Plant Regeneration from Suspension Cell Culture of *Delphinium*.

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Summary

Embryogenic calli were induced from cotyledons of 'Pacific Giant' *Delphinium hybridum* (white line) in MS medium containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D, $0.1 \text{ mg} \cdot \text{liter}^{-1}$ BAP and $30 \text{ g} \cdot \text{liter}^{-1}$ sucrose. Embryogenic cell suspension cultures were established from these embryogenic calli by using a liquid N6-based medium (N6 inorganic salts and MS organic constituents), containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D, $1.0 \text{ mg} \cdot \text{liter}^{-1}$ TDZ and $30 \text{ g} \cdot \text{liter}^{-1}$ sucrose. The cell aggregates increased about eight- to ten-fold after one week of culture under fluorescent light at 25°C . When suspension cell clusters were transferred to a N6-based medium that lacked plant growth regulators, many somatic embryos were formed. When these somatic embryos were transferred to the same medium, the shoots that differentiated were transferred to 1/2 MS medium to root. About 20 plantlets were produced from 1 g of suspension culture.

Key Words: *Delphinium*, embryogenic callus, regeneration, suspension culture.

Introduction

An efficient plant regeneration system is important for micropropagation and genetic manipulations, such as somatic hybridization and genetic transformation. Plant regeneration via organogenesis or somatic embryogenesis has been described for the genus *Delphinium* using tissue culture of leaf blades, petioles, tuberous roots, secondary roots, pistils, stamens and petals (Amagai et al., 1992; Amagai and Yonai, 1994; Hosokawa et al., 2001); and the production of transgenic plants through adventitious bud formation has been described (Hirose et al. 2002). However, there are no reports on cell suspension culture derived from embryogenic calli of *Delphinium*. In this study, we report on the establishment of an embryogenic suspension culture of 'Pacific Giant' *Delphinium hybridum* and a subsequent plant regeneration process.

Materials and Methods

Embryogenic callus induction and plant regeneration

Seeds of the 'Pacific Giant', purchased from Daiichi Seed (Tokyo), were grown and selfed over two or three generations from which plants with only white flowers (hereafter referred to as 'Pacific Giant') were selected. The seeds were sterilized in 0.25% sodium hypochlorite solution for 60 min, followed by rinsing with sterile water. They were sown in MS medium (Murashige and

Skoog, 1962) that contained $30 \text{ g} \cdot \text{liter}^{-1}$ sucrose and $2.5 \text{ g} \cdot \text{liter}^{-1}$ gellan gum and cultured at 20°C in the dark. After one to three weeks in culture and before the hypocotyls elongated to 5 mm, the seedlings were separated into cotyledons, petioles and hypocotyls. The separated explants were chopped into 1–3 mm pieces and then the pieces were cultured at 20°C in the dark on previously autoclaved MS medium containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), $0.1 \text{ mg} \cdot \text{liter}^{-1}$ benzylaminopurine (BAP), $30 \text{ g} \cdot \text{liter}^{-1}$ sucrose and $2.5 \text{ g} \cdot \text{liter}^{-1}$ gellan gum (Wako Pure Chemical Ind. Ltd., Osaka, Japan); the pH of the medium was adjusted to 5.8 prior to autoclaving. The concentration of 2,4-D and BAP used in this medium was based on the results of Amagai and Yonai (1994). Induced calli and somatic embryos were subcultured monthly on the same medium and embryogenic calli were selected from each subculture. After four to nine months from the initiation of the culture, embryogenic calli were transferred onto MS medium (pH 5.8) with $30 \text{ g} \cdot \text{liter}^{-1}$ sucrose and $2.5 \text{ g} \cdot \text{liter}^{-1}$ gellan gum and cultured at 20°C under fluorescent lights having a photosynthetic photon density (PPFD) of $5\text{--}45 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for 16 hr and $1\text{--}2 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for 8 hr for plant regeneration.

