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Plant Regeneration from Protoplasts in *Dianthus*: Comparison of Cultural Behavior of Different Donor Tissues

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Protoplast isolation, culture and shoot regeneration from protoplast-derived calli were compared among different organs of *Dianthus* species under the same conditions. Leaves, petals and seedling hypocotyls of *D. caryophyllus* cv. Chabaud (a seed-propagated cultivar) and *D. barbatus* were used as protoplast sources. Protoplasts isolated from petals of these two species showed only low yield and low division frequency. On the other hand, division frequency of hypocotyl protoplasts was higher than that of leaf protoplasts, although, in *D. caryophyllus*, protoplast yield in hypocotyls was slightly lower than in leaves. In both species, relatively high frequency of shoot regeneration was obtained in hypocotyl- and petal-derived protoplasts, while only low or no shoot regeneration occurred in leaf-derived protoplasts.

Introduction

Establishment of a protoplast culture system in flower crops is a prerequisite for improving their floral and marketable qualities by biotechnology such as somatic hybridization and genetic transformation. In the genus *Dianthus,* it has already been possible to regenerate plants from leaf mesophyll protoplasts of certain species and cultivars[1]. In addition, somatic hybrid plants have recently been obtained from protoplast fusion experiments between *D. chinensis* and *D. barbatus*[2], and between *D. caryophyllus* (carnation) and *D. chinensis*[3]. In these studies, it was demonstrated that shoot regeneration ability from leaf mesophyll protoplasts was markedly different among the species and that efficient regeneration was obtained only in *D. chinensis* and its interspecific hybrids. Despite the modification of culture media and conditions, high frequency shoot regeneration from leaf mesophyll protoplasts has not yet been achieved in most *Dianthus* species other than *D. chinensis* (unpublished results). For the wide application of biotechnology in this genus, however, it is necessary to develop a protoplast culture system in previously recalcitrant species such as *D. caryophyllus* and *D. barbatus.* In the present study, therefore, we screen various protoplast sources, in addition to leaves, such as hypocotyls and petals for obtaining an efficient plant regeneration from protoplasts in these two recalcitrant *Dianthus* species.

Materials and Methods

D. caryophyllus cv. Chabaud, a seed-propagated cultivar, and *D. barbatus* were used in this study.

Flower color of the plants used were yellow and red with white rim in *D. caryophyllus* and *D. barbatus,* respectively. *In vitro*-sown seedlings and *in vitro* shoot cultures were obtained and maintained as previously described[1]. As protoplast sources, hypocotyls harvested from 7-day-old seedlings and fully expanded leaves of plantlets 3 weeks after subculture were used. Immature flower buds (5 to 7 days before anthesis) were harvested from greenhouse-grown plants of both species, and surface-disinfected as previously described[4]. Receptacle, sepals and outermost petals were removed from the buds and the remaining petals were also used as a protoplast source.

Protoplasts were isolated and purified according to the method previously described[1]. Viability of purified protoplasts was assessed with fluorescein diacetate (FDA)[5]. Protoplasts were cultured in liquid KM8p medium[6] containing 5 mg/*l* α-naphthaleneacetic acid (NAA), 1 mg/*l* zeatin and 0.5 M glucose at a density of 1×10^5/m*l*. The pH of all culture media used in this study was adjusted to 5.8. For protoplast culture, plastic Petri dishes (6 cm in diameter) containing 3 m*l* of the medium were used. Cultures were maintained at 27°C in the dark for 2 months. To promote colony growth, one m*l* of the same fresh medium was added 3 times at 14-day intervals. Protoplast division frequency was recorded after 14 days of culture.

Protoplast-derived visible colonies (*ca.* 1 mm in diameter) were transferred for callus proliferation onto Murashige and Skoog's (MS) medium[7] containing 2% sucrose and 1 mg/*l* each of NAA and zeatin, and solidified with 0.2% gellan gum. Cultures during and after callus proliferation were maintained at 27°C under continuous illumination (35 μmol/m^2/s) with fluorescent light. After one month, protoplast-derived calli were transferred for shoot induction onto MS medium containing 2% sucrose, 1 mg/*l* NAA and 5 mg/*l* zeatin, and solidified with 0.2% gellan gum. Root induction from regenerated shoots and acclimatization of protoplast-derived plantlets were performed as previously described[1].

Results

Although protoplasts could be routinely isolated from all three organs examined in both species, protoplast yield was markedly different among the organs (**Table 1**). In both species, protoplast yields of more than 10^6 per g fresh weight (FW) were obtained from both leaves and hypocotyls, while petals yielded lower number of protoplasts (below 10^6 per g FW). In *D. caryophyllus*, protoplast yiled in hypocotyls was slightly lower than in leaves. Generally, leaf-derived protoplasts

Table 1. Differences in protoplast yield, viability, division and colony formation among different sources for protoplast isolation in two *Dianthus* species.

