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ORIGINAL ARTICLE

Regeneration of cell suspension derived *Apium graveolens* L. protoplasts

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Abstract Cytoplasmatic male sterility (CMS), which can be achieved by protoplast fusion and regeneration, has potential to greatly facilitate hybrid breeding of celery (*Apium graveolens* L.). Therefore as a first step we developed a simple and efficient protoplast isolation and regeneration protocol for three commercial *A. graveolens* varieties (green and white celery and celeriac). To this end, cell suspensions from independent cell lines of open pollinated cultivars and inbred lines were initiated as a source for protoplast isolation. Comparative analyses showed that culturing was most successful in modified Kao and Michayluk liquid medium supplemented with 0.3 mg l^{-1} 2,4-D. The cytokinin type (TDZ or zeatin) and concentration had no significant effect on regeneration efficiency. Microcalli were obtained within 15 days to 5 weeks after protoplast isolation. Supplementing the culture medium with 25% conditioned medium increased microcolony formation for some of the cultured lines. Plants were obtained within 2 months of microcallus culturing and these were all diploid, suggesting genetic inheritance consistency. The efficiency of regeneration mainly depended on the specific genotype, with outcrossing genotypes displaying high heterogeneity in regeneration responses whereas inbred lines did not regenerate. The protocol presented here enables to implement protoplast fusion in celery breeding.

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Abbreviations

CPP	Carrot petiole protoplast
2,4-D	2,4-Dichlorophenoxyacetic acid
DSS	Dextran sodium sulfate
MES	2-(N-morpholino)ethanesulfonic acid
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
PPFD	Photosynthetic photon flux density
TDZ	Thidiazuron

Introduction

Celery, (*Apium graveolens* L., 2n = 2x = 22) comprises three botanical varieties that are commercially exploited: var. *dulce* (white and green celery), var. *secalinum* (green celery) and var. *rapaceum* (celeriac). Celery cultivars are mainly the result of open-pollination (Rubatzky et al. 1999). Nevertheless, celery hybrid seeds are commercially available, although quantitative analysis of heterosis effects have hitherto not been reported. In general, the increase in yield and performance of F1 hybrids can vary depending on the crop species between 15%, as observed in maize, and 50% as observed in sunflower (Duvick 1999). The use of male sterile lines facilitates hybrid seed production. When the nuclear type of male sterility is implemented, the maternal lines are maintained by vegetative propagation which is costly. The cytoplasmic male sterility (CMS) system on the other hand, available in crops like sugar beet, carrot, maize, sunflower and rice (Kempe and Gils 2011), has the advantage that it is maternally inherited omitting requirement of vegetative propagation.

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CMS can be introduced into a crop via crossing with related plant species harbouring the CMS trait. Unfortunately, due to different types of reproduction barriers, crossing is not always successful and specialized techniques such as embryo rescue are required (de Nettancourt 2001). An alternative to crossing is to generate hybrid plants via asymmetric protoplast fusion (Waara and Glimelius 1995) followed by regeneration. Protoplast fusion is a promising approach for celery as it is highly responsive to in vitro cultivation and exhibits somatic embryogenesis (Chen 1976) and plant regeneration under suitable conditions (Williams and Collin 1976). In addition, celery cell suspension cultures are successfully used for the induction of somatic embryos (Alabta and Collin 1978; Dunstan et al. 1982; Nadel et al. 1989).

So far, celery protoplasts have been isolated from cell suspensions (Lynch et al. 1989) and from mesophyll (Etxeberria et al. 2007). Protoplasts from embryogenic cell suspension and embryogenic callus from the *dulce* -variety were regenerated (Han et al. 2007; Tan et al. 2009; Wan et al. 1988). Wang et al. (1989) describes the isolation of somatic hybrids obtained from fused protoplasts derived from carrot and mesophyll celery protoplasts. However, to our knowledge no further reports appeared regarding the viability and fertility of these hybrids. These earlier successes have prompted us to further develop methodology for introducing CMS into celery.

Carrot (*Daucus carrota* L. 2n = 2x = 18), is traditionally used as a resource for protoplast isolation and regeneration studies (Roest and Gilissen 1989). Protoplasts of carrot have been successfully regenerated from both cell suspensions (Grambow et al. 1972) and green tissue (Grzebelus et al. 2012, 2013). Celery protoplast regeneration from green tissue (leaf or petiole) has however to our knowledge not been reported.

Cell suspensions are generally considered an ideal starting material for protoplast regeneration purposes (Wang et al. 1993). Since celery is highly amenable for tissue culture from mesophyll cells, initiation of cell suspension cultures proved to be not very difficult (Alabta and Collin 1978; Dunstan et al. 1982).

Preliminary experiments in our laboratory revealed that celery mesophyll and callus derived protoplasts divide poorly and did not regenerate. We therefore decided to test cell suspensions cultures as a source for protoplast regeneration. In this paper we present a method to regenerate celery from protoplasts starting from cell suspension cultures and report plant regeneration in 3 commercially relevant celery varieties.