Induction of a suspension culture from embryogenic calli and plant regeneration

Embryogenic calli of 'Pacific Giant' were transferred to a 100 ml Erlenmeyer flask containing 20 ml of liquid medium to induce a suspension culture. The N6-based medium, containing N6 inorganic salts (Chu et al., 1975), MS organic constituents (vitamins, glycine and myo-inositol), $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D, $1.0 \text{ mg} \cdot \text{liter}^{-1}$

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Thidiazuron (TDZ) and $30 \text{ g} \cdot \text{liter}^{-1}$ sucrose, was used as the basal medium; the effects of $1.0 \text{ mg} \cdot \text{liter}^{-1}$ kinetin, $1.0 \text{ mg} \cdot \text{liter}^{-1}$ BAP or no cytokinin were examined. Suspension cultures were maintained on a rotary shaker (100 rpm) at 20°C or 25°C under fluorescent lights as above or in the dark and subcultured weekly. Each week, a 0.1 ml packed cell volume of embryogenic cell clusters was selectively drawn from the suspension culture into a callus pipette and transferred to a fresh medium. Embryogenic cell clusters were distinguishable from non-embryogenic clusters, as the former were bright white in color. To determine the packed cell volume, the embryogenic cell suspension was selected and transferred to a pipette, and the volume was measured after sedimentation. The proliferation of suspension cell clusters in one week was assessed by the determination of the packed cell volume.

Three to seven months after the initiation of the suspension culture, suspension cell clusters, after a five- to seven-day subculture, were transferred to a regeneration medium. Clumps of suspension cells, containing 20 mg of suspension cell clusters, were transferred to N6-based medium, that lacked plant growth regulators (pH 5.8) but contained $30 \text{ g} \cdot \text{liter}^{-1}$ sucrose and $2.5 \text{ g} \cdot \text{liter}^{-1}$ gellan gum for plant regeneration, and cultured at 20°C under the same light condition and duration as above. The somatic embryos that formed were subcultured monthly in the same medium and conditions. The regenerated shoots were transferred to 1/2MS medium that contained half-strength MS inorganic salts, full-strength MS organic constituents, $30 \text{ g} \cdot \text{liter}^{-1}$ sucrose and $2.5 \text{ g} \cdot \text{liter}^{-1}$ agar for rooting. The rooted plants were transplanted into pots and acclimatized in a greenhouse.

Data collection and analysis

Data for the growth rate of the suspension cell clusters were collected and processed for each duplicated set of three flasks, and each experiment was triplicated. For plant regeneration, 9–18 clumps that contained 20 mg of suspension cell clusters were transferred to the regeneration medium in one experiment. Data for the numbers of somatic embryos and shoots were collected and processed for each duplicated set of 9–18 clumps of suspension cell clusters; each experiment was triplicated. The data were subjected to analysis of variance (ANOVA) and Fisher's least significant difference test ($P < 0.05$).

Results and Discussion

Induction of embryogenic calli

One month after the initiation of the cotyledon, petiole and hypocotyl cultures, callus formation was observed on most explants (Table 1). These calli that were white and covered with viscous substances (Fig. 1 A, except for the arrows), easily turned brown. Somatic embryos

were only formed on calli derived from cotyledons (Fig. 1 A, arrows) (Table 1). Somatic embryos that were excised and subcultured in the same medium, formed secondary embryos and calli within two to three weeks after the transfer. Most of the calli induced in the somatic embryo cultures were white and covered with viscous substances (viscous type) (Fig. 1 B except for the arrow). One to two months after the initiation of the somatic embryo subcultures, a nodular type of callus appeared on the viscous type calli. These were also white but smooth and not covered with the viscous substances (Fig. 1 B arrow). On these calli, white, embryo-like structures sometimes appeared. These nodular type calli were regarded as embryogenic, because they produced many somatic embryos and plantlets upon transfer to MS medium (data not shown). Since the embryogenic calli obtained in this study turned brown easily and became necrotic, it was necessary to subculture them immediately into fresh medium upon the appearance of browning, and to remove any brown portions at every subculture. The embryogenic calli that proliferated were maintained by subculturing them monthly for a year or more.