Dianthus species	Protoplast source	Yield ($\times10^5$/gFW)	Viability[1] (%)	Division[2] (%)	Colony[3] (%)
D. caryophyllus cv. Chabaud	Leaves	42.6±6.3	87.5±3.5	6.0±1.2	0.07±0.01
	Hypocotyls	23.3±5.3	96.5±4.3	15.8±4.2	0.15±0.01
	Petals	8.1±2.8	82.2±5.6	1.2±1.1	0.02±0.01
D. barbatus	Leaves	10.6±2.1	92.5±4.2	8.7±3.6	0.09±0.01
	Hypocotyls	14.8±4.2	96.5±4.4	23.2±7.6	0.20±0.02
	Petals	3.3±1.2	81.7±7.7	0.8±0.6	0.02±0.01

Values represent the mean ±SE of at least five independent experiments. For examining frequencies of protoplast division and colony formation, each experiments consisted of at least five culture dishes in which 3×10^5 protoplasts were plated.

1) Percentage of protoplasts showing viability as assessed with FDA immediately after preparation.
2) Percentage of plated protoplasts showing at least one cell division after 14 days of culture.
3) Percentage of plated protoplasts which developed into visible colonies (*ca.* 1 mm in diameter) after 2 months of culture.

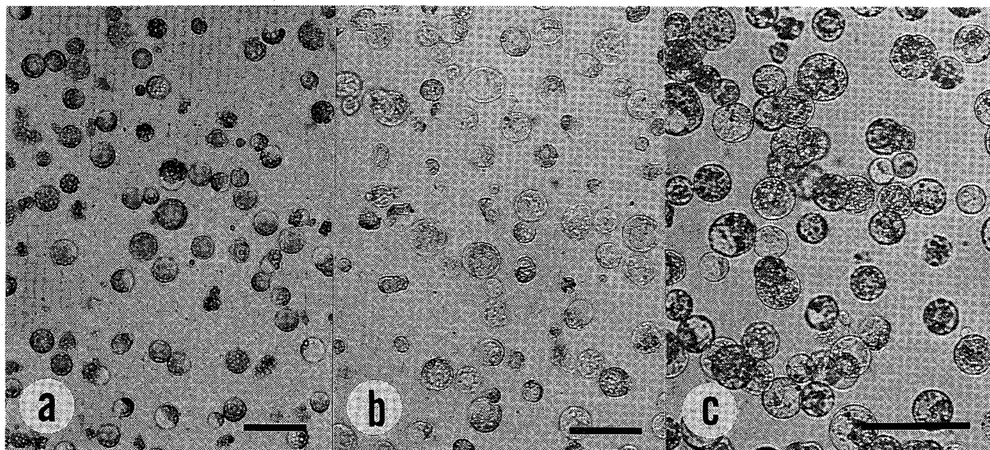

Fig. 1 Protoplasts of *D. caryophyllus* cv. Chabaud isolated from leaves (a), hypocotyls (b) and petals (c). Bars = 100 μm.

were vacuolated and contained many chloroplasts (**Fig. 1-a**) while those derived from hypocotyls were rich in cytoplasm (**Fig. 1-b**). Protoplasts isolated from petals were highly heterogeneous: most of them were highly vacuolated while the others were rich in cytoplasm as hypocotyl protoplasts (**Fig. 1-c**). Petal protoplasts were colorless in *D. caryophyllus* and red to colorless in *D. barbatus*. In both species, protoplasts had more than 80% viability irrespective of protoplast source (**Table 1**).

In both species, protoplasts isolated from all three organs started to divide after 3 to 6 days of culture (**Fig. 2-a**). In petal protoplasts, cell division occurred preferentially in those rich in cytoplasm, but highly vacuolated protoplasts also occasionally divided. Budding of petal protoplasts was frequently observed. Protoplast division frequencies recorded after 14 days of culture varied depending on the protoplast source (**Table 1**). In both species, the highest percentage of division was obtained from hypocotyl protoplasts: 15.8% and 23.2% in *D. caryophyllus* and *D. barbatus*, respectively. Leaf-derived protoplasts divided with lower frequencies (below 10%), and those derived from petals showed only low division frequencies (*ca.* 1%). Protoplasts sustained cell division, and cell proliferation was promoted by the addition of the fresh medium (**Fig. 2-b**). After 2 months of culture, visible colony formation frequencies of over 0.07 and 0.15% were obtained from leaf- and hypocotyl-derived protoplasts, respectively (**Table 1**). In petal protoplasts, only 0.02% of protoplasts formed visible colonies. Almost all of the protoplast-derived visible colonies of all three sources in both species grew vigorously and turned green upon transfer to the callus proliferation medium (**Fig. 2-c**).

