysis. Values with different letters are significantly different at $p<0.05$.

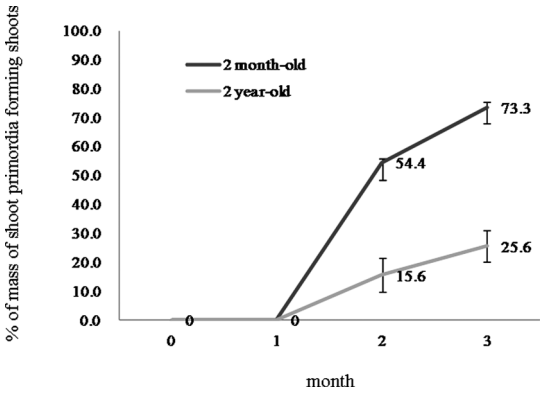

Figure 2. Difference in shoot regeneration ability between 2 month-old and 2 year-old MSP after establishment of MSP cultures. The MSP which had been kept on $10 mg l^{-1}$ TDZ-containing medium were transferred onto HF medium and subcultured on HF medium at one month intervals. Shoot regeneration was observed at the end of each subculture. Three repeats were performed with 30 MSP per repeat.

tissue, which had green coloration with numerous shoot primordia-like structures, was initiated to form from the nodular calli. Since this tissue showed close similarity to that initially developed by Tanaka and Ikeda (1983) in *Haplopappus gracilis* and now generally called as 'mass of shoot primordia' (Kondo et al. 1991; Matsumoto et al. 1997), we refer this meristematic tissue of dahlia as MSP thereafter. MSP was independently formed from some parts of nodular calli and it had a nature to liberate easily from a nodular callus, which did not continue to form MSP at the same part. MSP also showed differences not only in morphological characters but also in growth response from original nodular callus, i.e., it did not turn brown and grew vigorously on the same medium (Figure 1B). Although nodular callus died with total necrosis when it was kept for further one month on $10 mg l^{-1}$ TDZ-containing medium without subcultures, MSP survived and regenerated malformed adventitious buds (Figure 1C). Compared to the nodular calli induced from leaf explants, those induced from stem explants showed less browning during the subcultures but they rarely produced MSP (Table 1).

The concentration of TDZ in the medium also affected the formation of MSP. Although nodular calli were induced from all the leaf explants cultured at all the concentrations examined (1–$20 mg l^{-1}$), MSP was most frequently formed on medium containing $10 mg l^{-1}$ TDZ, whereas the lower concentrations gave much lower frequency of MSP induction. Although another type of nodular calli that did not turn brown were formed at the higher concentrations than $10 mg l^{-1}$, most of them did not produce MSP during the subsequent subcultures (Table 3).

Shoot regeneration

MSP could be proliferated during the subcultures at 2 week-intervals on MS medium containing $10 mg l^{-1}$ TDZ without showing appreciable changes in its morphology. When 2 month-old MSP after its isolation from nodular callus, i.e., 5 month-old after initiation of the culture of leaf explants were subcultured for one month on MS medium containing $10 mg l^{-1}$ TDZ without applying regular 2 week-interval subculture, they grew from 5 to 10–15 mm in diameter and regenerated malformed adventitious buds (Figure 1C). Since the malformed adventitious buds did not develop into normal shoots on TDZ containing medium, MSP with the buds were transferred onto 0.8% agar-solidified HF MS medium containing $30 g l^{-1}$ sucrose or maltose. On HF medium containing sucrose, MSP showed the change in color to light-green without shoot regeneration and some of them subsequently died with necrosis. In contrast, MSP on medium containing maltose kept green and formed new bud primordia 2 months after the transfer. Then, newly formed bud primordia developed into normal shoots on the same medium (Figure 1D). MSP could be subcultured for more than 2 years without loosing their property on medium containing $10 mg l^{-1}$ TDZ but showed reduced growth rate. When the 2 year-old MSP was transferred onto HF medium containing maltose, they formed normal shoots as is the case with 2 month-old MSP although shoot formation rate after 3 months of culture was 25.6%, which was almost 1/3 of that obtained in 2 month-old MSP (73.3%) (Figure 2). Moreover, the number of shoots produced per MSP formed more shoots (5–6) than 2 year-old MSP (1–2) after 3 months of culture, although 2 year-old MSP did not show appreciable changes in its morphology when compared with the 2 month-old MSP. Normally grown shoots were excised from MSP, and transferred onto HF

