Plant Cell Rep (2012) 31:2261–2269
DOI 10.1007/s00299-012-1335-8

ORIGINAL PAPER

Low melting point agarose beads as a standard method for plantlet regeneration from protoplasts within the *Cichorium* genus

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Received: 16 May 2012 / Revised: 7 August 2012 / Accepted: 9 August 2012 / Published online: 29 August 2012
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Abstract
Key message **A standard method has been developed with which we are able to fully regenerate protoplasts of different *Cichorium* species. For the first time, endive protoplasts have been regenerated into plantlets.**
Abstract Protoplast regeneration is essential for somatic hybridizations. In this study, a standard method for plantlet regeneration from *Cichorium* protoplasts was developed. We evaluated the effect of the low melting point agarose (LMPA) bead technique on the regeneration capacity of protoplasts of seven *C. intybus* and four *C. endivia* genotypes. The LMPA bead technique was more efficient than culture in liquid or solid medium and allowed us to obtain plating efficiencies up to 4.9 % in *C. intybus* genotypes and efficiencies of up to 0.7 % in *C. endivia* genotypes. Moreover, the LMPA bead technique offers great advantages over liquid and solid culture systems: the media can be readily refreshed, protoplasts can be monitored separately, and microcalli can easily be removed from the beads. This increased efficiency was observed for all of the 11 *Cichorium* genotypes tested. Shoot formation was induced more efficiently when using 0.5 mg l^{-1} indole-3-acetic acid-enriched medium (up to 87.5 % of the protoplast-derived calli started shoot development) compared to 1-naphthaleneacetic acid-enriched medium. The LMPA bead technique optimized in this study enabled for the first time the full plantlet regeneration from protoplasts of *C. endivia* genotypes and increased the protoplast regenerating ability in other *Cichorium* species. This fine-tuned LMPA bead technique can therefore be applied for protoplast regeneration after protoplast fusions of the genus *Cichorium*.

Keywords *Cichorium intybus* · *Cichorium endivia* · Low melting point agarose beads · Plantlet regeneration · Protoplasts

Communicated by F. Sato.

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Introduction

The genus *Cichorium* (Asteraceae) contains several important vegetable and industrial crops. Lucchin et al. (2008) divided the species into two types according to their application: the salad types (*C. endivia* L. and *C. intybus* L. var. *foliosum*) and the root type (*C. intybus* L. var. *sativum*), which is mainly used for the inulin extraction. Kiers et al. (2000) divided the salad genotypes into three subgroups: (1) the 'Witloof' cultivars, (2) the sugarloaf cultivars and (3) the 'Radicchio' cultivars. The salad type cultivars are mainly produced in Southern Europe and Asia. Belgium is a major exporter of the Witloof cultivars. The cultivation of root chicory is situated in the traditional production areas in northwestern Europe (Belgium, The Netherlands and Northern France) (Bais and Ravishankar 2001). Since 1990, root cultivars have played a major role in the production of inulin, which is important for food processing and cosmetics, as well as compounds such as sesquiterpene lactones, flavonoids and vitamins (Baert and

Van Bockstaele 1993; Baert 1997; Velayutham et al. 2006). Commercial breeding in chicory has traditionally been based on intercrossing a number of phenotypically superior parents selected for several commercial traits (Lucchin et al. 2008). *C. intybus* contains a self-incompatibility system that can be used to produce inbred lines and hybrids. However, none of the so-called F_1 hybrids can be appointed as 100 % true hybrids (Baert and Van Bockstaele 1993), which causes difficulties in setting up a reliable F_1 seed production scheme. In contrast with *C. intybus*, *C. endivia* is a self-pollinating species with less than 1 % spontaneous cross-fertilization (Rick 1953). Due to its low level of cross-pollination, the production of F_1 hybrids has not yet been extensively developed in endive (Lucchin et al. 2008). Cytoplasmic male sterility (CMS) can contribute to the creation of 100 % true hybrids. However, CMS does not naturally occur in *Cichorium* species, but can be introduced through inter or intraspecific crosses (Cappelle et al. 2007). As in any other breeding programme, the introduction of new features into chicory and endive is restricted by the reproductive barriers and the genetic structure of the populations. To address possible limitations in conventional commercial breeding programs, a protoplast-based approach such as somatic hybridization can be useful to circumvent sexual incompatibility and enable the direct transfer of both nuclear and cytoplasmic genome features into the plant cells. Somatic hybridization through protoplast fusion and regeneration in *C. intybus* has already been established. Efficient protoplast isolation and regeneration protocols of *C. intybus* var. *foliosum* cultivars have been described by several authors (Crepy et al. 1982; Saksi et al. 1986; Slabe and Bohanec 1989). The most obvious result obtained in those studies was the use of glutamine as the sole nitrogen source to improve plating efficiency. In the 1990s, protoplast regeneration in *C. intybus* was studied by Rambaud et al. (1990) and Varotto et al. (1997). Both authors reported that the use of a semisolid proliferation medium after a liquid culture phase improved the plating efficiency. Nenz et al. (2000) demonstrated that the protoplast regeneration cycle could be shortened after embedding the protoplasts in Ca-alginate droplets. More recently, protoplast fusion experiments have illustrated the capacity of somatic hybridization through protoplast fusion in *Cichorium* species. To obtain male-sterile asymmetric somatic hybrids, iodoacetic acid inactivated mesophyll chicory protoplasts (*C. intybus*) were chemically fused with irradiated hypocotyl sunflower protoplasts (*Helianthus annuus* L.) (Varotto et al. 2001). Cappelle et al. (2007) showed the possibility of regenerating an interspecific protoplast fusion between *C. intybus* and *C. endivia*.

