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EMBEDDING IN FILTER-STERILIZED ALGINATE ENHANCES *BRASSICA OLERACEA* L. PROTOPLAST CULTURE

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The influence of sodium alginate sterilization on the viability and mitotic activity of embedded protoplasts was studied in protoplasts of *Brassica oleracea* subsp. *alba* and *rubra* isolated from hypocotyl tissue and leaves of seedlings or plants grown in vitro. Both leaf and hypocotyl-derived protoplasts were more viable and divided more frequently when embedded in filtrated alginate. Division frequency was highest in cv. Reball F1 and the mitotic activity of its protoplasts was three times higher when embedded in filtrated alginate (36.1 ± 6.8%) than when cultured in autoclaved alginate (10.9 ± 5.0%). Protoplast-derived calli colonies were transferred to solid regeneration media and plants of all tested accessions were obtained.

Key words: *Brassica*, calcium alginate layers, mitotic division, protoplast viability, regeneration.

INTRODUCTION

Immobilization of protoplasts in a polymerizing matrix allows undisturbed rebuilding of the cell wall, prevents cell agglutination (Dovzhenko et al., 1998; Pati et al., 2005) and promotes completion of mitotic divisions (Schmitz and Schnabl, 1989; Isa et al., 1990; Niedz, 1993). There are two methods of gel formation for plant cell immobilization; hardening after cooling (thermal gels), or polymerization in the presence of a crosslinking agent (ionic gels). Ionic gels usually are stronger, as they are crosslinked by ionic or covalent bonds, whereas thermal gels have weaker hydrogen bonds (Soderquist and Lee, 2005). Agar is the thermal gelling agent most widely used in plant tissue culture. Melting of agar (40–50°C) for cell entrapment presents a high risk of thermal shock to the cultured cells, high light diffraction distorts observations (Hunter, 1994), and less purified agar types can be toxic to protoplasts (Shillito et al., 1983). Agar consists of a mixture of agarose and agaropectin with sulphate ester side groups, and when hydroxyethyl groups are introduced to its molecules during the extraction process a low-melting-point agarose (LMPA) is produced (Shillito et al., 1983; Deryckere et al., 2012). Immobilization in agarose was found to be far superior to agar, eliminating exposure to heat stress and preventing necrosis of *Cichorium* and *B. juncea* protoplasts, but it had no effect on *B. oleracea* protoplasts (Chen et al., 2004; Deryckere et al., 2012). Alternatively, semi-solid ionic gels such as alginates can be used. Alginates are polysaccharides derived from seaweed and crosslinked by divalent cations (Draget et al., 2005). Alginates have various applications in industry, medicine and agriculture (Smidsrød and Skjåk-Braek, 1990; Gontier et al., 1994). In plant tissue culture, alginic acid has been used mostly as a replacement for agarose for cell immobilization in bioreactors (Payne et al.,1988; Asada and Shuler, 1989; Draget et al., 2005) and for mass production of encapsulated plant material for various purposes such as cryopreservation or production of artificial seeds (Redenbaugh et al., 1991; Patel et al., 2000).

Protoplast culture conditions have been intensively studied in the Brassiceace family. The major obstacle to commercial application of protoplast fusion in *B. oleracea* is the lack of breeding lines with a high capacity for plant regeneration in vitro (Hansen et al. 1999; Holme et al. 2004). Positive results have been achieved using *B. oleracea* accessions selected for high regeneration capacity, but mitotic division and shoot regeneration generally remain problematic (Jourdan and Earle, 1989; Holme et al., 2004). Improvements in culture

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methodology are needed. *B. napus* protoplasts embedded in alginate layers showed 3–5% division frequency (Dovzhenko, 2001). There is only one report of its use for *B. oleracea*: embedding in alginate layers stimulated division in protoplasts but the process was strongly genotype-dependant (Kiełkowska and Adamus, 2012).