MS medium containing 30 g l^{-1} sucrose and 0.8% agar for rooting. Root formation occurred easily 1 to 2 weeks after the transfer (Figure 1E). Regenerated plantlets with roots were washed carefully to remove the medium and transferred to pots containing fine-grained soil. Each potted plant was kept in a poly bag and acclimatized for 2 to 3 weeks in a growth chamber at 22°C under a 14 h photoperiod. The acclimatized ten plants were then transferred to the greenhouse. They exhibited a normal phenotype and flowered normally 4 months after the transfer (Figure 1F).

Discussion

In *Dahlia*, the highest frequencies of callus induction (60%) and shoot regeneration (13%) were previously reported in the culture of cotyledon and hypocotyl explants, respectively, on medium containing 3 mg l^{-1} each of NAA and BA by Fatima et al. (2007). For the genetic transformation of dahlia, however, it is necessary to establish highly efficient plant regeneration protocols by using the explants obtained from the target cultivars, which are propagated vegetatively by cutting or division of tubers. In the present study, therefore, we tried to establish highly efficient plant regeneration system using leaf and stem segments of several commercial cultivars as explants. In the present study on dahlia, nodular calli were induced from leaf explants by single application of one of the cytokinins, either BA, TDZ or zeatin, without co-addition of auxins. Occurrence of callus induction on only cytokinin-containing medium has been reported in several species such as *Stylosanthes scabra* (Godwin et al. 1987) and *Cuscuta reflexa* (Das et al. 2011) with BA and okra (*Abelmoschus esculentus*) with either BA, kinetin or zeatin (Roy and Mangat 1989). In dahlia, almost 100% of the leaf explants showed nodular callus formation on medium with 10 mg l^{-1} TDZ or zeatin. The nodular calli induced on medium with zeatin formed shoots and a few multiple shoot were formed around the shoots. On this medium, however, shoot formation occurred at a very low frequency. On the other hand, the nodular calli induced on medium with 10 mg l^{-1} TDZ had an ability to produce MSP, which easily proliferate without loosing their regeneration ability and formed a lot of normal shoots by transferring onto HF medium with maltose. TDZ is known to exhibit the unique property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants, although structurally it is different from either auxins or purine-based cytokinins (Murthy et al. 1998). In several plant species, direct shoot formation (Canli and Tian, 2009; Malik and Saxena 1992) and callus induction (Chen et al. 2000; Jones et al. 2007) from cultured tissues were observed on MS medium supplemented with low concentration of TDZ singly. In dahlia, however, MSP was induced from leaf explants through nodular callus formation on MS medium supplemented with high concentration (10 mg l^{-1}) of TDZ, and shoots were regenerated from MSP by transferring onto HF MS medium. In several plant species, it is difficult to maintain the plant regeneration ability of long-term subcultured calli (Liu et al. 2009; Tsugawa and Suzuki 2000). In dahlia, however, 2 year-old MSP did not lose the ability to regenerate shoots although shoot regeneration frequency decreased significantly ($p<0.01$) compared to that of 2 month-old MSP. In dahlia, therefore, it is expected that even 2 year-old MSP could be used for micropropagation and transformation.

In this study, we have succeeded to develop a long term-sustainable *in vitro* plant regeneration system in dahlia, using cultivar 'Yamatohime' as a material. Among the other 8 cultivars tested, we have also successfully induced MSP from leaf explants through nodular callus formation on MS medium containing 10 mg l^{-1} TDZ and regenerated shoots on HF MS medium in the following 6 cultivars, 'Amy K', 'Black Cat', 'Hakusan', 'Kokucho', 'L'Ancresse' and 'Summer Firework'. However, two cultivars, 'Kamakura' and 'Sakurajyouou' failed to induce MSP after formation of nodular calli. From these results, it is highly possible that this protocol can be used for plant regeneration of many other dahlia cultivars and that it will be useful for micropropagation of plants and *Agrobacterium*-mediated transformation to introduce useful genes such as virus resistance and flower color genes, for increasing the commercial value of this important ornamental crop.

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