All protoplast research to date has focused on *C. intybus* varieties. However, the published protoplast regeneration protocols were cultivar-specific. Furthermore, no information is available on the regeneration of *C. endivia* protoplasts. Cappelle et al. (2007) mentioned the formation of callus after protoplast regeneration of *C. endivia*, but no plants could be obtained. An efficient plantlet regeneration system is the key to a successful protoplast-based breeding programme not only in chicory, but also in endive. It offers prospects towards the development of new varieties, the introgression of new traits and breeding time reduction compared to the time-consuming conventional breeding approach.

The main goal of this study was to develop a universal protocol for plantlet regeneration in *Cichorium* species. We therefore evaluated the low melting point agarose (LMPA) bead technique on different *C. intybus* and *C. endivia* genotypes.

Materials and methods

Plant material

Four industrial chicory cultivars, *C. intybus* var. *sativum* (‘VL52’, ‘K1093’, ‘K1729’ and ‘L4043’) bred at the Institute for Agricultural and Fisheries Research (ILVO) and provided by the COSUCRA-Groupe Warcoing S.A. Division Chicoline, three wild types *C. intybus* from Hungary (‘Ames22531’, ‘Ames22532’ and ‘Pi531291’) and four endive *C. endivia* genotypes (*C. endivia* var. *crispum* ‘Wallone Despa’, *C. endivia* var. *endivia* ‘CICH192’, *C. endivia* var. *latifolium* ‘nr.5’ and *C. endivia* var. *divaricatum* ‘CICH50’) were used in the experiments. Seeds of in vivo plants of the selected *Cichorium* genotypes were initiated in vitro. After rinsing for 1 min in 70 % ethanol, seeds were surface sterilized for 20 min in 6.5 % NaOCl and germinated in 60 mm petri dishes on solid Murashige and Skoog (1962) medium containing 150 mg l^{-1} casein hydrolysate and 30 g l^{-1} sucrose at pH 5.8. After germination, the plantlets were placed on solid Murashige and Skoog medium containing 20 g l^{-1} sucrose and grown in Meli jars (Meli NV Veurne, Belgium) at 23 ± 2 °C under a 16 h/8 h (light/dark) photoperiod at 40 µmol m^{-2} s^{-1} photosynthetic active radiation.

Protoplast isolation

Protoplasts were isolated from 2- to 4-week-old leaves. Leaves were chopped into small pieces and pre-incubated for 1 h in a 0.5 M mannitol solution P_0 (Table 1). Subsequently, the mannitol solution was replaced with an enzymatic mixture containing P_0 medium with 1 mg ml^{-1} cellulase Caylase 345 and 0.5 mg ml^{-1} pectinase Caylase M2 at pH 5.5 (Cappelle et al. 2007). Incubation was carried out in darkness at 23 ± 2 °C for 16 h with gentle agitation (25 rpm). After digestion, protoplasts were purified by

Table 1 Composition of washing solution and culture media for *Cichorium* protoplasts

	P_0	MC_1	MC_2	MC_3
Murashige & Skoog macro elements	½×	½× (no NH_4NO_3)	½× (no NH_4NO_3 or KNO_3)	½×
Heller micro elements	1×	1×	1×	1×
Heller KCl	–	–	1×	–
FeNa-EDTA (mg/l)	18.35	18.35	18.35	18.35
Morel & Wetmore vitamins	1×	1×	1×	1×
Inositol (mg/l)	100	250	100	100
Glutamine (mg/l)	–	375	750	–
Sucrose (g/l)	10	10	10	10
Mannitol (g/l)	90	90	60	–
NAA (mg/l)	–	2	0.5	0.5
BAP (mg/l)	–	1	0.5	0.5
Agar (g/l)	–	–	–	5
pH	5.5	5.5	5.5	5.5

filtration through a 100 µm pore size sieve and centrifuged at 100*g* for 10 min in a swing-out rotor. Protoplasts were pelleted and the supernatant was removed. Finally, the protoplasts were washed twice with MC_2 medium (Table 1) and centrifuged (100*g*, 10 min).

