

Plant regeneration from leaf-derived protoplasts within the *Daucus* genus: effect of different conditions in alginate embedding and phytosulfokine application

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Received: 29 October 2013 / Accepted: 25 January 2014 / Published online: 5 February 2014
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Abstract Protoplasts of four *Daucus carota* subspecies and three wild *Daucus* species were isolated from 2-week-old shoot cultures during overnight incubation in an enzyme mixture composed of 1 % (w/v) cellulase Onozuka R-10 and 0.1 % (w/v) pectolyase Y-23. Before the culture, they were embedded in autoclave- or filter-sterilized alginate solution. Modified thin alginate layer (TAL) and extra thin alginate film (ETAF) techniques were applied for protoplast immobilization. A rich mineral-organic medium based on the formulation of Kao and Michayluk supplemented with 0.1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid, 0.2 mg l⁻¹ zeatin, and optionally 100 nM phytosulfokine (PSK), a peptidyl plant growth factor, was used for protoplast culture. Plating efficiency was genotype-dependent and in 40-day-old cultures, it varied from 10 % for *Daucus pusillus* to 73 % for *D. carota* subsp. *sativus*. A considerably higher ability in colony formation was observed in the modified TAL culture system using filter-sterilized alginate and in the presence of PSK in the protoplast culture medium. Plant regeneration through somatic embryogenesis stimulated by PSK application occurred for five out of the seven *Daucus* accessions used in the present study. We believe our data may facilitate the use of wild *Daucus* in somatic hybridization with cultivated carrot.

Keywords Alginate sterilization · Culture technique · Plating efficiency · Protoplast regeneration · Wild *Daucus*

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
B5	B5 Gamborg medium (Gamborg et al. 1968)
CMS	Cytoplasmic male sterility
CPP	Carrot petiole protoplast medium
ETAF	Extra thin alginate film
FDA	Fluorescein diacetate
FW	Fresh weight
KM	Kao and Michayluk medium (1975)
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MS	Murashige and Skoog medium (1962)
NAA	α -Naphthaleneacetic acid
PEM	Proembryonic mass
PSK	Phytosulfokine
TAL	Thin alginate layer

Introduction

The genus *Daucus* belongs to the Apiaceae family, one of the most important families of angiosperms from an economical point of view. Within the genus, 24 *Daucus* species and nine *Daucus carota* subspecies are recognized, according to the most recent estimates (Grzebelus et al. 2011). Among them, the best known and agronomically important is cultivated carrot (*D. carota* L. subsp. *sativus* Hoffm., 2n = 2x = 18). It is grown for its edible storage root, which cumulates α - and β -carotene, the primary source of pro-vitamin A in the human diet. *Daucus* is widespread all over the world, being most frequently associated with the Mediterranean, Central Asia, and New World regions, and even Australia (Rubatzky et al. 1999). Wild *Daucus* germplasm, due to its worldwide origin and adaptation to a wide range of environmental conditions,

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could be a valuable natural source of genetic variability for developing new carrot cultivars with resistance/tolerance against adverse biotic and abiotic factors, as well as desirable culinary, nutritional, or processing traits (Camadro et al. 2008). Usually, to transfer germplasm from wild species to cultivated crops, intra- or inter-specific crosses are performed. Till now, two types of such generative hybridization have greatly contributed to the improvement of modern carrot cultivars, through the introduction of: (1) the petaloid type of cytoplasmic male sterility (CMS) from wild *D. carota* subspecies; and (2) resistance to carrot fly from *Daucus capillifolius* (Grzebelus et al. 2011). Despite these successful crosses, utilization of wild germplasm in carrot breeding programs is limited, probably due to the relatively poor characterization of *Daucus* genetic resources and different flowering biology, including usually shorter and only partially overlapping flowering periods and less inflorescences or very small flowers in comparison with carrot (Camadro et al. 2008; Grzebelus et al. 2011). It seems that somatic hybridization based on protoplast fusion could help to overcome these problems, but up to today, only protoplasts isolated from one wild species, i.e., *D. capillifolius*, have been fused in both a symmetric and an asymmetric way with carrot-derived protoplasts (Dudits et al. 1977; Ichikawa et al. 1987). The ability to regenerate plants from fused protoplasts is fundamental in somatic hybridization. Therefore, before applying this procedure to plant breeding programs, successful protocols for plant regeneration from protoplasts of both partners should be elaborated. For carrot, well known as a model species for plant tissue culture systems, efficient procedures for protoplast isolation and plant regeneration from different types of source tissue, such as suspension cultures, petioles, leaves, and hypocotyls have been presented (Grambow et al. 1972; Dirks et al. 1996; Grzebelus et al. 2012a). However, to our knowledge, similar studies with regard to wild *Daucus* species do not exist.

To stimulate or improve protoplast development into plants, different culture systems have been employed over the years. Among them, incubation of protoplasts in liquid medium is the most simple to establish but results in unfavorable cell agglutination (Davey et al. 2005a). Therefore, for many species, techniques ensuring physical separation of protoplasts based on their embedding in semi-solid media, with agar being first used as the gelling agent (Nagata and Takebe 1971), have been preferred (Davey et al. 2005b). Immobilization of protoplasts in such media before culture decreases the production of polyphenols that may prolong cell viability, support cell wall regeneration, and promote mitotic divisions. Besides agar, agarose was proved to be superior as a solidifying agent, possibly because of its neutrality and lower gelling temperature (Davey et al. 2005a, c). Alginate, a natural

binary copolymer of β -D-mannuronic acid and α -L-gulonic acid extracted from cell walls of brown algae (Draget 2000), is another and useful alternative to agar and agarose, especially for heat-sensitive protoplasts, since gelling is induced by exposure to calcium ions (Davey et al. 2005a). Additionally, in systems using alginate, the beneficial effect of calcium ions on cell wall re-synthesis may be observed. Protoplasts embedded in alginate matrix can be dispensed as beads (Damm and Willmitzer 1988), blocks (Pan et al. 2003), or layers with different thickness such as thin alginate layers (TAL, Damm et al. 1989) and extra thin alginate films (ETAF, Pati et al. 2005), and then cultured in liquid media. Alginate has been successfully used as a gelling agent in protoplast cultures of many species; among others, in *Lotus corniculatus* and *Nicotiana tabacum* (Pati et al. 2005), *Citrus sinensis* (Niedz 2006), *Helianthus annuus* (Rákósy-Tican et al. 2007), *D. carota* (Grzebelus et al. 2012a), *Beta vulgaris* (Grzebelus et al. 2012b), and *Brassica oleracea* (Kiełkowska and Adamus 2012).