Materials and methods

Plant material

Open pollinated *Apium graveolens* 'Daybreak' (white celery), 'Claret' (green celery) and 'Diamant' (celeriac) and inbred lines 'WL253' (white celery), 'VL391' (green celery) and 'L476' (celeriac) were cultivated *in vitro* from seed. Seeds were surface sterilized by immersion in 70% ethanol and 0.05% dextran sodium sulfate (DSS) for 5 min, rinsing in 95% ethanol for 5 and 20 min immersion in 5% NaOCl containing 2 drops of Tween 20, subsequently. Finally they were rinsed 3 times with sterile distilled water. The seeds were sown on 9 cm round Petri dishes containing solid Murashige and Skoog (MS) medium with vitamins (Murashige and Skoog 1962) supplemented with 20 g l^{-1} sucrose, 25 mg l^{-1} NaFeEDTA, 2 mg l^{-1} glycine and 6 g l^{-1} plant tissue culture agar (Duchefa). The pH of all culture media was adjusted to 5.8 prior to autoclaving unless stated otherwise. The plates were incubated at 22 ± 2 °C, with 16 h photoperiod (PPFD 40 µmol m^{-2} s^{-1}), to induce seed germination. Fifteen days old seedlings were transferred to glass jars containing the same solid medium.

Callus induction and cell suspension initiation

Leaf and petiole explants were cultured on MS medium supplemented with 30 g l^{-1} sucrose, 0.5 g l^{-1} enzymatic casein hydrolysate (Duchefa), 0.5 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 0.5 mg l^{-1} kinetin and 7 g l^{-1} agar. The cultures were maintained at 22 ± 2 °C in the dark and friable callus was selected and refreshed monthly.

The cell suspension cultures were initiated by culturing 250 mg of friable celery callus in 6 multi-well plates (CytoOne, Starlab) containing 5 ml/well of liquid suspension medium based on MS medium supplemented with 30 g l^{-1} sucrose, 0.5 g l^{-1} enzymatic casein hydrolysate, 0.6 mg l^{-1} 2,4-D and 0.55 mg l^{-1} kinetin. The cultures were incubated at 22 ± 2 °C in the dark with continuous agitation (100 rpm). One week after the initiation the callus was removed from the medium by sieving through a 100 µm sterile sieve. The cultures were refreshed weekly by substituting the medium with fresh liquid culture medium while maintaining a constant culture volume. Once the suspension stabilized and actively divided, the cultures were transferred to glass jars and subcultured weekly by diluting the cultures 1:1 with fresh liquid culture medium. The cultures were weekly examined using a Leica DMi8 inverted-microscope for detection of division status and possible contaminations.

Protoplast isolation and purification

Protoplasts were isolated from 5 to 9 week old suspension cultures at the 4th day after subculturing using the protocol of Grzebelus et al. (2012), with some modifications. About 1 g fresh weight of suspension cells was incubated in 10 ml enzyme solution that contained 0.5% (w/v) cellulase Onozuka R-10 (Duchefa), 0.05% (w/v) pectolyase Y-23 (Duchefa), 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES, Duchefa), 5 mM $CaCl_2$, and 0.6 M mannitol (Duchefa), and was filter-sterilized (0.2 µm, Whatman). The digestion was performed overnight at 22 °C by gently shaking (30 rpm) in the dark. After digestion, the protoplasts were subsequently sieved through 100 µm (Falcon) and 40 µm (SPL Life Sciences) nylon sieves, washed with 15 ml of W5 medium (Menczel et al. 1981) and centrifuged at 100 g for 5 min. The protoplasts in the pellet were resuspended in 10 ml of 0.6 M sucrose on top of which 1 ml W5 medium was overlaid. The samples were centrifuged at 80 g for 10 min, and the viable protoplasts localized in the interphase between the two solutions were collected and subsequently washed in 10 ml W5 medium and 10 ml culture medium and centrifuged at 100 g for 5 and 10 min. The purified protoplasts were counted, using a Bürker haemocytometer chamber and were diluted to a working concentration of 10^5 protoplasts per ml for all experiments.

Conditioned medium preparation

Four days after the weekly dilution, when cell suspensions selected for protoplast isolation had reached their exponential phase, they were centrifuged at 200 g for 10 min. The pellet was then used for protoplasts isolation as described above. The supernatant was filter-sterilized (0.2 µm, Whatman) to remove any traces of plant cell left in the medium and was subsequently used as conditioned medium for protoplast culturing. For each distinct protoplast line we used the conditioned medium corresponding to its cell suspension prior to protoplast isolation.

Protoplast culture and regeneration

Different protoplast culture set-ups were tested in 4 experiments. In all experiments the protoplast culture medium was based on CPP (carrot petiole protoplast) medium (Dirks et al. 1996) and consisted of macro-, micro-elements and organic acids according to Kao and Michayluk (1975), vitamins according to Gamborg et al. (1968), 74 g l^{-1} glucose and 250 mg l^{-1} enzymatic casein hydrolysate, (pH 5.6). For each experiment we started with different unique and independent cell lines. Each cell line had a unique code starting with the number of the experiment.

Experiment 1 was set up to test the culture conditions for celery protoplasts. Cell suspension derived 'Claret' protoplasts of cell line 1.1 were cultured in 12-multi-well plates (SPL Life Sciences) in volumes of 600 µl in CPP medium and CPP supplemented with 25% conditioned medium. The culture medium was supplemented with 0.03–1 mg l^{-1} 2,4-D and 0.06–2 mg l^{-1} zeatin or 0.012–0.4 mg l^{-1} TDZ (thidiazuron). In total 32 different hormone additions (Table 1) were tested in combination with either CPP or conditioned CPP.