Microscopical protoplast evaluation

The protoplast yield was determined using a Bürker counting chamber. For viability (% of viable protoplasts) staining, about 100 µl protoplast solution was mixed with 1 µl of a 0.5 % (w/v) fluorescein diacetate (FDA) stock solution (5 mg FDA dissolved in 1 ml acetone), incubated for 10 min at room temperature and observed with a fluorescence microscope. Microscopic detection was carried out using an inverted fluorescence microscope (Leica DMIRB) equipped with a Leica Camera System (Leica DFC320). Cell wall regeneration was studied by using Calcofluor White M2R (CFW). CFW binds strongly to cellulose, carborylated polysaccharides and callose (Hughes and McCully 1975) and thus can be used as an indicator of cell wall formation in protoplasts. Freshly isolated protoplasts were mixed with a stock solution of CFW resulting in a final dye concentration of 0.01 % (w/v). Cell wall formation of the protoplasts was then analyzed. Protoplast diameters were measured by ImageJ Software (NIH, National Institutes of Health).

Protoplast regeneration systems

Experiment 1 was set up to evaluate three culture systems on the regeneration ability of protoplasts of the industrial chicory *C. intybus* var. *sativum* 'VL52', the wild type chicory *C. intybus* 'Pi531291' and the endive *C. endivia* var. *crispum* 'Wallone Despa'. In each culture system, the formation of tetrads, microcolonies, calli and plants was evaluated for the three genotypes. The media used in this experiment (Table 1) were based on Saksi et al. (1986), Cappelle et al. (2007) and Rambaud et al. (1990). Protoplasts were cultured at 23 ± 2 °C in both light and dark conditions (16 h/8 h (light/dark) photoperiod at 40 µmol m^{-2} s^{-1} photosynthetic active radiation).

The first culture system was based on solid medium. In this system, 5 g l^{-1} (Duchefa Biochemie B.V.) was added to both liquid MC_1 and MC_2 medium (Table 1). After heating (60 °C) and cooling (35 °C), equal volumes of this liquified MC_1 and MC_2 were mixed with liquid MC_1 and MC_2, containing 1×10^5 protoplasts ml^{-1}, respectively. Petri dishes (60 mm) were filled with 5 ml of the protoplast solution (final protoplast concentration was 5×10^4 protoplasts ml^{-1}). Because the protoplasts were embedded in this solid matrix, the medium was not refreshed.

The second culture system was based on a liquid medium culture consisting of 5 ml of starting medium (MC_1 or MC_2) containing 5×10^4 protoplasts ml^{-1} in a petri dish (60 mm) with gentle shaking (10 rpm). At day 5 of the regeneration process, the medium was fully replaced by fresh medium: MC_1 was replaced by either MC_1 or MC_2; MC_2 was replaced by MC_2 only.

The third culture system was based on LMPA beads. Protoplasts were embedded in LMPA beads and surrounded by liquid media. For culture in LMPA beads, a protoplast suspension (containing 1×10^5 protoplasts ml^{-1} liquid MC_2) was mixed with an equal volume of liquified solid MC_2 (containing 5 g LMPA l^{-1} liquid MC_2). Six beads of 50 µl of this mixture were dispensed in a petri dish (60 mm). After solidification of the beads, 5 ml of liquid MC_1 or MC_2 was added. At day 5 of the regeneration process, the liquid medium was fully replaced: MC_1 was replaced by fresh MC_1 or MC_2 and MC_2 was replaced by fresh MC_2.

When the microcolony phase was reached in the liquid culture systems and in the LMPA bead culture system, the liquid medium was refreshed each week. After 4 weeks, the mannitol concentration was stepwise reduced using liquid MC_2, containing 30 g l^{-1} mannitol instead of 60 g l^{-1}. After 6 weeks, protoplast-derived microcalli were transferred onto solid regeneration medium MC_3 (Table 1) to induce callus growth.

In experiment 2, the influence of different initial protoplast densities (1, 2, 5, 10 or 20 $\times$ 10^4 protoplasts ml^{-1}) in the LMPA beads were tested. All protoplasts were cultured in petri dishes (60 mm diameter) sealed with parafilm. The cultures were kept under 16 h/8 h day/night conditions at 23 $\pm$ 2 °C.

Experiment 3 was set up to evaluate the impact of different auxins, either 1-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA) in combination with cytokinin 6-benzylaminopurine (BAP), on shoot formation. Regenerating calli obtained in experiment 1 were placed on solid MC_3 containing 0.5 mg l^{-1} BAP and 0.1, 0.5 or 1.0 mg l^{-1} NAA or IAA. Subsequently, regenerated shoots were placed on 100 ml solid medium (Murashige and Skoog medium containing 2 % sucrose, pH 5.7) in Meli jars for rooting and further growth.