Protoplasts from different species and even from different tissues of the same species generally vary in their nutritional requirements. Over the years, different medium formulations with defined and undefined supplements such as coconut milk have been tested. Among them, the most important are growth regulators, particularly auxins and cytokinins, normally essential for sustained divisions of protoplast-derived cells and their further differentiation into plants (Davey et al. 2005b). Recently, oligopeptides have been recognized as signaling molecules playing an important role in plant cell growth and development (Matsubayashi et al. 1997; Ryan and Pearce 2001). One of them is phytosulfokine (PSK), a plant-specific disulfated pentapeptide isolated for the first time from conditioned medium of an *Asparagus officinalis* mesophyll cell culture (Matsubayashi and Sakagami 1996). Since PSK is involved in nanomolar concentrations in the initial steps of cellular dedifferentiation, proliferation, and re-differentiation, its biological function is similar to that of plant hormones (Igasaki et al. 2003; Matsubayashi et al. 2004). PSK can not only induce cell division in suspension and protoplast cultures (Matsubayashi and Sakagami 1996; Matsubayashi et al. 1997), but appears to play a role in diverse aspects of plant development and reproduction, including the formation of tracheary elements and adventitious roots (Yamakawa et al. 1998; Matsubayashi et al. 1999), somatic embryogenesis (Kobayashi et al. 1999; Igasaki et al. 2003), and pollen germination (Chen et al. 2000). It seems that besides the widely used auxins and cytokinins, PSK can be a promising supplement for in vitro plant cultures, especially in the case of recalcitrant species. To date, PSK has been applied to *Oryza sativa* and *Beta vulgaris* protoplast cultures and, as a result, increased plating efficiency was

observed (Matsubayashi et al. 1997; Grzebelus et al. 2012b).

The main objective of the present work was to evaluate the effect of: (1) alginate sterilization method; (2) different systems of cell culture in alginate; and (3) PSK application on plating efficiency and plant regeneration from leaf-derived protoplasts, for the first time, for some *Daucus* species, to facilitate their subsequent use in somatic hybridization with cultivated carrot.

Materials and methods

Plant material

Seven accessions of *Daucus* genus were used as a source of protoplasts, including three wild subspecies of *D. carota* (*D. carota* subsp. *azoricus*, subsp. *drepanensis*, subsp. *maritimus*), three wild species of *Daucus* (*D. aureus*, *D. pusillus*, *D. montevidensis*) and, as a control, cultivated carrot (*D. carota* L. subsp. *sativus* Hoffm., breeding line 9304B). Seeds were derived from research centers and gene banks (Table 1). Protoplasts were isolated from in vitro grown shoots produced from seeds. In order to obtain aseptic material, donor seeds were disinfected using a three-step procedure, as described by Grzebelus et al. (2012a). To make the germination of wild accessions possible, decontaminated seeds were left to swell in sterile water. After 1 day of incubation, seed coats were removed under a stereomicroscope and the embryos in endosperm tissue were placed in 9 × 1.5 cm Petri dishes on solid Murashige and Skoog (MS) medium with vitamins

(Murashige and Skoog 1962), supplemented with 30 g l⁻¹ sucrose and 6.5 g l⁻¹ plant agar (Biocorp, Poland) at 18 ± 2 °C in the dark. Seven or 30 days later, seedlings derived from cultivated and wild *Daucus* accessions, respectively, were transferred to glass jars with regeneration medium (R) composed of MS macro- and micro-elements, 0.1 mg l⁻¹ thiamine HCl, 0.1 mg l⁻¹ pyridoxine HCl, 0.5 mg l⁻¹ nicotinic acid, 3.0 mg l⁻¹ glycine, 100 mg l⁻¹ myo-inositol, 20 g l⁻¹ sucrose, and 2.5 g l⁻¹ phytigel (Sigma) and kept in a climate room at 26 ± 2 °C under a 16-h photoperiod and light intensity of 55 μmol m⁻² s⁻¹. Because of the limited number of seeds, for wild accessions, shoot cultures were established by transfer of leaf rosettes to fresh R medium every 2 weeks. In the case of carrot (line 9304B), new aseptic plantlets were regularly produced from seeds before each isolation experiment.

Protoplast isolation

Protoplasts of cultivated and wild *Daucus* accessions were isolated from expanded leaves with petioles of 2-week-old in vitro grown plantlets or shoot cultures (Fig. 1a), respectively. All steps of protoplast isolation were performed according to a previously described protocol (Grzebelus et al. 2012a). Briefly, about 1 g of source tissue was cut in 9 × 1.5 cm glass Petri dish into small pieces in the presence of a 0.5 M mannitol solution (pH = 5.6–5.8), and incubated for 1 h at 26 ± 2 °C in the dark to plasmolyze the cells. After that, the mannitol solution was replaced by an enzyme mixture consisting of 1 % (w/v) cellulase Onozuka R-10 (Duchefa Biochemie, Haarlem, The Netherlands), 0.1 % (w/v) pectolyase Y-23 (Duchefa Biochemie), 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma-Aldrich, St. Louis, MO, USA), 5 mM CaCl₂, and 0.6 M mannitol (Sigma-Aldrich), pH = 5.6, filter-sterilized (0.22 μm, Millipore, Billerica, MA, USA). Leaf tissue was digested for 12–16 h at 26 ± 2 °C in the dark on a gyratory shaker (30 rpm). The released protoplasts were initially purified by filtration through a 100-μm nylon mesh (Millipore) and centrifuged at 100g for 5 min in order to remove the enzyme solution. The pellet was resuspended in 8 ml of 0.5 M sucrose with 1 mM MES, very slowly overlaid with 2 ml of W5 salt solution (Menczel et al. 1981), and centrifuged at 145g for 10 min. Viable protoplasts localized at the interface between two solutions of different concentrations as a green band were transferred into a fresh tube using a Pasteur pipette and washed twice by centrifugation at 100g for 5 min in carrot petiole protoplast medium (CPP), which consisted of macro- and micro-elements, and organic acids according to Kao and Michayluk (1975), vitamins according to Gamborg B5 medium (Gamborg et al. 1968), 74 g l⁻¹ glucose,

Table 1 Origin, seed source, and somatic chromosome number (2n) of *Daucus* accessions used for protoplast cultures

Accession	Origin, seed source	Reference	2n
<i>D. carota</i> subsp. <i>sativus</i>	USA, USDA carrot inbred line	9304B	18
<i>D. carota</i> subsp. <i>azoricus</i>	Germany, HRI 6667	WD102	18
<i>D. carota</i> subsp. <i>drepanensis</i>	Denmark, PI 279762	D19	18
<i>D. carota</i> subsp. <i>maritimus</i>	Germany, HRI 7996	WD114	18
<i>D. aureus</i>	Israel, PI 319403	D4	22
<i>D. montevidensis</i>	South America, JKI AS162	WD28	22
<i>D. pusillus</i>	Uruguay, PI 349267	D16	22