Experiment 2 assessed the effect of the initial conditioning of the medium with 25% suspension medium in combination with two cytokinin treatments. Four independent cell suspension lines of 'Claret' (2.1, 2.2, 2.3 and 2.4) and 'Daybreak' (2.1, 2.2, 2.3 and 2.4) were subjected to 4 culture treatments. The protoplasts at the working densities were cultured in 12 multi-well plates in volumes of 600 µl in CPP medium or CPP supplemented with 25% conditioned medium from the suspension. The culture medium was supplemented with 0.3 mg l^{-1} 2,4-D and 0.2 mg l^{-1} zeatin or 0.012 mg l^{-1} TDZ.

Experiment 3 compared the early regeneration and the protoplast-to-plant regeneration potential of different celery cultivars and lines. Three independent suspension lines of 'WL253' (3.1, 3.2 and 3.3), 3 of 'Diamant' (3.1, 3.2 and 3.3) and 4 of 'Daybreak' (3.1, 3.2, 3.3 and 3.4) were used. Protoplasts at working density were cultured in 1 ml volume in 35 mm petri plates (Greiner bio-one). The culture medium consisted of CPP supplemented with 25% conditioned medium and 0.3 mg l^{-1} 2,4-D and 0.012 mg l^{-1} TDZ.

Experiment 4 evaluated the protoplasts-to-plant regeneration capacity of different celery cultivars and lines. Protoplasts of 4 independent cell suspension lines of 'WL253' (4.1, 4.2, 4.3 and 4.4), 'Daybreak' (4.1, 4.2, 4.3 and 4.4), 'VL391' (4.1, 4.2, 4.3 and 4.4), 'Claret' (4.1, 4.2, 4.3 and 4.4), 'L476' (4.1, 4.2, 4.3 and 4.4) and 'Diamant' (4.1, 4.2, 4.3 and 4.4). The purified protoplasts at working densities were cultured in CPP medium supplemented with 0.3 mg l^{-1} 2,4-D and 0.012 mg l^{-1} TDZ.

In all experiments protoplast cultures were incubated in the dark at 22 ± 2 °C. A volume of 25% of the initial culture volume of fresh CPP medium, supplemented with the hormones corresponding to each experiment, was added at day 7 and 14. Experiment 2 was terminated after the data collection at day 15. In experiments 1, 3 and 4, the glucose concentration was reduced to 0.2 and 0.13 M at day 21 and day 28 respectively. In experiment 1, microcalli that could be picked with tweezers were plated on solid CPP medium supplemented with 23 g l^{-1} glucose, 0.1 mg l^{-1} NAA (1-naphthaleneacetic acid) and 0.2 mg l^{-1} zeatin, 10 weeks

Table 1 Claret' cell suspension derived protoplast culture of line 1.1 (experiment 1): number of cultured calli and regenerated shoots after 17 weeks

Medium	Cytokinin (mg l^{-1})		# calli cultured (# regenerated shoots)			
			2,4-D (mg l^{-1})			
			0.03	0.1	0.3	1
CPP medium	Zeatin	0.06	0(–)	23(0)	22(0)	11(0)
		0.2	0(–)	38(0)	27(1)	33(0)
		0.6	0(–)	24(0)	13(1)	0(–)
		2	0(–)	4(0)	45(4)	0(–)
	TDZ	0.012	0(–)	30(0)	15(0)	0(–)
		0.04	0(–)	0(–)	36(0)	0(–)
		0.12	0(–)	0(–)	0(–)	0(–)
		0.4	0(–)	0(–)	15(0)	0(–)
CPP + 25% conditioned medium	Zeatin	0.06	0(–)	13(0)	0(–)	0(–)
		0.2	33(0)	29(0)	5(0)	0(–)
		0.6	0(–)	8(0)	10(0)	0(–)
		2	0(–)	0(–)	0(0)	0(–)
	TDZ	0.012	31(0)	3(0)	8(0)	0(–)
		0.04	0(–)	16(1)	9(0)	0(–)
		0.12	0(–)	0(–)	0(–)	0(–)
		0.4	0(–)	12(0)	4(0)	0(–)

after protoplast isolation. In experiments 3 and 4, the auxin 2,4-D was replaced with 0.1 mg l^{-1} NAA, 5 weeks after protoplast isolation. All microcalli produced during experiments 3 and 4 were plated 7 and 8 weeks after protoplast isolation respectively, on sterile meshes with 50 µm pore size, on solid CPP medium, supplemented with 23 g l^{-1} glucose and 0.1 mg l^{-1} NAA while the cytokinin was maintained as in the initial culture.

The shoots formed in experiment 1, 3 and 4 were transferred, 2–4 months after protoplast isolation, in glass jars containing the same MS hormone free medium used for seed germination. The ploidy level of the regenerated plants was determined by flow cytometry. The samples and internal controls were prepared according to Galbraith et al. (1983) and Otto (1990). The 2C-values of the stained nuclei were determined with a Partec Cyflow Space equipped with a green solid state laser (100 mW, 532 nm) (Partec, Münster, Germany). The ploidy level of the samples was established comparing the ratio of the 2C peak of regenerated celery plants to internal control (*Zea mays* 2C = 5.67 pg) with the ratio of the 2C peak of diploid celery plants to internal control (Dolezel et al. 1998; Lysak and Dolezel 1998).

Data collection

All cultures were examined weekly using a Leica DMi8 inverted-microscope. Pictures were taken using a Leica DFC450C camera and the LAS V3.8 software. In experiment 2 and experiment 3 the regeneration frequencies were determined, 15 days after protoplasts isolation, by counting the number of two-, four- and multi-cell colonies. The parameters used for assessing the variance between treatments were the microcolony frequencies, defined as the number of multi-cell colonies, and the frequencies of total response, defined as the sum of the two-, four- and multi-cell colonies.