Induction of a suspension culture from embryogenic calli and plant regeneration

Five to six months after the initiation of the culture, embryogenic calli were transferred to a liquid N6-based medium, containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D and $1.0 \text{ mg} \cdot \text{liter}^{-1}$ TDZ that yielded three types of cell clusters: vacuolated cell clusters, browned cell clusters and bright white cell clusters rich in cytoplasm (Fig. 1 C). At first, the proliferation rate of the suspension cell clusters was low and unstable. The vacuolated cell clusters and browned cell clusters were removed at every subculture because these cell clusters appeared to be inhibitory to the growth of suspension cell clusters. Their elimination led to a stable, proliferating suspension cell cluster system that took four to six months. In this stable culture, cell clusters grew eight- to ten-fold in one week and most of the cell clusters consisted of bright white cells rich in cytoplasm, although small numbers of vacuolated cell clusters and browned cell clusters still remained. The selection of the cell clusters was an important step for the induction of the suspension culture as previously reported for *Hordeum vulgare* (Jahne et al., 1991), *Paspalum notatum* (Akashi et al., 1993) and *Iris germanica* (Shimizu et al., 1997). When suspension cell cultures were transferred to the regeneration medium, most clumps of suspension cells formed somatic embryos.

A study on the effects of temperature and light exposure on the growth rate of the suspension cell clusters revealed that the fluorescent lights had no significant effect on the cell growth at either 20°C or 25°C . However, culture at 25°C , supplemented with fluorescent light proliferated more rapidly than did

Fig. 1. Plant regeneration from suspension cultures of the white line of the 'Pacific Giant'. A: Viscous calli (except for the somatic embryos indicated by arrows) derived from a cotyledon in MS medium containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D and $0.1 \text{ mg} \cdot \text{liter}^{-1}$ BAP. B: Viscous calli with a white embryogenic callus (arrow) induced in a somatic embryo culture. C: Cell clusters rich in cytoplasm induced in a suspension culture of embryogenic calli. Suspension cell clusters were cultured in N6-based medium containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D and $1.0 \text{ mg} \cdot \text{liter}^{-1}$ TDZ at 25°C under fluorescent light conditions. D: Somatic embryos (arrows) and shoots induced from suspension cell clusters in a N6-based medium lacking plant growth regulators. E and F: Regenerated plants derived from somatic embryos.

Table 1. Effects of explants on callus and somatic embryos formation in tissue culture of the white line of the 'Pacific Giant'.

Explant source	No. of explants cultured	Callus induction rate (%)	Somatic embryo induction rate (%)
Cotyledon	75	97.3	6.7
Petiole	22	100.0	0
Hypocotyl	16	100.0	0

cultures at 20°C in the dark (Fig. 2). No significant differences were observed between the somatic embryo

formation rates from the clumps of suspension cell clusters grown under these conditions (data not shown). Tissue culture of *Delphinium* has previously been performed at 20°C (Amagai and Yonai, 1994; Hirose et al., 2002; Hosokawa et al., 2001). Amagai et al. (1992) reported that shoot proliferation of *Delphinium* was inhibited at 25°C . In our study, somatic embryo formation from suspension cells and shoot formation from somatic embryos seemed to be inhibited at 25°C (data not shown). In contrast, low temperature conditions were not required for the proliferation of the suspension cell clusters of *Delphinium* used in this study.

The growth rate of suspension cell cultures in media

Fig. 2. Effects of temperature and light conditions on the growth of suspension cultures of the white line of the 'Pacific Giant' on a N6-based medium containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D and $1.0 \text{ mg} \cdot \text{liter}^{-1}$ TDZ. Vertical bars represent the SD ($n=3$). The same letter shows no significant difference at the 5% level by Fisher's least significant difference test.