In experiment 4, the optimal conditions obtained for the model genotypes were tested for the regeneration of other *Cichorium* cultivars: *C. intybus* var. *sativum* 'K1093', 'K1729' and 'L4043', for wild *C. intybus* 'Ames22531' and 'Ames 22532' and for *C. endivia* var. *endivia* 'CICH192', *C. endivia* var. *latifolium* 'nr.5' and *C. endivia* var. *divaricatum* 'CICH50'.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's Post Hoc test were used to analyze the effect of different media sequences on protoplast regeneration in the LMPA beads, the influence of different initial protoplast densities in the LMPA beads and the effect of the combination of either NAA or IAA with BAP on the shoot formation of regenerating calli. All calculations were obtained using the statistical software package Statistica v.10.

Results

Protoplast isolation

On average, 1 $\times$ 10^6 highly chloroplast-rich protoplasts of 10–70 μm diameter were isolated per gram of fresh-weight leaves from all of the *Cichorium* genotypes tested. Their viability varied between 85 and 95 %.

Protoplast regeneration systems

In experiment 1, the efficiency of the three culture systems with regard to tetrad and microcolony formation was tested. In solid medium, the protoplasts did not divide and all genotypes showed a high mortality rate within 5 days after isolation (Table 2). The liquid media induced initial divisions in the *C. intybus* 'Pi531291' protoplasts during the first week. Tetrads were formed, but further development was limited. FDA tests on day 5 showed a significantly improved protoplast viability when using MC_1 as the initial medium (56.9 $\pm$ 2.8 % viable protoplasts) compared to using MC_2 as the initial medium (32.9 $\pm$ 6.3 % viable protoplasts). In all liquid culture systems, however, anthocyanin production was observed, indicating stress. Monitoring cell wall formation with CFW in the liquid cultures revealed cell wall regeneration 1 day after protoplast isolation in 15 % of the initiated protoplasts on average. After 5 days, up to 40 % of the protoplasts had formed a new cell wall, suggesting the possibility of lowering the mannitol concentration after 5 days. However, replacing MC_1 with MC_2 after 5 days of culturing did not enable further divisions. Protoplasts of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' did not initiate first divisions in the liquid culture system and subsequently died (Table 2).

Protoplasts cultured in the LMPA beads were able to form tetrads and developed further into microcolonies 14 days after isolation for all genotypes (Table 2).

Testing of different media compositions in the LMPA bead technique showed that for both *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' protoplasts, an initial 5-day culture in liquid MC_1 medium produced twice as many tetrads compared to the beads in liquid MC_2 medium (Table 3). Microcolony development increased significantly when MC_1 was replaced by MC_2 after 5 days of culturing. Refreshing the MC_1 medium with fresh MC_1 limited further development in the tetrad phase. Although an initial incubation of the beads in liquid MC_2 produced a significantly lower number of tetrads than in liquid MC_1, further incubation in fresh MC_2 resulted in a significant higher number of microcolonies in the beads (Table 3). Consequently, the highest number of microcolonies was obtained by initial incubation of the beads for 5 days in liquid MC_1 medium and subsequent replacement of MC_1 with MC_2. Moreover, a higher percentage of microcolonies was observed at day 14 than the percentage of tetrads after 5 days, suggesting a beneficial effect of the replacement of MC_1 with MC_2. For *C. endivia* var. *crispum* 'Wallone Despa' protoplasts, no microcolonies were obtained under a 16/8 h light/dark photoperiod. However, when culturing the endive protoplasts continuously in the dark, microcolony formation could be observed. The percentage of microcolonies produced was

Table 2 Effect of different culture systems and media composition on the regeneration of *C. intybus* var. *sativum* 'VL52', *C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa' protoplasts

Culture system	Genotypes	Medium sequence[a]	5 days (tetrad phase)	14 days (microcolony)
Solid	*C. intybus* var. *sativum* 'VL52'	MC1	–	–
		MC2	–	–
	C. intybus 'Pi531291'	MC1	–	–
		MC2	–	–
	C. endivia var. *crispum* 'Wallone Despa'	MC1	–	–
		MC2	–	–
Liquid	*C. intybus* var. *sativum* 'VL52'	$MC_1 \rightarrow MC_1$	–	–
		$MC_1 \rightarrow MC_2$	–	–
		$MC_2 \rightarrow MC_2$	–	–
	C. intybus 'Pi531291'	$MC_1 \rightarrow MC_1$	+	–
		$MC_1 \rightarrow MC_2$	+	–
		$MC_2 \rightarrow MC_2$	–	–
	C. endivia var. *crispum* 'Wallone Despa'	$MC_1 \rightarrow MC_1$	–	–
		$MC_1 \rightarrow MC_2$	–	–
		$MC_2 \rightarrow MC_2$	–	–
LMPA beads	*C. intybus* var. *sativum* 'VL52'	$MC_1 \rightarrow MC_1$	+	–
		$MC_1 \rightarrow MC_2$	+	+
		$MC_2 \rightarrow MC_2$	+	+
	C. intybus 'Pi531291'	$MC_1 \rightarrow MC_1$	+	+
		$MC_1 \rightarrow MC_2$	+	+
		$MC_2 \rightarrow MC_2$	+	+
	C. endivia var. *crispum* 'Wallone Despa'	$MC_1 \rightarrow MC_1$	+	–
		$MC_1 \rightarrow MC_2$	+	+
		$MC_2 \rightarrow MC_2$	–	–