The treatments were set up in four replicates. Per Petri dish 100–300 cells were counted for determining the microcolonies and total response frequencies. The mean values and standard errors were calculated. Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests were performed using Statistica V12.7 software in order to assess the effect of the treatments.

Results

Cell suspension initiation

Callus derived from leaf blades and petioles was cultured in liquid medium. Cells started dividing during the first week and cultures reached the stationary phase after 4 weeks. From then on they were subcultured every week (Fig. 1b). The cultures typically produced cell clumps of up to 2–3 mm in size and contained very few single cells (Fig. 1a). After 4 subculture cycles, the suspensions were stabilized and suitable for protoplast isolation. The cell suspension of cell line 4.2 from inbred line 'WL253' generated only single cells that did not engage in cell division. This

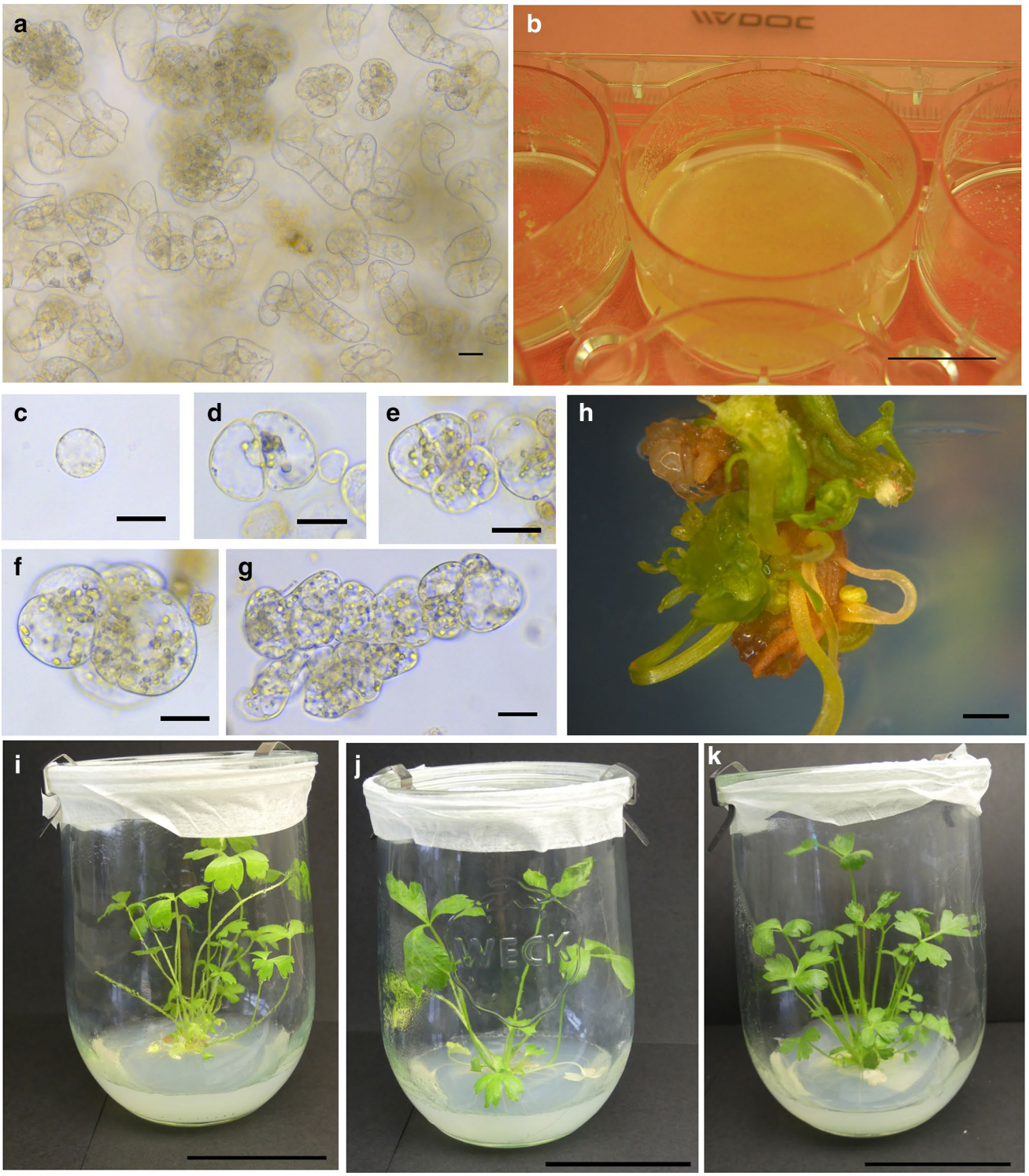

Fig. 1 Plant regeneration from cell suspension derived protoplasts of 'Claret' cell line 1.1, 'Diamant' cell line 3.1 and 'Daybreak' cell line 4.2. 'Claret' cell suspension before protoplasts isolation **a** microscopic, **b** macroscopic view; **c** freshly isolated protoplast; **d** first division; **e** second division; **f** microcolony 14 days after isolation; **g** microcolony before transferring on solid medium; **h** shoot 17 weeks after isolation; **i** 'Daybreak' plant 5 months after protoplast isolation; **j** 'Claret' plant 17 months after protoplast isolation; **k** 'Diamant' plant 9 months after protoplast isolation (*bars* in **a, c**–**g** 30 µm; **b** 2 cm; **h** 2 mm; **i**–**k** 5 cm)

culture did not survive subculturing. The suspension culture of cell line 4.4 of the inbred line 'L476' and cell line 4.4 of cultivar 'Diamant' were not suitable for protoplast isolation because they produced large compact clumps that did not yield enough protoplasts.