USDA = United States Department of Agriculture; PI = USDA Plant Introduction numbers; HRI = Horticulture Research Institute, Genetic Resources Unit (Wellesbourne, UK) numbers; JKI = Julius Kuhn Institute (Germany) numbers

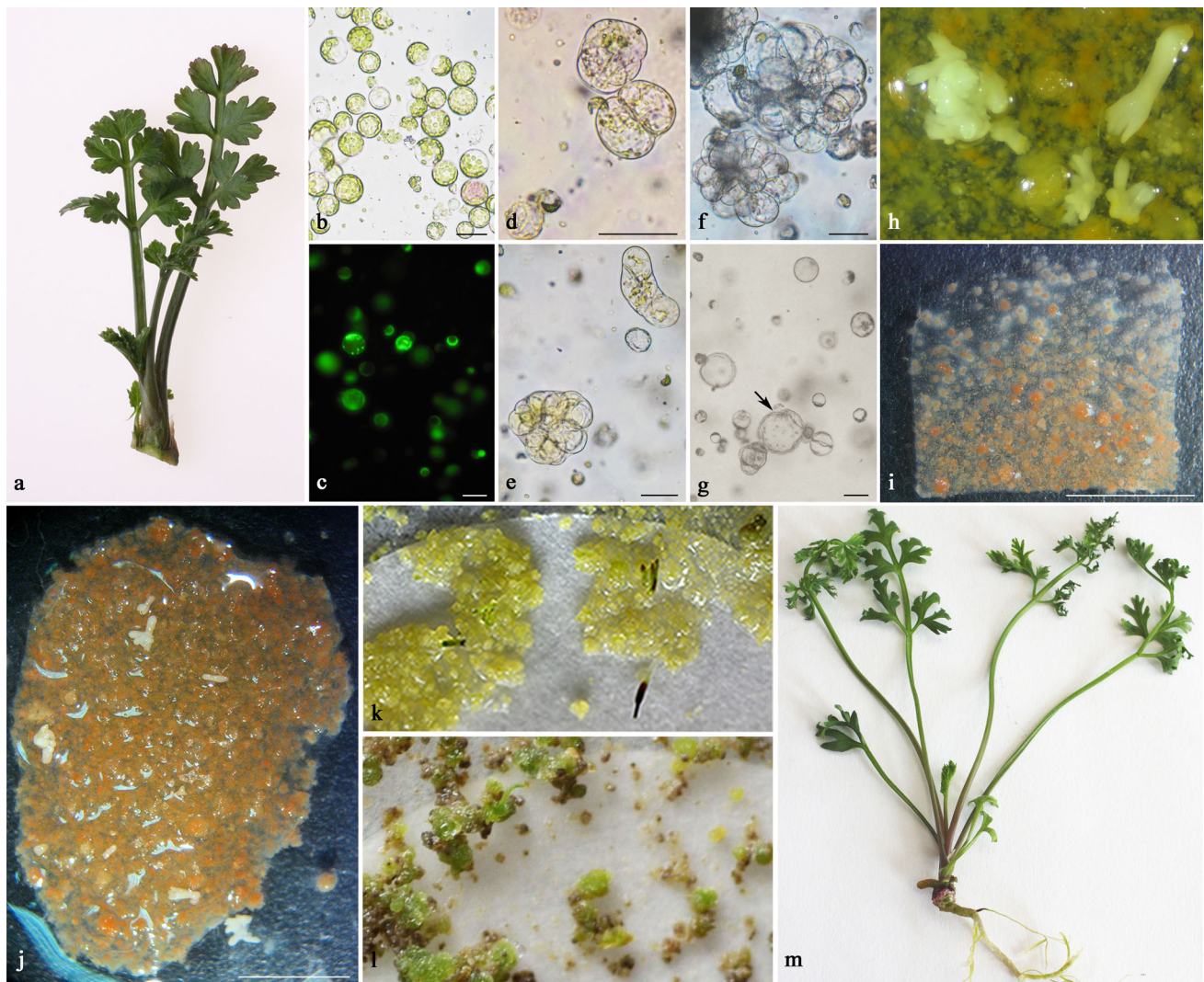


Fig. 1 Plant regeneration from leaf-derived protoplasts within the *Daucus* genus. **a** Source tissue of *D. carota* subsp. *maritimus*; **b** leaf protoplasts; **c** apple-green fluorescence of viable protoplasts after fluorescein diacetate (FDA) staining; **d–f** first mitotic divisions and multi-cell protoplast-derived aggregates in 10-, 20-, and 40-day-old cultures of *D. aureus*, respectively; **g** plasmolysis (arrow) of protoplast-derived cells in 40-day-old cultures of *D. pusillus*; **h** somatic embryos overgrowing alginate matrix; **i, j** protoplast-

derived cells of *D. carota* subsp. *sativus* cultured in extra thin alginate film (ETAF) and thin alginate layer (TAL), respectively; **k** protoplast-derived proembryonic masses and green somatic embryos in the cotyledonary stage, on filter paper placed on solid regeneration medium, 1 week after release from the alginate matrix; **l** protoplast-derived non-regenerating callus of *D. carota* subsp. *sativus*; **m** completely regenerated plant of *D. carota* subsp. *azoricus* (bars in **b, c, g** 50 μ m, **d–f** 100 μ m, **i, j** 1 cm)

250 mg l⁻¹ casein enzymatic hydrolysate (Sigma-Aldrich), 0.1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.2 mg ml⁻¹ zeatin (pH = 5.6, filter-sterilized). After purification, protoplasts were resuspended in 1 ml of culture medium and counted using a Fuchs-Rosenthal hemocytometer. The working density before embedding in alginate matrix was adjusted to 8×10^5 cells per ml.