Tetrad and microcolony formation were analyzed 5 and 14 days after protoplast isolation, respectively

[a] For the liquid and LMPA bead culture: at day 5 of regeneration, MC_1 was replaced by either MC_1 or MC_2. MC_2 was replaced by MC_2 only

significantly lower compared to *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' (Table 3). When transferring the endive microcalli on solid medium for callus growth and shoot induction, standard light conditions were used.

After 4 weeks, the LMPA beads contained a high number of developing microcalli and the mannitol concentration was gradually decreased. After 6 weeks, protoplast-derived microcalli were transferred to solid regeneration medium MC_3 to induce callus growth. For *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' cultures, 4 % of the initiated protoplasts on average could be regenerated to calli. For the *C. endivia* var. *crispum* 'Wallone Despa' culture, this was less than 1 % (Table 3).

Experiment 2 showed that for the three genotypes tested, a density of 5×10^4 protoplasts ml^{-1} in the agarose beads resulted in the highest plating efficiencies (Fig. 1). When using lower densities, fewer divisions occurred. The use of higher densities led to high frequency of first mitotic cell divisions, but further development stopped once tetrads were formed. The protoplast density of 5×10^4 protoplasts ml^{-1} was used in further protoplast regeneration experiments with the LMPA beads.

Experiment 3 demonstrated that shoots were induced more efficiently when callus was cultured in IAA-enriched MC_3 medium instead of in NAA-enriched MC_3 medium (Fig. 2). An IAA concentration of 0.5 mg ml^{-1} combined with 0.5 mg ml^{-1} BAP yielded the highest shoot formation for either *C. intybus* var. *sativum* 'VL52' (67.8 ± 8.8 %), *C. intybus* 'Pi531291' (87.5 ± 4.0 %) and *C. endivia* var. *crispum* 'Wallone Despa' (26.8 ± 2.6 %). At higher IAA concentrations, the shoots were translucent and exhibited hyperhydric symptoms. Those shoots did not develop further upon isolation. Lower IAA concentrations induced fewer shoots (Fig. 2).

In experiment 4, protoplast regeneration was tested on a higher number of genotypes using the most optimal culture conditions from former experiments. Protoplasts of *C. intybus* var. *sativum* 'K1729' and 'L4043' could be regenerated as efficient as *C. intybus* var. *sativum* 'VL52'. *C. intybus* var. *sativum* 'K1093' yielded even a higher plating efficiency (PE) (Table 4). The wild type genotype

Table 3 Effect of different media sequences on *C. intybus* var. *sativum* 'VL52', *C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa' protoplast regeneration in LMPA beads (% of the initial number of cultured protoplasts)

Genotypes	Medium sequence[z]	5 days (tetrad phase)	14 days (microcolony)[y]	Callus	Plants
C. intybus var. *sativum* 'VL52'	$MC_1 \rightarrow MC_1$	34.5 ± 3.2a[x]	0c	/	/
	$MC_1 \rightarrow MC_2$	39.5 ± 3.9a	47.5 ± 1.0a	4.1 ± 0.2a	+
	$MC_2 \rightarrow MC_2$	18.5 ± 2.2b	26.5 ± 2.5b	2.3 ± 0.2b	+
C. intybus 'Pi531291'	$MC_1 \rightarrow MC_1$	37.5 ± 3.0a	9.0 ± 2.7c	0c	/
	$MC_1 \rightarrow MC_2$	39.5 ± 2.9a	56.5 ± 2.1a	4.4 ± 0.2a	+
	$MC_2 \rightarrow MC_2$	19.5 ± 1.0b	25.5 ± 1.7b	2.0 ± 0.1b	+
C. endivia var. *crispum* 'Wallone Despa'	$MC_1 \rightarrow MC_1$	4.5 ± 0.5*a	0	/	/
	$MC_1 \rightarrow MC_2$	4.5 ± 1.3*a	6.5 ± 2.2*	0.7 ± 0.1	+
	$MC_2 \rightarrow MC_2$	0*b	/	/	/

Tetrad formation and microcolony formation were analyzed after 5 and 14 days, respectively. Callus development was analyzed on solid MC_3 medium

Data are mean ± SE ($n = 9$)

* Regeneration under dark conditions

[x] a, b, c significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results compared within each genotype for the three medium sequences

[y] Microcolonies: cell clumps of 10–20 cells

[z] At day 5 of regeneration, MC_1 was replaced by either MC_1 or MC_2; MC_2 was replaced by MC_2