Protoplast regeneration

Experiment 1

During this experiment we screened several culture parameters in order to build a protocol for protoplast culture that leads to plant regeneration. Protoplasts derived from 'Claret' suspension culture 1.1 were cultured in liquid CPP medium in which they became enlarged after 3 days. After 5 days of culture, cells started to divide in most tested conditions (Fig. 1c–e). At day 10, secondary divisions were observed, and after 3 weeks, microcolonies were formed (Fig. 1f–g). Five weeks after protoplast culture initiation, microcalli had formed. Microcallus formation was dependent on the type of cytokinin. Zeatin was the most potent stimulator of callus growth with 7 out of 16 protoplast cultures forming microcalli whereas TDZ induced microcalli in 3 out of 16 cultures. The inclusion of filtered cell suspension medium enhanced microcallus formation and was most effective when TDZ was used. After 10 weeks of cultivation, microcalli were transferred to shoot induction medium and 2 weeks later the first shoots appeared. The exact number of independent shoots formed for each condition could not be estimated because calli fell apart in liquid medium. Shoots formed from the calli derived from the protoplasts cultured in CPP supplemented with 0.3 mg l^{-1} 2,4-D and 0.2 mg l^{-1} or 2 mg l^{-1} zeatin and CPP supplemented with conditioned medium and 0.1 mg l^{-1} 2,4-D and 0.04 mg l^{-1} TDZ (Table 1; Fig. 1f). Shoots that were transferred to hormone free medium developed into plants (Fig. 1j).

Experiment 2

In this experiment we compared the impact of the cytokinin type and the addition of conditioned medium on the regeneration of protoplasts derived from cell suspensions of cultivars 'Claret' and 'Daybreak'.

The regeneration frequencies varied significantly between different cell lines in both cultivars (Table 2). Microcolony formation varied between 2.3 and 4.8% for 'Claret', while this was 2.1–3.5% for 'Daybreak' (Table 3). For 'Claret', the total response frequency was on average between 15.5 and 19.5% and for 'Daybreak' between 10.7 and 14.9% (Table 3).

The cytokinin type did not influence the number of microcolonies formed and the total response frequencies were the same (Fig. 2; Table 2).

Addition of 25% conditioned medium increased significantly both the total response (from average 17.6–23.8%) and the microcolony formation frequencies (from average 2.2–5.4%) for 'Claret' but not for 'Daybreak'. The increase in microcolony formation frequency caused by addition of conditioned medium varied between cell lines for both

Table 2 Anova results for total response and microcolony formation of 'Claret' and 'Daybreak' celery cell suspension derived protoplasts (experiment 2): significance level of genotype, cytokinin (0.012 mg l^{-1} TDZ or 0.2 mg l^{-1} zeatin), adding conditioned medium and their interaction

'Claret' total response	P values	'Claret' microcolonies	P values
Cell line	0.011*	Cell line	0.003**
Cytokinin type	0.500	Cytokinin type	0.365
Conditioning	0.000***	Conditioning	0.000***
Cell line × cytokinin type	0.261	Cell line × cytokinin type	0.641
Cell line × conditioning	0.151	Cell line × conditioning	0.038*
cytokinin type × conditioning	0.459	Cytokinin type × conditioning	0.474
Cell line × cytokinin type × conditioning	0.599	Cell line × cytokinin type × conditioning	0.707
'Daybreak' total response	P values	'Daybreak' microcolonies	P values
Cell line	0.001**	Cell line	0.091
Cytokinin type	0.844	Cytokinin type	0.712
Conditioning	0.164	Conditioning	0.161
Cell line × cytokinin type	0.740	Cell line × cytokinin type	0.139
Cell line × conditioning	0.210	Cell line × conditioning	0.036*
Cytokinin type × conditioning	0.224	Cytokinin type × conditioning	0.387
Cell line × cytokinin type × conditioning	0.654	Cell line × cytokinin type × conditioning	0.936

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Table 3 Total response and microcolony formation frequencies (%) of different independent cell lines of 'Claret' and 'Daybreak' cell suspension derived protoplasts, 15 days after protoplast isolation (experiment 2)

Cultivar	Cell line	Total response (%)	Microcolony formation (%)
Claret'	2.1	20.85ab	4.26ab
	2.2	17.91a	2.28a
	2.3	23.39b	4.86b
Daybreak'	2.1	16.84ac	3.50a
	2.2	13.52b	3.15a
	2.3	14.04bc	2.72a
	2.4	11.94b	2.12a

Data shown are means (n = 16)

a, b, c significant differences based on Tukey's honestly significant test, $p < 0.05$

cultivars (Table 2, interaction term). For instance, the average microcolony formation for 'Daybreak' protoplasts increased by addition of conditioned medium in line 2.1 (from 2.74 to 4.26%) and line 2.2 (from 2.26 to 3.92%), decreased in line 2.3 (from 3.37 to 2.96%) and remained stable in line 2.4 (1.94% without conditioned medium and 2.29 with conditioned medium).

Experiment 3

This experiment aimed to test the regenerability of cell lines derived from 'WL253', 'Diamant' and 'Daybreak'. Protoplast cultures isolated from all lines displayed dividing cells after 7–15 days of cultivation. For the 'WL253' inbred line, protoplasts from cell line 3.3 were contaminated and protoplasts from cell line number 3.1 did not respond.