Alginate gels have an open pore structure, allowing free diffusion of small molecules (Tricoli et al., 1986; Draget et al., 1988; Smidsrød and Skjåk-Braek, 1990). Cells entrapped in alginate are easily manipulated with less risk of physical damage (Draget et al., 1988; Niedz, 1993). The physiological responses of embedded cells are linked to the physical and chemical properties of alginates (Gontier et al., 1994). The preferred alginate for plant tissue culture is a linear copolymer of L-guluronic (G) and D-mannuronic (M) acid residues. The ratio of guluronic/mannuronic blocks, the length of the guluronic acid residues and their molecular weight all significantly affect the crosslink characteristics of the gel, its mechanical stability and porosity. Other factors affecting the gel's properties include the concentration of the alginate solution (1–4% w/v), the duration of gel complexation and the molarity of the solution (Wong et al., 2001). Larkin et al. (1988) noted that high temperature during sterilization causes depolymerization of the chains, which affects the stability of the alginate gels.

In this study we evaluated the effect of alginate sterilization on the quality of the formed gels by testing two culture parameters (viability and division frequency) of Ca-alginate-embedded *Brassica oleracea* protoplasts.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of *Brassica oleracea* var. *capitata f. alba* 'Kamienna Głowa' (PlantiCo, Poland), 'Sława z Gołębiewa' (PlantiCo, Poland), and *B. oleracea* var. *capitata f. rubra* cv. Reball F1 (Syngenta Seeds, Netherlands) were surface-disinfected in 70% (v/v) ethanol for 2–3 min and 10% (w/v) chloramine T (Biochemie Poland, Katowice) for 20 min, then washed three times in sterile distilled water for 5 min. Seeds were germinated on MS (Murashige and Skoog, 1962) medium supplemented with 0.8% (w/v) agar (Biocorp, Warsaw) in sterile 250 ml glass jars. Cultures were kept at 26±2°C under a 16 h photoperiod (55 μmol m^{-2} s^{-1} flux). Hypocotyl explants were obtained from etiolated seedlings. For this purpose, seeds were placed in 9 cm Petri dishes with 30 ml MS medium and kept in the dark at 26±2°C.

PROTOPLAST CULTURE

Protoplasts were isolated from young leaves of 4-week-old plants, and hypocotyls from 1-week-old seedlings. Protoplasts were isolated and cultured according to the protocol of Kiełkowska and Adamus (2012). The enzyme solution for tissue digestion consisted of 0.5% (w/v) Onozuka R-10 cellulase (Duchefa Biochemie, Netherlands), 0.1% (w/v) pectolyase Y-23 (Duchefa Biochemie, Netherlands), 3 mM $CaCl_2$ and 0.4 M mannitol, and was filter-sterilized (0.22 μm, Millipore, Durham, UK).

The density of cultured protoplasts was adjusted to 8×10^5 per ml culture medium, and the protoplasts were embedded in a calcium alginate layer. The alginate solution consisted of 2.8% (w/v) Na alginate (Sigma, low viscosity) dissolved in 0.4 M mannitol and stirred (150 rpm) overnight. Two methods of sterilizing the alginate solution were employed; autoclaving (20 min at 121°C, 0.1 MPa) and filter sterilization (0.22 μm; Millipore, Durham, UK). Protoplast suspension and sterile alginate solution were mixed in equal volumes to obtain a final culture density of 4×10^5 protoplasts per ml. Approximately 0.5 ml protoplast-alginate mixture was spread onto 1% (w/v) agar containing 40 mM $CaCl_2$. After 1 h incubation at room temperature, alginate discs with embedded protoplasts were formed. The discs were transferred to 6 cm Petri dishes containing 4 ml culture medium. The culture medium was based on Kao and Michayluk (1975), with organic acids, 250 mg dm^{-3} casein hydrolysate, 0.1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg dm^{-3} zeatin, pH 5.6, filter-sterilized. Culture media were renewed once after 10 days. The cultures were incubated in the dark at 26±2°C.

Protoplast viability was estimated on the first and fifth days of culture by staining with fluorescein diacetate (FDA) and observing with an Axiovert S 100 microscope (Carl Zeiss, Göttingen, Germany). Staining was done according to Anthony et al. (1999).