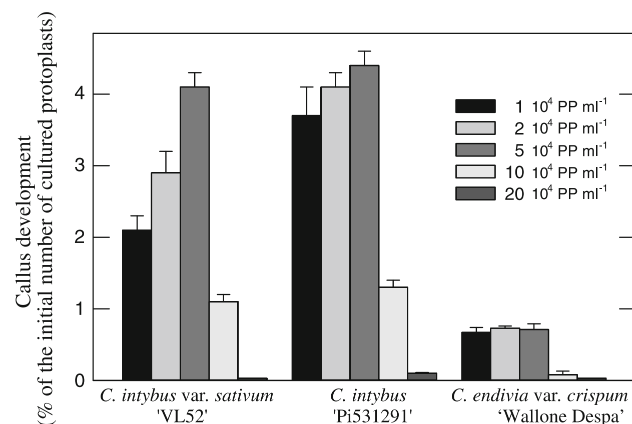

Fig. 1 The effect of different initial protoplast densities [1, 2, 5, 10 or 20×10^4 protoplasts (PP) ml^{-1}] on callus development in the LMPA beads for three *Cichorium* genotypes (% of the initial number of cultured protoplasts)

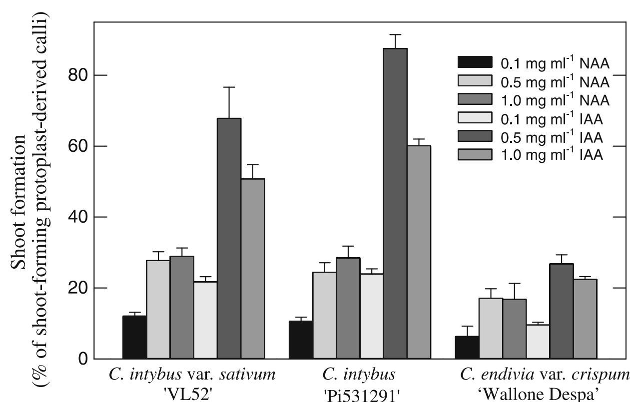

Fig. 2 Percentage of shoot-forming *Cichorium* protoplast-derived calli on MC_3 medium supplemented with 0.5 mg l^{-1} BAP and 0.1, 0.5 or 1.0 mg l^{-1} NAA or IAA concentrations

C. intybus 'Ames22532' yielded comparable results as *C. intybus* 'Pi531291'. *C. intybus* 'Ames22531' protoplasts formed as many microcolonies as the model genotype protoplasts. However, the frequency of callus formation was significantly lower compared to the other *C. intybus* cultivars (Table 4). The three endive genotypes yielded similar PEs as the endive model genotype. Continuously dark conditions were needed to observe microcolony formation within the endive genotypes. When transferring the endive microcalli on solid medium for callus growth and shoot induction, standard light conditions were used. However, the endive group produced significantly fewer calli than the *C. intybus* genotypes. Full plantlet regeneration of protoplast-derived calli could be obtained for all genotypes tested.

Discussion

Protoplasts were successfully isolated from leaves of four industrial chicory cultivars, *C. intybus* var. *sativum* 'VL52', 'K1093', 'K1729' and 'L4043', three wild types from Hungary, *C. intybus* 'Ames22531', 'Ames22532' and 'Pi531291' and the endive genotypes *C. endivia* var. *crispum* 'Wallone Despa', *C. endivia* var. *endivia* 'CICH192', *C. endivia* var. *latifolium* 'nr.5' and *C. endivia* var. *divaricatum* 'CICH50' using the method reported in Cappelle

Table 4 Callus formation of several *Cichorium* cultivars after protoplast regeneration in LMPA beads using the $MC_1 \rightarrow MC_2$ medium sequence (% of the initial number of cultured protoplasts)

Species	Genotypes	Callus
C. intybus var. *sativum*	'VL52'	4.1 ± 0.2bc[x]
	'K1093'	4.9 ± 0.1a
	'K1729'	4.6 ± 0.1ab
	'L4043'	3.9 ± 0.1c
C. intybus	'Pi531291'	4.4 ± 0.2abc
	'Ames22531'	1.7 ± 0.2d
	'Ames22532'	3.9 ± 0.1c
C. endivia var. *crispum*	'Wallone Despa'	0.7 ± 0.1e
C. endivia var. *endivia*	'CICH192'	0.5 ± 0.04e
C. endivia var. *latifolium*	'nr.5'	0.5 ± 0.03e
C. endivia var. *divaricatum*	'CICH50'	0.3 ± 0.05e

Data are mean ± SE ($n = 8$)

[x] a, b, c, d, e significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. The results for callus formation were compared between each genotype

et al. 2007. No further optimization of protoplast isolation was needed for the genotypes studied.