After 15 days of cultivation, cell line 2.2 of 'WL253' showed a total response rate of 16.5% and a microcolony formation rate of 3.2% (Table 4).

For the cultivars 'Diamant' and 'Daybreak' there was no significant difference between the different cell lines for either total response rate or microcolony formation. The total response frequency and microcolony formation of 'Diamant' protoplasts were 22.3 and 12.3%, while for 'Daybreak' protoplasts the respective results were 16.5 and 4.7% (Table 3).

The microcolonies derived from 'Daybreak' cell lines 3.1 and 3.3 further developed into callus while microcalli from cell lines 2 and 4 died. Surviving calli were cultured on solid medium and were subcultured the following 9 weeks. No shoots formed and henceforth no plants were generated (Table 4).

The microcolonies derived from the 'Diamant' cell lines showed a similar behaviour. Microcalli continued to develop and formed calli that were transferred to solid medium. For all the tested cell lines, shoots appeared 9 weeks after protoplast isolation (Table 4). As in experiment 1, the number of independent shoot formation events per culture could not be determined because calli disaggregated. The shoots transferred to the hormone free medium developed into plants (Fig. 1k).

Experiment 4

Based on the previous experiments, we were confident on the protoplast isolation and culturing conditions to allow a larger experiment comparing several genotypes.

Seven days after protoplast isolation we observed cell divisions in most of the tested cell lines which developed microcolonies (Table 5). Differences in development were observed both between the different cultivars and inbred lines as well as between individual cell lines of the same cultivar or inbred line. For example, within the white celery accession 'WL253' cell line 4.3 did not react, the number of living cells declined and 3 weeks after isolation the protoplasts appeared dead. Cells of line 4.1 slowly started to divide in the first week and the culture further produced microcolonies 3 weeks after isolation. Most of these colonies died and the few microcalli that still developed at a later stage showed no further development. In cell line 4.4, in only one of the technical replicates few microcolonies developed into calli.

Protoplasts from 'WL253', 'Daybreak', and 'VL391', produced microcalli and finally callus. However, only for 'Daybreak' also shoots were formed after transfer to solid medium. The regenerated shoots developed into plants when cultured on the hormone free medium (Fig. 1i).

Ploidy level determination

To determine whether the regenerated protoplasts showed somaclonal ploidy deviations from the original germplasm, we analysed the ploidy of regenerated plants.

We analysed 28 'Claret' plants regenerated from experiment 1. From experiment 2, we analysed 29, 40 and 9 'Diamant' plants belonging to lines 2.1, 2.2 and respectively 2.3. 9 'Daybreak' plants belonging to line 4.2 from experiment 4 were as well analysed. For all the analysed samples the ratio of the 2C peak of the regenerated plants to the internal control was between 2.15 and 2.32 while the ratio of the 2C peak of diploid celery plants to the internal control was in the same range (between 2.23 and 2.3).