Fig. 3. Effects of combinations of plant growth regulators on the growth of suspension cell clusters of the 'Pacific Giant'. Suspension cell clusters were cultured in N6-based medium at 25°C under fluorescent lights. Vertical bars represent the SD ($n=3$). The same letter shows no significant difference at the 5% level by Fisher's least significant difference test.

that contained four different combinations of plant growth regulators is shown in Fig. 3. The suspension cell cultures proliferated in all media. However, in the medium containing the synthetic auxin 2,4-D only (2,4-D medium), the growth of the suspension cell culture was slow (Fig. 3), and the cell clusters developed

Fig. 4. Comparison of the somatic embryo and shoot formation rates from 20 mg of suspension cell clusters of five suspension cell lines (A, B, C, D and E) of the 'Pacific Giant'. Suspension cell clusters were cultured in N6-based medium containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D and $1.0 \text{ mg} \cdot \text{liter}^{-1}$ TDZ at 25°C under fluorescent lights. Vertical bars represent the SD ($n=3$). The same letter shows no significant difference at the 5% level by Fisher's least significant difference test.

browning and subsequently necrosis. No significant differences were observed between the somatic embryo formation rates from the suspension cell cultures grown under these conditions except for the 2,4-D medium (data not shown). It was difficult to transfer the suspension culture in the 2,4-D medium to the regeneration medium, because they were highly viscous and we could not completely isolate the suspension cells from the medium. When the mixture of suspension cell clusters and 2,4-D medium was transferred to the regeneration medium, somatic embryos were not formed. Generally, auxin and cytokinin or auxin only are added to the medium for somatic embryo formation and embryogenic callus proliferation. Amagai and Yonai (1994) reported that the combined supplementation with auxin and cytokinin promote somatic embryogenesis in *Delphinium*. In this study, we found that auxin and cytokinin were also required for the proliferation of suspension cell clusters. The suspension cell clusters increased about eight- to ten-fold in the medium containing 2,4-D and cytokinin after one week under fluorescent lights at 25°C .

Six months after the initiation of the suspension culture, somatic embryogenesis and the shoot formation capability were compared among five suspension cell lines (Fig. 4). These cell lines were derived from the same seedling, separated into independent flasks from the initiation of the suspension culture and named A, B, C, D and E. Substantial differences were found among the cell lines in the numbers of somatic embryos and shoots formed per 20 mg of suspension cell clusters

Fig. 5. Effect of the culture duration on somatic embryo formation and shoot regeneration from suspension cell clusters of the 'Pacific Giant'. Suspension cell clusters were cultured in N6-based medium containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D and $1.0 \text{ mg} \cdot \text{liter}^{-1}$ TDZ at 25°C under fluorescent lights. Vertical bars represent the SD ($n=3$). The same letter shows no significant difference at the 5% level by Fisher's least significant difference test.

(Fig. 4). It is thought that these differences are caused by somaclonal or physiological variations that occurred during the subculture. Such differences in the culture response among suspension cell lines derived from the same cultivar have previously been reported by Kihara and Funatsuki (1994) who examined the plant regeneration ability of protoplasts isolated from different cell lines of barley.

When suspension cell clusters of cell line B (Fig. 3) were transferred to the same medium that lacked a plant growth regulator, 19.6 embryos per 20 mg suspension cell clusters were produced (Fig. 1 D) in one month. Somatic embryos that were subcultured monthly into the same medium initiated many shoots. However, the majority of these shoots were morphologically abnormal and showed vitrification. Two months after the transfer of the somatic embryos, 5.8 normal shoots were produced from 20 mg of suspension cell clusters. The shoots were transferred to 1/2MS medium to root, since root formation was inhibited in the N6-based medium. About 10% of the shoots formed roots (Fig. 1 E and F) within four weeks, and about 20 plantlets were obtained from 1 g of suspension cell clusters. Regenerated plants were successfully transferred to pots in a greenhouse where they are being assessed for uniformity.

Generally, suspension cultures are more suitable for physiological and biochemical investigations than callus cultures grown in solid media because the former provide a more homogeneous system than the latter (Ozawa and Komamine, 1989). Moreover, millions of cells or cell aggregates can be maintained in a single

vessel and used for protoplast culture and genetic transformation (Wang et al., 1999). However, in some species, plant regeneration from suspension cultures has been difficult. We were able to maintain successfully the regeneration ability of the suspension cultures of 'Pacific Giant' for more than a year by culturing them in N6-based medium containing $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D and $1.0 \text{ mg} \cdot \text{liter}^{-1}$ TDZ at 25°C under fluorescent lights (Fig. 5).