The development of a suitable culture method is a key factor in the final efficiency of a protoplast regeneration protocol (Davey et al. 2005; Eeckhaut and Van Huylenbroeck 2011). In this study, the regeneration ability of *Cichorium* protoplasts was tested using the gelating agent, LMPA. The first gelating agent used for protoplast regeneration was agar. This was successfully performed on tobacco mesophyll protoplasts (Nagata and Takebe 1971; Davey et al. 2005). However, the use of agarose significantly improved the plating efficiency of regenerating protoplasts in numerous species. Due to its neutral charge and lower degree of chemical complexity, fewer interactions between biomolecular nutrients and agarose occured. Furthermore, the larger pore size of agarose gels promoted a higher degree of biomolecule exchange within the agarose gel and between the gel and the external environment. The LMPA with a low gelling temperature of 24–30 °C, used in this study, allowed protoplast mixture with the LMPA solution without exposing the cells to damaging temperatures as the LMPA solution remained fluid at 35 °C and gelation occured below 26 °C (Lörz et al. 1983; Shillito et al. 1983; Lian et al. 2012).

We have evaluated the regeneration efficiency of protoplasts of various *Cichorium* genotypes in LMPA beads in comparison to liquid and solid cultures.

Culture in exclusively solid medium resulted in no divisions. These protoplasts soon burst and subsequently died. Stress factors typically linked with the use of solid media for protoplasts are the probable cause of this failure. Without the possibility of regular refreshment, the medium dehydrates, and thus lowers the osmotic potential. Moreover, toxic compounds of dying neighboring protoplasts may accumulate in the medium and inhibit division of other protoplasts (Vanslogteren et al. 1980; Davey et al. 2005; Duquenne et al. 2007).

Liquid medium was not optimal for *Cichorium* protoplast culture either. This was demonstrated by the overall decrease of protoplast viability, the production of anthocyanins [also observed in liquid culture of petunia protoplast, Frearson et al. 1973) and the limited division of protoplasts during the first week of culture. Similar results were obtained in the regeneration of protoplasts of *C. intybus* 'Rosso do Chioggia' in liquid conditions (Nenz et al. 2000). Two possible explanations are the low accessibility of gases at the bottom of the petri dish, where the protoplasts are located (Duquenne et al. 2007), or the exposure of healthy cells to high concentrations of toxic substances of dying neighboring cell due to the clustering of protoplasts in liquid cultures (Yu et al. 2000).

Regeneration of protoplasts of all the *Cichorium* genotypes tested was achieved by embedding the protoplasts in LMPA beads surrounded by liquid medium. Compared to the solid medium culture, the LMPA bead technique enabled regular refreshment, preventing medium dehydration and toxic compounds accumulation. Unlike culturing in liquid media, protoplasts in the LMPA beads were homogeneously spread, inhibiting cluster formation. Moreover, due to the low concentration of the LMPA used for the formation of the beads, nutrients and gasses could be exchanged easily between the liquid and solid phase. The use of LMPA in semisolid protoplast regeneration culture systems for chicory has been described previously (Saksi et al. 1986; Slabe and Bohanec 1989; Rambaud et al. 1990). Those systems required that the protoplasts first be cultured in liquid medium and subsequently centrifuged before starting culture in a semisolid medium. Protoplast loss can therefore be expected, and clustering can hinder protoplast separation on the semisolid medium. In the Ca-alginate nurse-cultures described by Nenz et al. (2000), protoplasts are embedded in Ca-alginate beads surrounded by liquid medium. This technique requires supplementary steps, however; at the start of the culture, beads need to be formed through the merger of an alginate solution, in which protoplasts are suspended, as well as a Ca solution. Once microcalli have been formed in the beads, they need to be released by chelating the calcium from the matrix and centrifuging the released colonies, resulting in the aforementioned problems. Our LMPA bead-based system has two major advantages compared to these culture systems: (1) it offers a high regeneration capacity for a greater number of genotypes, (2) its simplicity avoids the necessity of supplementary steps such as centrifugation and/or chemical bead breakdown that can be expected to