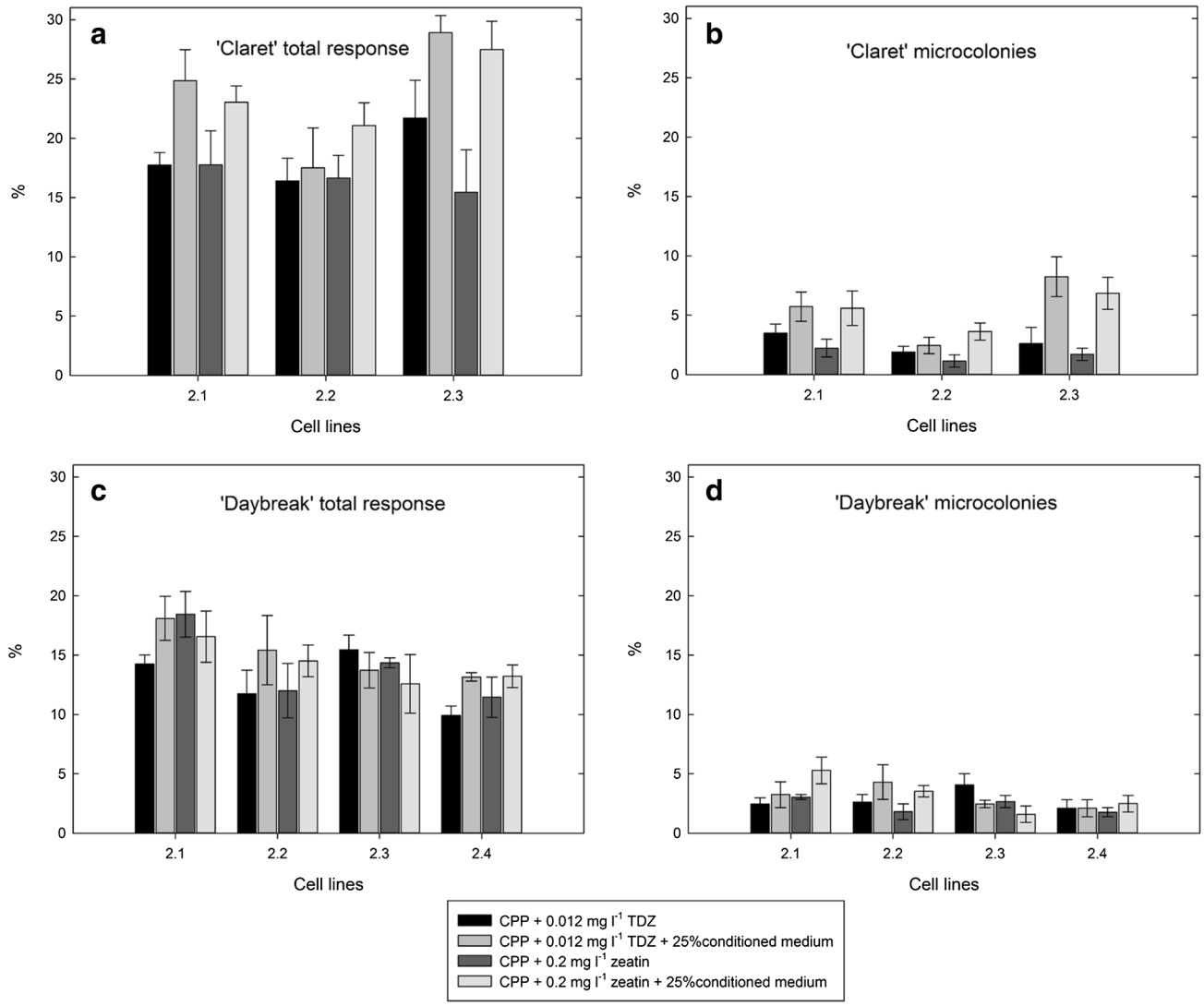

Fig. 2 Early (15 days) total response and microcolony formation frequencies (%) of 'Claret' and 'Daybreak' cell suspension derived protoplast cultured in CPP or conditioned CPP medium supplemented with 0.012 mg l^{-1} TDZ or 0.2 mg l^{-1} zeatin (experiment 2); **a** and **c** total response; **b** and **d** microcolony formation. Data shown are means ± SE (n = 4)

Table 4 Early (15 days) total response and microcolony formation and late regeneration status (4 months) of cell suspension derived protoplasts of different cell lines belonging to 'WL253', 'Daybreak' and 'Diamant' inbred lines and cultivars (experiment 3). Data shown are means ± SE (n = 16)

Inbred line/cultivar	Cell line	Protoplast culture started	Total response (% ± SE)	Microcolony formation (% ± SE)	Regeneration status
'WL253'	3.1	Yes	–	–	–
	3.2	Yes	16.55 ± 2.99	3.24 ± 1.35	Callus
	3.3	No	–	–	–
'Daybreak'	3.1	Yes	16.39 ± 2.84	4.27 ± 0.97	Callus
	3.2	Yes	13.88 ± 2.93	3.25 ± 0.82	Microcolonies
	3.3	Yes	20.77 ± 2.60	4.92 ± 1.06	Callus
	3.4	Yes	10.98 ± 3.69	5.41 ± 1.82	Microcolonies
'Diamant'	3.1	Yes	22.20 ± 1.27	11.04 ± 0.93	Shoots
	3.2	Yes	22.56 ± 2.11	14.50 ± 1.05	Shoots
	3.3	Yes	22.09 ± 1.14	10.30 ± 1.03	Shoots

Table 5 Overview of regeneration status evolution in time (week number after protoplast isolation) of cell suspension derived protoplasts of various cell lines belonging to 'WL253', 'Daybreak', 'VL391', 'Claret', 'L476' and 'Diamant' inbred lines and cultivars (experiment 4)

Cultivar	Line	Protoplast culture started	1st division	Microcolonies	Plated microcalli	Callus	Shoots
'WL253'	4.1	Yes	w1	w3	w6	–	–
'WL253'	4.2	No	–	–	–	–	–
'WL253'	4.3	Yes	–	–	–	–	–
'WL253'	4.4	Yes	w1	w3	w6	w10	–
'Daybreak'	4.1	Yes	w1	w2	w6	w10	w10
'Daybreak'	4.2	Yes	w1	w2	w6	w10	w10
'Daybreak'	4.3	Yes	w1	w3	–	–	–
'Daybreak'	4.4	Yes	w1	w2	w6	w10	–
'VL391'	4.1	Yes	w2	w3	–	–	–
'VL391'	4.2	Yes	–	–	–	–	–
'VL391'	4.3	Yes	w1	–	–	–	–
'VL391'	4.4	Yes	w1	w2	w6	w10	–
'Claret'	4.1	Yes	w1	w1	–	–	–
'Claret'	4.2	Yes	–	–	–	–	–
'Claret'	4.3	Yes	w1	–	–	–	–
'Claret'	4.4	Yes	w1	–	–	–	–
'L476'	4.1	Yes	w1	–	–	–	–
'L476'	4.2	Yes	w1	w2	–	–	–
'L476'	4.3	Yes	–	–	–	–	–
'L476'	4.4	No	–	–	–	–	–
'Diamant'	4.1	Yes	w1	w2	–	–	–
'Diamant'	4.2	Yes	w1	w2	–	–	–
'Diamant'	4.3	No	–	–	–	–	–
'Diamant'	4.4	Yes	w3	w3	–	–	–

Discussion

Protoplast regeneration involves several consecutive steps and depends on multiple parameters including resource material and culture type, protoplast isolation method, protoplast density, medium composition, cultivation conditions, method of medium replacement etc. (Davey et al. 2005; Eeckhaut et al. 2013). Development of a robust protocol for successful protoplast regeneration requires testing of various conditions for each of the steps executed (Chupeau et al. 1993). Here we report on the development of a protocol for the regeneration of protoplasts derived from 3 celery varieties, var. *dulce* (white and green celery), var. *secalinum* (green celery) and var. *rapaceum* (celeriac). Hitherto, regeneration has been reported in the *dulce* variety (Han et al. 2007; Tan et al. 2009). Our protocol is based on earlier reports describing the regeneration of protoplasts from *Daucus carota*, a model for protoplast culturing. (Dirks et al. 1996). The results showed that the type of cytokinin used is not critical, however supplementation of the culture medium with 25% conditioned medium appeared to improve regeneration efficacy.