Protoplast embedding and culture conditions

For protoplast culture, two embedding systems in alginate matrix were applied, i.e., TAL and ETAF techniques, as

described by Grzebelus et al. (2012a) and Pati et al. (2005), respectively, with some modifications. In both cases, a filter- or autoclave-sterilized (20 min at 121 °C, 0.1 MPa) alginate solution containing 2.8 % (w/v) Na-alginate (Sigma-Aldrich) and 0.4 M mannitol (pH = 5.8) was used. Before filtration through syringe disposable membrane filters (0.22 μ m, Millipore), Na-alginate was dissolved in mannitol solution by overnight stirring on a gyratory shaker (100 rpm). Before forming TALs or ETAFs, equal volumes of sterile alginate solution and protoplast suspension at working density were gently mixed, reducing the density of embedded protoplasts to a final value of

4×10^5 per ml. In both techniques, polymerization of the protoplast-alginate mixture took place in 6×1.5 cm Petri dishes on Ca-agar plates [1 % (w/v) agar (Biocorp), 20 mM CaCl_2 , 0.4 M mannitol]. TALs were obtained by spreading 300 μl of protoplast-alginate suspension on the surface of Ca-agar plates following gentle rotation of Petri dishes to minimize layer thickness, while to prepare ETAFs, 100 μl of protoplast-alginate suspension was placed on Ca-agar plates and immediately covered by a sterile coverglass (22×22 mm). After 1-h or 15-min incubation at room temperature, solidification of alginate matrix with embedded protoplasts occurred for TAL (Fig. 1j) and ETAF (Fig. 1i), respectively. Solid alginate sheets with immobilized protoplasts were gently transferred to 6×1.5 cm Petri dishes containing 4 ml of liquid culture medium. Two types of culture media were used: CPP medium and CPP supplemented with 100 nM PSK (phytosulfokine- α , PeptaNova GmbH, Germany). In order to maintain aseptic conditions of the cultures, 400 mg l^{-1} cefotaxime (Polfa Tarchomin SA, Poland) and 200 mg l^{-1} timentin (GlaxoSmithKline, Poland) were applied to each Petri dish. Cultures were incubated at 26 ± 2 °C in the dark. Protoplast medium with all supplements was renewed for the same one after 10 days of culture.

Plant regeneration

After 2 months of culture in liquid medium, both protoplast-derived tissue [callus tissue and proembryonic masses (PEM)] and somatic embryos overgrown or emerged from alginate matrix, respectively, were used in the regeneration process. The regeneration procedure was slightly different for the two protoplast embedding techniques. In the case of TALs, protoplast-derived material was freed from alginate gel by its depolymerization through exposure to a 20 mM sodium citrate solution with 0.2 M mannitol (pH = 5.8) for 1 h at room temperature (Grzebelus et al. 2012a). Then, the released tissue was washed twice by centrifugation at 100g for 5 min. After a second wash in CPPD medium containing 0.1 mg l^{-1} α -naphthaleneacetic acid (NAA), 0.2 mg l^{-1} zeatin, pH = 5.6 (Dirks et al. 1996), 1 ml aliquots of protoplast-derived tissue and somatic embryos were spread on filter paper placed on solidified R medium in glass jars. The procedure for regeneration from ETAFs was performed without the alginate depolymerization step. ETAFs were washed in CPPD medium and transferred to glass jars on R medium. During the regeneration process, cultures were maintained in a climate room at 26 °C with a 16-h photoperiod under a light intensity of 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Two weeks later, material from TAL-derived cultures was transferred to fresh R medium without filter paper and kept in the same environmental conditions. After 5 weeks of

culture on solid R medium, regeneration capacity in TAL and ETAF protoplast embedding systems was evaluated.

Data collection and statistical analysis

Protoplast yield was determined using a hemocytometer and presented as the protoplast number per gram fresh weigh (FW). The viability of protoplasts was evaluated just after immobilization of the cells in alginate matrix, by staining with fluorescein diacetate (FDA), as described by Grzebelus et al. (2012a), and expressed as a percentage of protoplasts with green fluorescence out of total observed cells. Plating efficiency, defined as the ability of single cells to form colonies through continuous mitotic divisions, was assessed on the 10th, 20th, and 40th days of culture and presented as the number of cell colonies per the total number of observed protoplasts ($\times 100$). All microscopic observations were performed under an inverted Axiovert S100 microscope (Carl Zeiss, Göttingen, Germany) equipped with the filter set appropriate for fluorescein visualization ($\lambda_{\text{Ex}} = 485$ nm, $\lambda_{\text{Em}} = 515$ nm). In order to describe regeneration efficiency after 5 weeks of culture on R medium, the number of normal and completely formed plants, plants with morphological abnormalities, and non-regenerating callus clumps were counted.

As repetitions, at least two to eight independent protoplast isolation experiments with a single treatment represented by three Petri dishes were carried out. Microscopic observations were performed in four or five microscopic fields on 100–200 cells per Petri dish. The overall effect of treatments was determined using multivariate analysis of variance (ANOVA) in Statistica ver. 9.0 (StatSoft, Inc. 2009). Duncan honestly significant difference test was used for mean separation.

Results

Protoplast yield and viability

Highly chloroplast-rich protoplasts (Fig. 1b) were successfully isolated from the leaves of 2-week-old shoot cultures in all accessions, reaching on average $2.8 \pm 0.3 \times 10^6$ protoplasts per g FW (Table 2). The mean yield of *D. carota* subspecies-derived protoplasts was over two times higher ($4.0 \pm 0.4 \times 10^6$) than that from wild *Daucus* species ($1.8 \pm 0.3 \times 10^6$, $P < 0.001$). The applied enzyme mixture led to the release of large and workable cell populations (i.e., $>10^6$) for all tested accessions. The efficiency of isolation varied from 1.4×10^6 (*D. montevicensis*) to 4.5×10^6 (*D. carota* subsp. *sativus* and *D. carota* subsp. *drepanensis*) (Table 2). The differences in protoplast yield

Table 2 Yield and viability of leaf-derived protoplasts within the *Daucus* genus

Accession	Protoplast yield ($\times 10^6$ /g FW)		Protoplast viability (%)	
	Mean \pm SE	n	Mean \pm SE	n
<i>D. carota</i> subsp. <i>sativus</i>	4.5 \pm 1.2 a	2	66.6 \pm 2.6 bc	4
<i>D. carota</i> subsp. <i>azoricus</i>	2.9 \pm 1.2 ab	2	92.6 \pm 3.2 a	2
<i>D. carota</i> subsp. <i>drepanensis</i>	4.5 \pm 0.6 a	3	73.9 \pm 6.6 ab	6
<i>D. carota</i> subsp. <i>maritimus</i>	4.0 \pm 0.4 a	4	75.8 \pm 9.6 ab	5
<i>D. aureus</i>	2.6 \pm 0.7 ab	3	63.0 \pm 0.3 bc	3
<i>D. montevidensis</i>	1.4 \pm 0.4 b	5	48.9 \pm 1.6 c	4
<i>D. pusillus</i>	1.7 \pm 0.3 b	5	72.2 \pm 5.7 ab	6
Mean/total	2.8 \pm 0.3	24	69.7 \pm 2.9	30

FW fresh weight, *n* number of independent protoplast isolations. Means within columns with the same letters were not significantly different at $P \leq 0.05$

observed between tested accessions were significant ($P < 0.01$).