One month after transfer, protoplast-derived calli were further transferred to the shoot induction medium in order to determine the regeneration ability of each protoplast source. After 2 to 4 months, shoot primordia of green nodular structure appeared on the surface of the calli and developed into adventitious shoots (**Fig. 2-d**). Shoot regeneration frequency was significantly different among the organs used as protoplast sources in both species (**Table 2**). Only low frequency (0.2% in *D. barbatus*) or no (in *D. caryophyllus*) shoot regeneration occurred in calli derived from leaf protoplasts even 6 months after transfer of the calli onto the induction medium. On the other hand, relatively high frequency shoot regeneration was obtained from the other two protoplast sources of both species. Calli derived from hypocotyls, in particular, showed the highest percentage of regeneration: 4.5% and 7.6% in *D. caryophyllus* and *D. barbatus*, respectively.

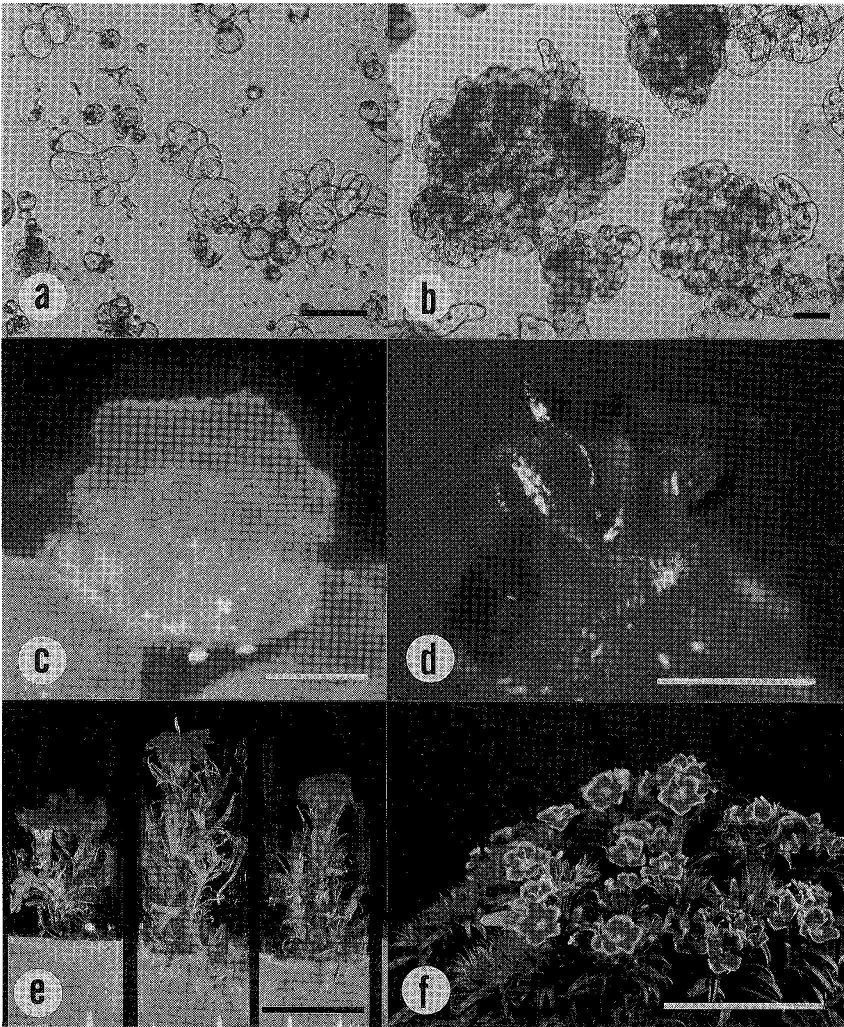

Fig. 2 Plant regeneration from hypocotyl protoplasts of *D. barbatus*
(a) Protoplast division after 6 days of culture. Bar=100 μm. (b) Protoplast-derived visible colonies after 2 months of culture. Bar=50 μm. (c) Protoplast-derived calli obtained one month after transfer to the callus proliferation medium. Bar=5 mm. (d) Regeneration of adventitious shoots from protoplast-derived callus 2 months after transfer to the shoot induction medium. Bar=5 mm. (e) Protoplast-derived plantlets precociously flowered *in vitro*. Bar=3 cm. (f) A normal protoplast-derived plant growing in the greenhouse (flowering stage). Bar=5 cm.

Most of the regenerated shoots developed roots by excising from the calli and transferring to plant growth regulator-free medium. Some abnormalities including dwarf shoots, and difficulty in development and rooting were observed in protoplast-derived plantlets of both species irrespective of protoplast source. In *D. barbatus*, in addition, almost all plantlets derived from all three protoplast sources exhibited precocious flowering in the *in vitro* condition (**Fig. 2-e**), which was rarely observed in those of *D. caryophyllus*. However, in both species, plantlets occasionally recovered normal morphology from abnormal ones during prolonged culture on plant growth regulator-free medium. These normal plantlets were successfully transferred to the greenhouse after they completed acclimatization (**Fig. 2-f**).