SHOOT REGENERATION

Colonies of protoplast-derived calli were freed from alginate layers according to the protocol of Damm and Willmitzer (1988). Callus colonies were transferred to 9 mm Petri dishes containing 30 ml MS regeneration medium or B5B regeneration medium consisting of B5 (Gamborg et al., 1968) micro-, macro-elements and vitamins with 750 mg dm^{-3} $CaCl_2$ $2H_2O$, 1.0 mg dm^{-3} BA and 20 g dm^{-3} sucrose. Both media were adjusted to pH 5.7–5.8 and 0.25% (w/v) Phytagel was added (Sigma) prior to autoclaving. The developing shoots were transferred to fresh media every 3 weeks. Cultures were maintained at 26±2°C under a 16 h photoperiod (55 μmol m^{-2} s^{-1} flux).

STATISTICAL ANALYSIS

A single treatment consisted of 20 Petri dishes. Each experiment was replicated twice. Protoplast viability was scored as the number of green-fluorescing protoplasts per total number of observed cells (×100). Means were calculated from a minimum 200 cells per Petri dish. Division frequency was expressed as the number of dividing protoplasts per total number of observed protoplasts (×100). Regeneration frequency was calculated as the number of shoots developed from callus per total number of calli cultured on the regeneration media (×100). Analyses employed the ANOVA module in Statistica ver. 10.0 (Statsoft Poland Inc.). Means were separated using Tukey's HSD test.

RESULTS

PROTOPLAST VIABILITY

We observed effects of cultivar, protoplast source and alginate sterilization method on cabbage protoplast viability (Tab. 1). Protoplast viability was high on the first day following isolation, ranging from 68.3±3.1% for cv. Sława z Gołębiewa up to 82.2±2.7% for cv. Kamienna Głowa. Viability was higher among hypocotyl-derived protoplasts (78.1±2.9%). Protoplasts embedded in filtrated alginate were ~20% more viable than those embedded in autoclaved alginate. On day 5 of culture, protoplast viability decreased 25–30% in all treatments and all cultivars. Over this period there were no differences in protoplast viability between cultivars or protoplast sources but viability decreased less among protoplasts embedded in filtrated alginate.

TABLE 1. Effect of accession, protoplast source and alginate sterilization method on *B. oleracea* protoplast viability

Factor	Protoplast viability [% ± SE]	
	1st day	5th day
Cultivar		
Kamienna Głowa	82.2±2.7[a]	45.1±2.8[a]
Sława z Gołębiewa	68.3±3.1[b]	45.2±1.7[a]
Reball F1	71.9±3.7[ab]	41.6±4.7[a]
Source of protoplasts		
leaves	71.0±2.4[b]	42.5±2.2[a]
hypocotyls	78.1±2.9[a]	46.6±2.1[a]
Alginate sterilization		
filtration	82.1±2.2[a]	48.3±1.7[a]
autoclaving	64.8±2.9[b]	39.7±2.6[b]

Values within column bearing the same letter do not differ significantly (p≤0.05, HSD).

PROTOPLAST DIVISION FREQUENCY

On day 4 of culture the protoplasts began to change in shape and the first mitotic divisions occurred. On day 5 the percentage of dividing protoplasts was highest in for cv. Sława z Gołębiewa (10.9±1.4%); only 0.3–2% of the protoplasts of the other cultivars underwent division (Tab. 2). Up to day 5 of culture the mitotic activity of protoplasts did not differ between protoplast sources or alginate sterilization methods, but later there were noticeable differences. On day 15 of culture, mitotic activity was highest in protoplasts of cv. Sława z Gołębiewa and Reball F1. Over this period, division was highest among protoplasts isolated from hypocotyls (21.3±1.6%) and those embedded in filtrated alginate (21.7±2.2%).

The interaction between the tested factors also affected the frequency of protoplast division (Tab. 3). Generally, the frequency of division was highest for cv. Reball F1, and the mitotic activity of its protoplasts embedded in filtrated alginate was more than three times higher (36.1±6.8%) than those cultured in autoclaved alginate (10.9 ±5.0%). Filtration of alginate also benefitted protoplasts of cv. Kamienna Głowa, which showed more than six times higher division frequency. Protoplasts of cv. Sława z Gołębiewa were less sensitive to the applied treatments: mitotic activity was similar in the two treatments and was relatively high (26–30%). Both leaf and hypocotyl-derived protoplasts divided more frequently (21.0 ± 2.6% and 22.4 ± 3.7% respectively) when embedded in filtrated alginate.