Protoplasts stained with FDA (Fig. 1c) just after embedding in alginate matrix exhibited an acceptable level of viability, yielding on average 70 % (Table 2). A slightly higher proportion of viable cells ($P < 0.01$) was observed for protoplasts isolated from *D. carota* subspecies (74.9 \pm 3.9 %) in comparison with wild *Daucus* species (62.9 \pm 3.8 %). Different numbers of living cells were also recorded for individual accessions ($P < 0.05$). The highest viability, ranging from 72 to 93 %, was observed for *D. pusillus*, *D. carota* subsp. *drepanensis*, *D. carota* subsp. *maritimus*, and *D. carota* subsp. *azoricus*, while the lowest was observed for *D. montevidensis* (49 %), *D. aureus* (63 %) and, unexpectedly, for *D. carota* subsp. *sativus* (67 %).

Plating efficiency

Protoplasts of all accessions revealed to ability to undergo cell division. The first cell divisions (Fig. 1d) occurred between the 4th (*D. carota* subsp. *sativus* and *D. carota* subsp. *drepanensis*) and 8th days after protoplast plating (*D. carota* subsp. *azoricus*) (data not shown). In the subsequent days, continuous mitotic divisions took place and multi-cell aggregates were formed (Figs. 1e, f), reaching on average 32, 41, and 46 % in 10-, 20- and 40-day-old cultures, respectively. At subsequent points of evaluation, the proportion of colony formation highly depended on the accession, method of alginate sterilization, culture technique, and PSK application (Table 3, $P < 0.001$). In 10-, 20-, and 40-day-old cultures, mitotic division of protoplast-derived cells of *D. carota* subspecies was two- to three-fold more frequent in comparison with that of cells isolated

Table 3 Mean effect of accession, method of alginate sterilization, culture technique, and phytosulfokine (PSK) application on plating efficiency in protoplast cultures of different *Daucus* species

Treatment	Plating efficiency (% \pm SE)		
	Age of culture (days)		
	10	20	40
Accession			
<i>D. carota</i> subsp. <i>sativus</i>	50.4 \pm 3.6 a	61.5 \pm 3.2 a	72.4 \pm 2.5 a
<i>D. carota</i> subsp. <i>azoricus</i>	30.0 \pm 7.0 c	52.6 \pm 9.3 ab	54.0 \pm 9.5 c
<i>D. carota</i> subsp. <i>drepanensis</i>	44.5 \pm 4.8 ab	58.6 \pm 5.5 a	67.3 \pm 5.6 ab
<i>D. carota</i> subsp. <i>maritimus</i>	33.4 \pm 4.0 c	46.0 \pm 5.0 bc	55.1 \pm 5.2 bc
<i>D. aureus</i>	35.5 \pm 5.0 bc	40.5 \pm 5.2 c	46.2 \pm 7.2 c
<i>D. montevidensis</i>	16.4 \pm 2.1 d	14.8 \pm 2.5 d	13.7 \pm 2.9 d
<i>D. pusillus</i>	11.2 \pm 2.0 d	11.6 \pm 1.9 d	9.7 \pm 2.1 d
Alginate sterilization			
Autoclave	23.8 \pm 2.3 b	30.0 \pm 2.9 b	35.1 \pm 3.4 b
Filter	34.9 \pm 2.5 a	43.5 \pm 3.0 a	46.9 \pm 3.5 a
Culture technique			
ETAF	18.1 \pm 2.1 b	24.6 \pm 2.7 b	27.7 \pm 3.3 b
TAL	38.2 \pm 2.3 a	46.5 \pm 2.9 a	51.6 \pm 3.3 a
PSK concentration (nM)			
0	22.5 \pm 2.2 b	29.3 \pm 2.8 b	34.9 \pm 3.4 b
100	36.5 \pm 2.5 a	44.8 \pm 3.0 a	47.6 \pm 3.5 a

In each section of the table, means with the same letters did not differ significantly ($P \leq 0.001$) within the columns

TAL thin alginate layer; ETAF extra thin alginate film

from wild *Daucus* species ($P < 0.001$). In 40-day-old cultures, the highest numbers of cell aggregates were recorded for *D. carota* subsp. *sativus* and *D. carota* subsp. *drepanensis* (72 and 67 %, respectively), while the lowest numbers were recorded for *D. montevidensis* and *D. pusillus* (14 and 10 %, respectively). At all points of evaluation, on average, over 10 % more cell aggregates were developed after immobilization of protoplasts in a filter-sterilized than in an autoclave-sterilized alginate solution (Table 3). A positive influence of alginate filter-sterilization on plating efficiency was recorded for all tested accessions but significant differences between the two methods of sterilization were confirmed for *D. carota* subsp. *drepanensis*, *D. carota* subsp. *maritimus*, and *D. pusillus* (Fig. 2). The culture system with embedding of protoplasts in TALs better promoted division of protoplast-derived cells, resulting in a plating efficiency about 20 % higher in comparison with the ETAF system (Table 3). Again, such a tendency was observed for all accessions; however, for *D. aureus*, the differences were not