Our regeneration system may facilitate micropropagation, protoplast culture and genetic transformation by *Agrobacterium tumefaciens* or particle bombardment in *Delphinium*. Currently, we are attempting to apply our culture conditions to other cultivars.

Literature Cited

- Akashi, R., A. Hashimoto and T. Adachi. 1993. Plant regeneration from seed-derived embryogenic callus and cell suspension culture of bahiagrass (*Paspalum notatum*). Plant Sci. 90: 73–80.
- Amagai, M., Y. Okabe and S. Yonai. 1992. *In vitro* mass propagation of *Delphinium* by tissue culture of flowering plants. Bull. Tochigi Agr. Exp. Stn. 39: 43–52.
- Amagai, M. and S. Yonai. 1994. *In vitro* propagation of *Delphinium* (*Delphinium elatum* L.) by tissue culture. 2. Efficient culture condition inducing somatic embryogenesis. Bull. Tochigi Agr. Exp. Stn. 42: 45–52.
- Chu, C. C., C. C. Wang, C. S. Sun, C. Hsu, K. C. Yin, C. Y. Chu and F. Y. Bi. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sinica 18: 659–668.
- Jähne, A., P. A. Lazzeri, M. Jäger-Gussen and H. Lörz. 1991. Plant regeneration from embryogenic cell suspensions derived from anther culture of barley (*Hordeum vulgare* L.). Theor. Appl. Genet. 82: 74–80.
- Hirose, Y., R. Aida and M. Shibata. 2002. *Agrobacterium tumefaciens*-mediated transformation of *Delphinium* spp. Plant Biotechnol. 19: 377–382.
- Hosokawa, K., H. Koiwa and S. Yamamura. 2001. *In vitro* adventitious shoot formation on petioles of commercial cultivars of delphinium. Scientia Hort. 90: 143–150.
- Kihara, M. and H. Funatsuki. 1994. Fertile plant regeneration from barley (*Hordeum vulgare* L.) protoplasts isolated from long-term suspension culture. Breed. Sci. 44: 157–160.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473–479.
- Ozawa, K. and A. Komamine. 1989. Establishment of system of high-frequency embryogenesis from long-term cell suspension culture of rice (*Oryza sativa* L.). Theor. Appl. Genet. 77: 205–211.
- Shimizu, K., H. Nagaike, T. Yabuya and T. Adachi. 1997. Plant regeneration from suspension culture of *Iris germanica*. Plant Cell Tiss. Org. Cul. 50: 27–31.
- Wang, Y., Z. Jeknić, R. C. Ernst and T. H. H. Chen. 1999.

Delphinium における懸濁培養細胞からの植物体再生

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摘 要

デルフィニウム (*Delphinium hybridum*) のパシフィック・ジャイアントシリーズの白色花系統の子葉を $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D, $0.1 \text{ mg} \cdot \text{liter}^{-1}$ BAP を含む MS 培地で培養し, embryogenic カルスを得た. これを N6 無機塩類, MS 有機栄養素 (ビタミン類, グリシン, ミオイノシトール), $1.0 \text{ mg} \cdot \text{liter}^{-1}$ 2,4-D, $1.0 \text{ mg} \cdot \text{liter}^{-1}$ TDZ 及び $30 \text{ g} \cdot \text{liter}^{-1}$ ショ糖を含む液体培地 (N6-based 培地) で培養することにより, 懸濁培養系を確立することに成功した. 懸濁培養細胞塊は

25°C 明条件において回転培養することによって, 1 週間あたり圧縮細胞容積で 8-10 倍の増殖を示した. さらに, ゲランガムで固化した植物生長調節物質を含まない N6-based 培地に移植し, 継代培養することで, 体細胞胚形成を経てシュートが形成された. シュートからの発根は植物生長調節物質を含まない 1/2MS 培地で行い, 懸濁培養細胞塊 1 g 当たりの小植物体再生数は約 20 個体であった.