The cytokinins TDZ and zeatin are frequently used in protoplast regeneration protocols. Zeatin induces successful protoplast regeneration in carrot (Dirks et al. 1996; Grzebelus et al. 2012. 2013), *Solanum* (Borgato et al. 2007), *Allium* (Hansen et al. 1995) and *Stevia* (Lopez-Arellano et al. 2015). TDZ shows strong shoot regeneration potential, especially in protoplasts of woody species including *Populus* (Chupeau et al. 1993) and *Malus* (Wallin and Johansson 1989). TDZ also efficiently induces shoots from protoplast derived callus of *Vicia* (Tegeder et al. 1995). Although protoplast culture in some species is cytokinin specific, we observed that both TDZ and zeatin induced protoplast division and at a later stage shoot regeneration. We concluded that the cytokinin type is not critical for the regeneration competence of celery.

Feed-layers and nursing cells are commonly used in protoplast cultures to stimulate division and regeneration in species like *Hordeum* (Funatsuki et al. 1992), *Lilium* (Horita et al. 2002) *Gossypium* (Sun et al. 2004), or *Musa* (Assani et al. 2006). This method also promotes growth of *Allium* somatic hybrids cultures (Buiteveld et al. 1998). Microalgal conditioned medium improves in vitro plant growth in *Arabidopsis* and *Nicotiana* (Zielinska et al. 2014). We adopted this technique and added conditioned medium to the celery protoplast cultures. We observed that addition of 25% conditioned medium increased

regeneration success of celery protoplasts in a genotype dependent manner. The interaction between genotype and treatment is a common phenomenon in protoplast regeneration experiments. For example, in carrot, the growth promoting effects of phytosulfokine is genotype dependent (Mackowska et al. 2014). The conditioned medium used in the present study was cultivar and cell line specific. This implies that different mother cell lines harbor varying levels of stimulatory compounds. The conditioned medium is a complex solution and the macro-, microelements and hormones left as well as other possible cell secretions probably depends on the plant material that initially grew in it. Further investigation of the conditioned medium is required to identify the active ingredients that stimulate protoplast division and microcallus formation. The use of conditioned medium could be beneficial for somatic hybridization to compensate the decreased cell viability that results from fusion.

Protoplast regeneration of different cultivated and wild accessions that have been reported for *Lycopersicon* (Muhlbach 1980), *Helianthus* (Bohorova et al. 1986), *Arabidopsis* (Damm and Willmitzer 1988), *Musa* (Assani et al. 2002), *Cichorium* (Deryckere et al. 2012) and *Daucus* (Mackowska et al. 2014), underline the genotype dependent variability on protoplast culture development. Studies with *Picea* showed that two embryogenic suspension lines derived from the same cultivar displayed different regeneration capacity (Attree et al. 1988).

We observed a different behaviour of individual cell lines within every experiment. Between experiments, different results were obtained per cultivar. Both observations can be explained by the use of independent cell lines, indicating that regeneration is highly genotype dependent. We were able to select highly regenerative cell lines for 'Claret' (line 1.1), 'Diamant' (lines 3.1, 3.2 and 3.3) and 'Daybreak' (lines 4.1 and 4.2). However, this was not the case for the inbred lines 'WL253', 'VL391' and 'L476'. This could be explained by the fact that the cultivars used are open pollinated and hence each cell line is genetically distinct from one another. The variability in regeneration capacity may therefore be caused by genotype differences. The cell lines obtained from inbred lines on the other hand showed limited genetic variability. Our findings underline that testing individual cell lines within a cultivar and selecting the most responsive ones is crucial for successful celery protoplast regeneration. It is thus easier to identify responsive lines when working with open pollinated cultivars compared to inbred lines. On the other hand if one has sufficient resources to test many inbred lines it is possible to select a line that always maintains its regeneration potential.

The source material for protoplast isolation is an important factor for the success of protoplast regeneration into plants. In general, cell suspensions are often a suitable source for the isolation of protoplast with good regeneration capacity. Indeed, cell suspensions have been shown to be much more suitable for protoplast regeneration than other tissues in the cases of *Zea* (Prioli and Sondahl 1989), *Hordeum* (Funatsuki et al. 1992), *Oriza* (Jain et al. 1995), *Allium* (Buiteveld et al. 1998) or *Musa* (Assani et al. 2002). Similar to these studies, our preliminary experiments with celery, showed difficulties in inducing cell divisions in protoplasts derived from mesophyll tissue (unpublished data). This result is consistent with those of Han et al. (2007) and Tan et al. (2009) who also regenerated celery protoplasts from cell suspensions.

In conclusion, we presented here a simple protocol for celery protoplast culture and regeneration. We provide evidence that regeneration is strongly genotype dependent and that cell suspensions are most suitable for protoplast regeneration. The addition of cell suspension filtered medium has a significant promoting effect. To our knowledge, this is the first report on successful celery protoplast culture and regeneration in 3 commercially important celery varieties. The selected responsive genotypes are currently used for somatic hybridisation with the purpose of CMS introduction in celery.

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Compliance with ethical standards

Conflict of interest We have no conflict of interest to declare.

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