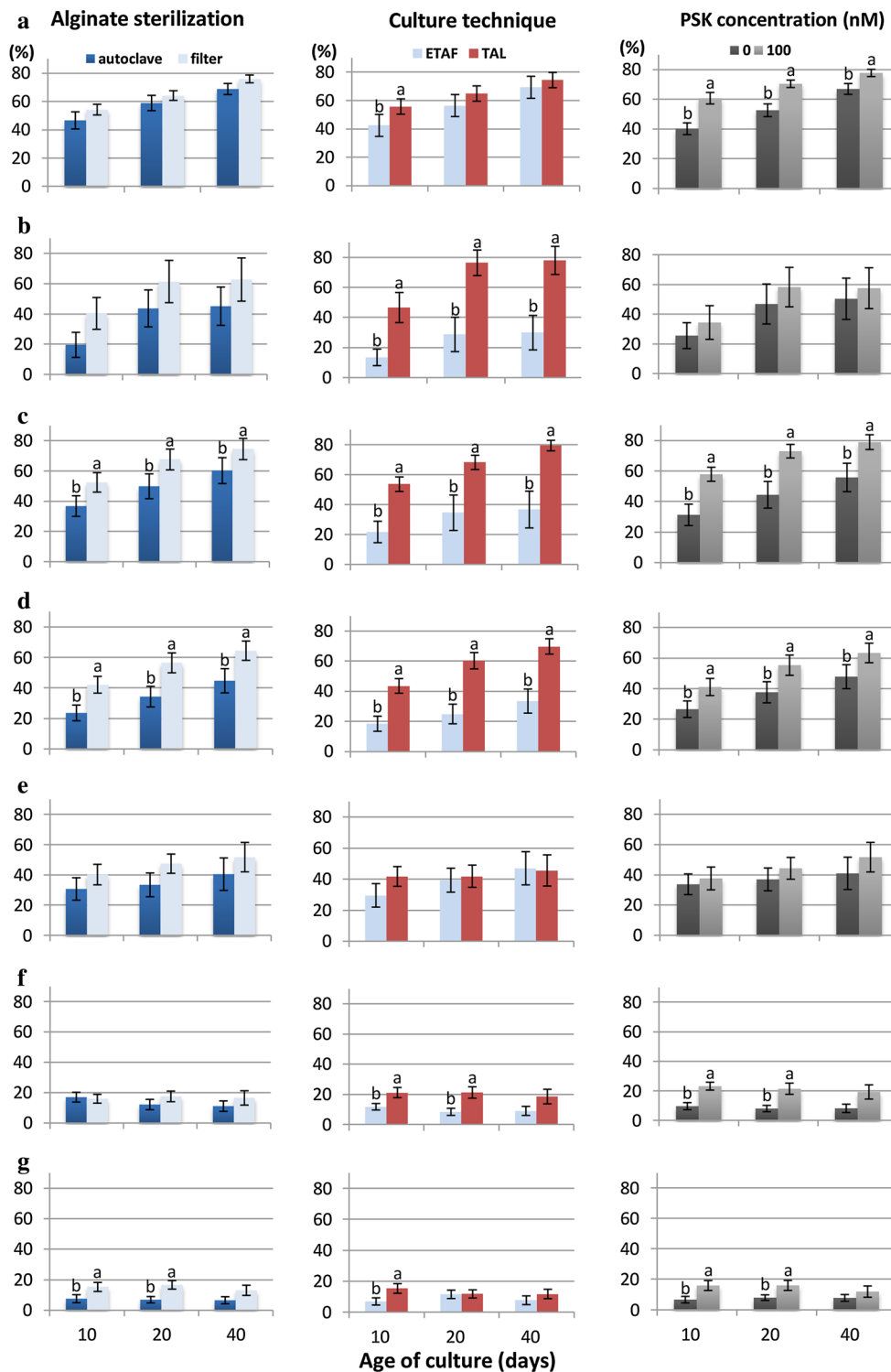


Fig. 2 Effect of alginate sterilization method (left column), culture technique (middle column), and phytosulfokine (PSK) application (right column) on plating efficiency in protoplast cultures of *Daucus carota* subsp. *sativus* (a), *D. carota* subsp. *azoricus* (b), *D. carota*

subsp. *drepanensis* (c), *D. carota* subsp. *maritimus* (d), *Daucus aureus* (e), *Daucus montevidensis* (f), and *Daucus pusillus* (g). Bars represent standard error. For subsequent points of evaluation, means denoted with different letters are significantly different ($P \leq 0.05$)

statistically significant (Fig. 2). Supplementation of protoplast culture medium with PSK at the concentration of 100 nM showed a beneficial effect on plating efficiency in

comparison with the control (Table 3). PSK application stimulated cell division in protoplast-derived cells in all analysed accessions and for most of them, excluding *D.*

Table 4 Effect of accession and different conditions during protoplast culture on regeneration capacity from 2-month-old protoplast-derived tissue within the *Daucus* genus

Treatment	No. (mean ± SE)		
	Normal plants	Abnormal plants	Clumps of non-regenerating callus
Accession			
<i>D. carota</i> subsp. <i>sativus</i>	20.5 ± 4.4 b	12.2 ± 2.5 b	45.4 ± 16.3 a
<i>D. carota</i> subsp. <i>azoricus</i>	9.2 ± 3.7 b	1.3 ± 0.6 b	9.7 ± 5.8 b
<i>D. carota</i> subsp. <i>drepanensis</i>	18.3 ± 4.2 b	30.7 ± 9 a	10.1 ± 3.1 b
<i>D. carota</i> subsp. <i>maritimus</i>	8.3 ± 2.4 b	0.5 ± 0.4 b	7.3 ± 2.8 b
<i>D. aureus</i>	73.2 ± 33.7 a	6.5 ± 1.9 b	17.6 ± 7.7b
<i>D. montevidensis</i>	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
<i>D. pusillus</i>	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
Alginate sterilization			
Autoclave	13.7 ± 4.3	4.5 ± 1.0	8.6 ± 3.8
Filter	17.1 ± 6.1	10.2 ± 3.3	13.9 ± 3.1
Culture technique			
ETAF	20.2 ± 8.3	2.0 ± 0.5 b	21.3 ± 5.3 a
TAL	11.7 ± 1.7	11.7 ± 3.0 a	3.5 ± 0.9 b
PSK concentrations (nM)			
0	6.2 ± 2.9 b	8.0 ± 3.0	6.6 ± 1.8
100	24.9 ± 6.8 a	6.8 ± 1.9	16.1 ± 4.6

In each section of the table, means with the same letters did not differ significantly ($P \leq 0.05$) within the columns
TAL thin alginate layer; *ETAF* extra thin alginate film

carota subsp. *azoricus* and *D. aureus*, the increase of plating efficiency was significant ($P \leq 0.05$, Fig. 2).

Plant regeneration

During 2 months of culture in liquid medium, some of the protoplast-derived cells showed plasmolysis symptoms such as contraction of the cytoplasm before first division or after several mitotic cycles (Fig. 1g), while others underwent sustained divisions, leading to micro- and macro-colony development following the formation of micro- and macro-calli of up to 1 mm in size. At that time, somatic embryos were also observed occasionally (Fig. 1h). After transfer of protoplast-derived tissues to solid regeneration medium, yellow-green and friable PEMs or green and gray compact calli were produced (Figs. 1k, l). From PEM somatic embryos developed, which usually regenerated into morphologically normal and rooted plants (Fig. 1m). However, some disorders in development pattern were recorded. Compact callus tissue failed to differentiate into plants.