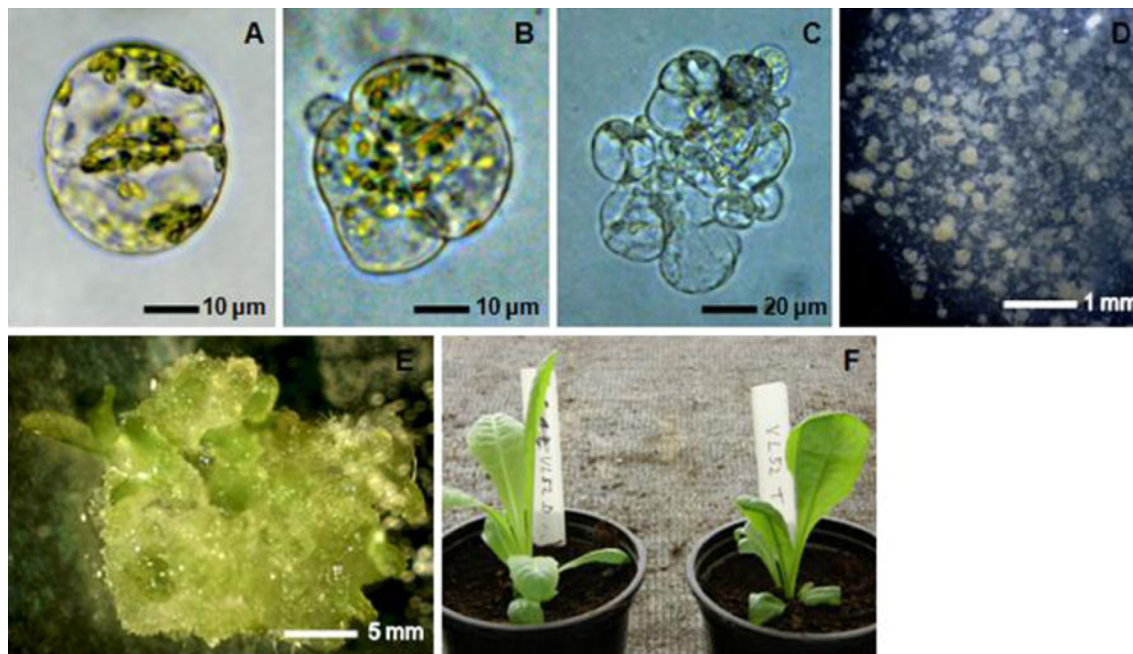

Fig. 3 *Cichorium intybus* var. *sativum* 'VL52' protoplast culture: regeneration steps following the LMPA bead technique. First mitotic division (**a**); tetrad formation after the second mitotic division (**b**); microcolony (**c**); microcalli development (**d**); shoot development (**e**); in vivo regenerants (**f**)

significantly reduce the overall efficiency of the protocol. Developing microcalli can easily be removed from the soft LMPA beads with tweezers and placed on solid medium without an extra centrifugation step. Also, the proliferation of particular cells or colonies can be followed up on a daily basis. Changing or refreshing the medium at any time without disturbing the protoplasts is also possible. This is particularly important when culturing fused protoplasts, because supplementary toxic waste molecules produced by the fusion event must be more rapidly diluted. Our results demonstrate that the LMPA bead technique is a very efficient tool for protoplast regeneration, and makes it possible to change culture media to fit the suitable environmental conditions for many genotypes.

After microcalli formation in the LMPA beads and callus development on solid MC_3 medium, shoot development was more efficiently induced when the regenerating calli were cultured in IAA-enriched MC_3 medium. Although, previous studies on shoot induction on protoplast-derived calli reported the use of NAA as a shoot inducing hormone (Saksi et al. 1986; Rambaud et al. 1990; Cappelle et al. 2007), we found IAA to be more effective. This is in accordance with findings in in vitro plant regeneration through organogenesis from cotyledon, petiole, leaf and root explants derived calli, which used IAA in favor of shoot formation (Park and Lim 1999; Velayutham et al. 2006; Choi et al. 2009).

When using the LMPA bead technique with the medium sequence $MC_1 \rightarrow MC_2$, followed by an incubation of the protoplast-derived calli on IAA-enriched MC_3 medium, total protoplast regeneration was possible within 14 weeks for the *Cichorium* species under study (Fig. 3).

In our experiments, a protoplast density of 5×10^4 protoplasts ml^{-1} in the agarose beads resulted in the highest plating efficiencies. Compared to previously reported results in chicory protoplast regeneration, which showed the highest plating efficiencies when using 2×10^4 protoplasts ml^{-1} in semisolid culture systems (Rambaud et al. 1990; Varotto et al. 1997) and in alginate culture systems (Nenz et al. 2000), our technique enables regeneration of more protoplasts in a single experiment.

For the first time, plantlets of several genotypes of as well industrial chicory (*C. intybus* var. *sativum*), wild chicory (*C. intybus*) and endive (*C. endivia*) were successfully regenerated from protoplasts, using the LMPA bead technique. To our knowledge, this technique is the only one that induces sustained protoplast division and complete regeneration in such a wide *Cichorium* genotype range. A second innovation presented in this study is the first full plantlet regeneration from endive protoplasts. Consequently, our findings can contribute to the further development of somatic hybridization within *C. endivia* or between different *Cichorium* species. Indeed, the presence of an effective regeneration protocol is indispensable for the development of protoplast-based breeding tools, including both symmetric and asymmetric somatic fusion. The development of the LMPA technique therefore offers significant potential for interspecific *Cichorium* breeding

and subsequent genetic variation broadening and introgression of new traits.

Acknowledgments We thank COSUCRA-Groupe Warcoing S.A. Division Chicoline for kindly providing seeds and plants and as financial supporters of the project. Also, we are grateful to Ronald van den Oord and Joost Baert for their practical help.

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