Table 2. Differences in shoot regeneration from protoplast-derived calli among different sources for protoplast isolation in two *Dianthus* species.

Dianthus species	Protoplast sources	Shoot regeneration (%)[1]
D. caryophyllus cv. Chabaud	Leaves	0
	Hypocotyls	4. 5**
	Petals	1. 2*
D. barbatus	Leaves	0. 2
	Hypocotyls	7. 6*
	Petals	5. 5*

[1] Percentage of protoplast-derived calli which regenerated shoots by 4 months after transfer to the shoot induction medium. Values represent the mean of at least five independent experiments each of which consisted of at least 100 protoplast-derived calli.

* and ** = significantly different from the leaves in each species at the 5. 0% and 1. 0% levels, respectively (Duncan's New Multiple Range Test).

Discussion

It has already been reported that shoot regeneration from leaf mesophyll protoplasts of several *Dianthus* species such as *D. caryophyllus* and *D. barbatus* was very difficult[1], which was again confirmed in this study. Although leaf mesophyll protoplasts have been shown to be a suitable material in a number of dicotyledonous species[8,9], they sometimes gave a low yield of regenerated plants as in *Brassica*[10]. In these cases, other donor tissues were alternatively used and often provided a better result than leaves. In this study, an efficient regeneration system from protoplasts of the previously recalcitrant *Dianthus* species also could be established without modification of culture media and conditions by using alternative donor tissues such as hypocotyls and petals, both of which have never been used as protoplast sources in *Dianthus*. This is the first report to describe the regeneration of plants from protoplasts of *D. caryophyllus* cv. Chabaud.

In both *Dianthus* species, hypocotyls provided the best result among the donor tissues examined not only on shoot regeneration but also on protoplast division. Hypocotyls have already proved to be a good source for protoplast isolation in several plant species including *Brassica*[10] and *Helianthus annuus*[11]. In the latter species, in particular, only hypocotyl-derived protoplasts divided and gave rise simultaneously to microcalli and somatic embryos, whereas no cell division was observed in cotyledon- or leaf-derived protoplasts. The usefulness of hypocotyls as a protoplast source should be confirmed in other *Dianthus* species.

Although hypocotyls found to be a suitable source for protoplasts with a high regeneration ability in certain recalucitrant *Dianthus* species, it should be noted that many *Dianthus* cultivars, particularly those in *D. caryophyllus*, are vegetatively propagated. In these cultivars, therefore, petals may be suitable as a protoplast source, although only low division frequency was obtained from them under the conditions used in this study. Petals have already been shown to have a higher regeneration ability of adventitious shoots than leaves and stems of *D. caryophyllus* cv. Scania[4]. It has also been reported that shoots were regenerated only from the proximal region of petals[4,12,13], which possibly associated with the zone of cell division and cell elongation nearest the receptacle[14]. Therefore, petal protoplasts rich in cytoplasm which preferentially divided in this study may be derived from the cells of this region. The origin of these cytoplasm-rich protoplasts should be identified. Also, further experimentation should be directed to increase the division frequency of petal-derived protoplasts, for example, by separating cytoplasm-rich protoplasts using density gradient centrifugation[15], and by applying a nurse culture technique as used for petal protoplast

culture in *Nicotiana alata*[16].

In the present study, some morphological abnormalities were observed in protoplast-derived plantlets as in plantlets derived from leaf mesophyll protoplasts[1] and petals[4]. However, most of these abnormalities seems to be induced by physiological disorders rather than to arise as a results of genetic variations, because plantlets with normal morphology occasionally developed from abnormal ones thereafter. Detailed characterization of regenerated plants obtained in this study is now in progress under greenhouse conditions.

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《和文要約》

ナデシコ属植物のプロトプラストからの植物体再生に及ぼすプロトプラスト単離組織の影響

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二種のナデシコ属植物，カーネーション(*Dianthus caryophyllus*)およびビジョナデシコ(*D. barbatus*)について，葉，花弁および胚軸から同一条件でプロトプラストを単離，培養し，プロトプラストの収量，生存率，分裂率およびプロトプラスト由来カルスからの不定芽分化率を比較した．胚軸由来プロトプラストの分裂率は葉肉由来プロトプラストと比較して高かったが，花弁由来プロトプラストの分裂率は低かった．プロトプラスト由来カルスからの不定芽の分化は，材料に葉を用いた場合にはほとんどあるいは全く観察されなかったが，花弁および胚軸を用いた場合には比較的高頻度で観察された．以上の結果から，ナデシコ属植物においては，プロトプラストの材料として胚軸，次いで花弁が適していることが示唆された．