For five out of seven accessions, normal plants, abnormal plants, and non-regenerating calli were all obtained (Table 4). The number of normal plants depended highly on the accession ($P = 0.001$) and varied on average from 8 (*D. carota* subsp. *maritimus*) to 73 (*D. aureus*, Table 4). Accessions differed also in the production of abnormal plants ($P = 0.01$)—the highest number of such plants (31) developed from protoplast-derived tissue of *D. carota*

subsp. *drepanensis*. Compact and non-regenerating callus tissue most frequently formed in cultures of *D. carota* subsp. *sativus* (45), while for the other four accessions, the number of such callus clumps was lower, reaching from 7 (*D. carota* subsp. *maritimus*) to 18 (*D. aureus*). In *D. montevidensis* and *D. pusillus*, protoplast cultures led to the formation of micro-calli of up to 140 µm in size after several cycles of cell division; than mitoses were arrested and further development was not observed. The method of alginate sterilization had no influence on the regeneration capacity of protoplast-derived tissue. The two applied systems of protoplast embedding in alginate matrix (i.e., TAL and ETAF) affected the growth of abnormal plants and compact calli ($P < 0.01$, Table 4). Less deformed plants and callus clumps were obtained from tissues produced in ETAFs and in TALs, respectively. On the other hand, supplementation of protoplast cultures with PSK increased the yield of regenerated plants four-fold in comparison with the control experiments ($P = 0.01$).

Discussion

Since genetically modified organisms are not fully accepted by the public, there is an interest in exploiting protoplast-to-plant technologies in the production of novel germplasm for plant breeding (Eeckhaut et al. 2013). One of the most common protoplast-based approaches uses protoplast fusion to generate unique plants in commercial

crops with desirable agronomically relevant traits such as resistance to pathogens, stress tolerance, or CMS (Matsumoto 1991; Akagi et al. 1995; Kisaka and Kameya 1998). Somatic hybridization through protoplast fusion overcomes pre- and post-zygotic barriers among incompatible species, allowing plants with novel genetic combinations within the species, between different species of the same genus, or even between different genera to be obtained (Davey et al. 2005a). However, the application of somatic hybridization in practical breeding processes requires the establishment of a protoplast-to-plant system for the fusion parents. Such studies are reasonable, since it is known that the regenerative capacity of different genotypes varies significantly even for closely related species or individual lines within the same species (e.g., Davey et al. 2005c). To our knowledge, the present study is the first attempt to assess the regeneration ability of leaf protoplasts isolated from wild *Daucus* species and *D. carota* subspecies other than cultivated carrot (*D. carota* subsp. *sativus*).

The availability of a large number of protoplasts with high viability is the first step for successful protoplast culture. The efficiency of protoplast isolation and their quality, expressed by cell viability, are governed by several factors including: genotype; type of source tissue; mechanical procedures on source tissue (slicing or removal of the epidermis); conditioning of source tissue before enzyme maceration (dark or osmotic treatment); conditions of tissue digestion (i.e., composition of enzyme mixture, temperature and duration of enzyme incubation, pH of the enzyme solution, gentle agitation), and the method of protoplast isolation (Frearson et al. 1973; Rao and Prakash 1995; Ortin-Parraga and Burgos 2003; Sinha et al. 2003; Davey et al. 2005c; Kielkowska and Adamus 2012). When large populations of protoplasts are required, which is the norm for fusions, from 1 g of FW, between 10^5 and 10^7 viable cells should be released (Davey et al. 2010). Though the protoplast yield obtained in the present study, varying from 1.4 to 4.5×10^6 , was genotype-dependent, for all accessions it was even high enough to perform protoplast fusion. Additionally, these results showed that the protocol of protoplast isolation based on slicing and plasmolysis of source tissue, overnight digestion with gentle shaking, and gradient centrifugation for protoplast purification, elaborated for protoplast isolation from leaves and hypocotyls of cultivated carrot (Grzebelus et al. 2012a), may be also applied to their wild relatives. The induction and maintenance of shoot cultures for wild *Daucus* are a long and laborious process; nevertheless, the isolation of protoplasts from short-term hypocotyl cultures seems to be impossible, due to the limited number of available seeds. The viability of protoplasts released from wild accessions was satisfactory and not significantly lower than that of cultivated carrot, but

varied greatly, ranging from 50 to 93 %. Slightly differences in physiological status of source tissue or seasonal variation may explain the different numbers of viable protoplasts observed in analyzed accessions, since shoot cultures were maintained over the year. Keskitalo (2001) reported that protoplast isolation from cultured shoots of *Tanacetum* was most successful during winter and spring, suggesting the persistence of the seasonal clock in vitro. We observed a different dependence, with higher values of isolation efficiency during spring and summer and lower values at autumn and winter time (data not shown). Moreover, protoplast isolation from shoot cultures even 1 day older than 2 weeks resulted in reductions in both their yield and viability.

Synthesis of the cell wall, cell cycle re-entry, and sustained mitotic divisions are key steps in the production of daughter cells and further plant regeneration from protoplast-derived tissues (Eeckhaut et al. 2013). Improvements in protoplast-to-plant systems have been achieved by testing different culture techniques and medium formulations with various supplements (Anthony et al. 1997; Matsubayashi et al. 1997; Oh and Clouse 1998; Davey et al. 2005a). Since over 40 years ago, when fertile plants from protoplast-derived cells of tobacco were regenerated for the first time (Nagata and Takebe 1971), many systems of protoplast culture have been verified to improve regeneration ability from a wide range of plant species (reviewed by Davey et al. 2005a, b, c; Eeckhaut et al. 2013). Generally, a higher rate of cell colony formation was noted in the case of protoplasts embedded before culture, especially in alginate matrix, as compared to non-embedded protoplasts. Such a positive effect of embedding on cells may be explained by membrane stabilization through lipid peroxidase inhibition, the prevention of leakage of cell wall precursors or other metabolites, lower ethylene level (Bajaj 1989), or improved signaling cascade before the first cytokinesis (Sheahan et al. 2007; Zaban et al. 2013). Moreover, it was documented that protoplast separation ensured a better oxygen supply, resulting in avoidance of browning (Majewska-Sawka et al. 1994; Pati et al. 2005; Eeckhaut et al. 2013). The beneficial effect of protoplast immobilization in alginate matrix on plating efficiency was recorded for, among others, soybean (Tricoli et al. 1986), sweet orange (Niedz 1993), *N. tabacum* (Dovzhenko et al. 1998), and *Arabidopsis thaliana* (Dovzhenko et al. 2003). According to very early studies on some properties of alginate solutions and gels, the method of sterilization, a necessary step when working with in vitro cultures, may influence polymerization of the alginate molecules (Leo et al. 1990). The commonly used method of sterilization of heat and high pressure treatment (autoclaving) can modify such properties of the gel as mechanical strength, abrasion resistance, diffusion within the gel, and development of

entrapped biomass. It seems that only few authors working with alginate-embedded protoplasts took this fact into consideration, applying filter-sterilization (Draget et al. 1988; Hall et al. 1997; Dovzhenko et al. 1998; Dovzhenko et al. 2003). Very often, the method of alginate solution sterilization is not given in the papers. Moreover, it is difficult to find numerical data confirming the beneficial effect of filter-sterilized versus autoclave-sterilized alginate on protoplast development. Nevertheless, several authors suggested the positive role of filter-sterilized alginate on plating efficiency, e.g., for *Brassica napus* (Draget et al. 1988), *B. vulgaris* (Hall et al. 1997), or *N. tabacum* (Dovzhenko et al. 1998). Although filter sterilization of alginate solution is quite a difficult and long process (preceded by overnight stirring on a gyratory shaker to dissolve), the values noted for plating efficiency in the present study confirmed its stimulatory effect on mitotic activity of protoplast-derived cells. Additionally, due to better clarity of filter-sterilized alginate, as compared to autoclave-sterilized, microscopic observations were made easily and precisely.

Immobilization of protoplasts in gel matrices not only enhances plating efficiency but is sometimes crucial for mitotic activity by the protoplast-derived cells (Ebrahimzadeh et al. 2001; Grzebelus et al. 2012b). A general conclusion was proposed based on data from many reports, suggesting that the thinner the matrix, the higher the frequency of colony formation, probably as a consequence of increased compound diffusion from the medium to the protoplasts (Dovzhenko et al. 1998, 2003; Pati et al. 2005; Eeckhaut et al. 2013). Reduction of the alginate gel thickness from droplet to layer was achieved originally by inserting, before polymerization, a polypropylene grid or coverglass in the TAL or ETAF system, respectively (Golds et al. 1992; Pati et al. 2005). In the present study, we used a modified TAL system, described in previous reports (Grzebelus et al. 2012a, b), based on: (1) application of a reduced volume of protoplast-alginate mixture to form the alginate layer (300 μ l instead the 625 μ l proposed in earlier studies); (2) excluding polypropylene mesh grid from the system, as it is difficult to obtain; and (3) minimizing layer thickness by rotation of protoplast-alginate mixture during application. In the ETAF technique, in comparison with the original protocol (Pati et al. 2005), we modified the polymerization step—this was performed on Ca-agar plates, as in the TAL technique, instead of on microscope slides. The proposed modifications for both TAL and ETAF simplify the systems, reducing handling and costs. The values of plating efficiency observed in *N. tabacum* and *Lotus corniculatus* protoplast cultures indicated that ETAF was on a par with the TAL technique (Pati et al. 2005). In all *Daucus* accessions evaluated in the present study, mitotic activity of protoplast-derived cells was observed both in TAL and

ETAF cultures; however, contrary to expectation, using ETAF led to an approximately two-fold decrease of plating efficiency at all points of evaluation. It could be due to fact that the ETAFs, in comparison with TALs, were produced from a three-fold smaller volume of protoplast-alginate mixture (100 vs. 300 μ l, respectively) and were represented by one-cell layers. Although in both systems the density of embedded protoplasts was the same, the cross-stimulating effect of dividing adjacent cells was higher in the multi-cell layers obtained in the TAL technique.

Lately, PSK has been recognized as a chemical nursing agent that drastically promotes cell divisions in rice and sugar beet protoplast cultures (Matsubayashi et al. 1997; Grzebelus et al. 2012b). Similarly, protoplast cultures of all seven *Daucus* accessions analyzed in this study responded positively to PSK supplementation at the concentration of 100 nM, leading to, on average, an above 10 % higher level of plating efficiency in comparison with PSK-free control. However, for two wild *Daucus* species, stimulation of cell activity was observed only in early cultures; it gradually stopped and, as a result, calli were not formed. This may suggest that the applied concentration of PSK was too low for these two accessions to promote sustained division of the protoplast-derived cells, while for the remaining *Daucus* species, it was sufficient to stimulate a higher level of somatic embryogenesis, since four-fold fewer plants were regenerated from PSK-free cultures. Similar promotion of somatic embryogenesis as a result of PSK supplementation was observed in suspension cultures of plants as diverse as carrot (dicotyledons) and *Cryptomeria japonica* (conifers) (Hanai et al. 2000; Igasaki et al. 2003).

The differential response of wild relatives in comparison with cultivated crop to the same culture conditions is a known phenomenon. In protoplast cultures, both plating efficiency and plant regeneration may be genotype-dependent. In an early study on mesophyll protoplasts of *A. thaliana*, Damm and Willmitzer (1988) noticed between 10- and 20-fold differences in the level of plating efficiency among the analyzed races. Similarly, differences in the ability to form colony and/or plant regeneration between wild and cultivated representatives of the same genus were observed in protoplast cultures of tomato (Mühlbach 1980), *Helianthus* (Bohorova et al. 1986) and, lately, *Cichorium* (Deryckere et al. 2012). In the present study, all wild *D. carota* subspecies showed high potential both for colony formation and plant regeneration, while two out of three wild *Daucus* species exhibited recalcitrant status, since mitotic divisions of protoplast-derived cells were arrested in early cultures. The factors preventing protoplasts from expressing totipotency may: (1) be related to the oxidative stress generated during protoplast isolation and subsequent culture;

and/or (2) result from a lack of signaling molecules synthesized in plants and involved in the developmental process. The oxidative stress induced during the isolation procedure may be long-term and individual species may respond to it differently, among others, producing and cumulating peroxides and other active oxygen species (Watanabe et al. 2002; Davey et al. 2005c). It was postulated that suppressed expression of totipotency in protoplasts was correlated with reduced activity of the cellular antioxidant machinery (Papadakis et al. 2001). Protection from oxidative damage and, in consequence, overcoming protoplast recalcitrance may be possibly achieved by application of an exogenous antioxidant such as ascorbic acid, glutathione, tocopherol, polyamines, or n-propyl gallate.

In conclusion, to our knowledge, this is the first report to show successful plant regeneration from leaf protoplast cultures of wild representatives within the *Daucus* genus. We demonstrated that high efficiency of cell colony formation and plant regeneration could be induced after protoplast embedding in a modified thin layer of filter-sterilized alginate and supplementation of culture medium with PSK. The protocol can be easily applied in rarely exploited protoplast fusion between cultivated carrot and wild *Daucus* species.

Acknowledgments The authors thank Prof. P. W. Simon, Prof. D. Grzebelus, Prof. R. Baranski, Dr. C. Allender, and Dr. T. Nothnagel for providing seeds. This work was supported by the Polish National Science Centre, Grant No. NN310 440 238, awarded to E. G.

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