TABLE 2. Effect of accession, protoplast source and alginate sterilization method on *B. oleracea* protoplast division frequency

Factor	Division frequency [% ± SE]	
	5th day	15th day
Cultivar		
Kamienna Głowa	1.8±0.5[b]	8.0±1.5[b]
Sława z Gołębiewa	10.9±1.4[a]	27.5±2.4[a]
Reball F1	0.3±0.1[b]	17.4±4.6[ab]
Source of protoplasts		
Leaves	4.5±1.0[a]	15.4±2.0[b]
Hypocotyls	6.2±1.0[a]	21.3±1.6[a]
Alginate sterilization		
Filtration	6.2±1.0[a]	21.7±2.2[a]
Autoclaving	4.5±1.0[a]	13.7±2.3[b]

Values within column bearing the same letter do not differ significantly (p≤0.05, HSD).

TABLE 3. Effect of interaction between alginate sterilization method and *B. oleracea* cultivar or protoplast source on division frequency at day 15 of culture

Factor	Division frequency [% ± SE] of protoplasts immobilized in alginate	
	filtered	autoclaved
Cultivar		
Kamienna Głowa	12.6±2.3[c]	2.1±0.8[d]
Sława z Gołębiewa	30.6±2.4[b]	26.0±3.4[b]
Reball F1	36.1±6.8[a]	10.9±5.0[c]
Source of protoplasts		
leaves	21.0±2.6[a]	9.0±2.9[c]
hypocotyls	22.4±3.7[a]	13.8±3.4[b]

Values bearing the same letter do not differ significantly (p≤0.05, HSD).

TABLE 4. Shoot regeneration from protoplast-derived callus colonies of *B. oleracea*

Cultivar	Shoots [% ± SE] regenerated from protoplast isolated from:	
	leaves	hypocotyls
Kamienna Głowa	7.7±0.6[b]	12.9±1.0[b]
Sława z Gołębiewa	0.0±0.0[b]	5.2±2.2[b]
Reball F1	21.2±1.8[a]	13.0±1.8[b]
Mean	8.1±0.9[a]	8.6±1.5[a]

Values bearing the same letter do not differ significantly (p≤0.05, HSD).

SHOOT REGENERATION

Multiple-cell colonies became visible on the plates to the unaided eye at around week 4 of culture and then were transferred to regeneration media. Colonies started to differentiate and shoots to form about 4 weeks later. We found no differences in shoot regeneration between the two regeneration media used (p = 0.31), nor any differences in shoot regeneration between protoplast sources; for both leaf- and hypocotyl-derived callus colonies the overall shoot regeneration frequency was near 8% (Tab. 4). Shoot development frequency was highest for cv. Reball F1 from leaf protoplasts (21.2±1.8%). Leaf-derived callus colonies of cv. Sława z Gołębiewa failed to regenerate. For cv. Kamienna Głowa we found no differences in shoot regeneration between protoplast sources.

DISCUSSION

In plant protoplast culture, alginates are used in the form of beads and layers (Adaoha Mbanaso and Roscoe; 1982; Shillito et al., 1983). Further work has led to the development of thin alginate layers (TAL), successfully used in *Arabidopsis*, *Nicotiana*, *Daucus* and *Beta* (Dovzhenko et al., 1998; Dovzhenko et al., 2003; Maćkowska et al., 2014), and extra-thin alginate film (ETAF) used in *Rosa* sp. (Pati et al., 2008). The available reports focus mainly on the concentration of alginate and calcium ions determining polymerization, while sterilization methods are not clearly specified (see: Linsefors and Brodelius, 1985; Damm and Willmitzer, 1988; Asada and Shuler, 1989; Dirks et al., 1996). Leo et al. (1990) compared different methods of sodium alginate sterilization (autoclaving at 121°C for 15 min, ethylene oxide sterilization, γ-radiation) to assess its effect on gel strength, viscosity, particle diameter and *Penicillium chrysogenum* growth. They found high depolymerization and decreased gel viscosity at temperatures above 100°C. The alginate samples exposed to γ-radiation formed weak deformed beads. Autoclaving and ethylene oxide sterilization yielded similar alginate polymer integrity but doubts about the safety of ethylene oxide sterilization favored autoclaving. *P. chrysogenum* growth in ethylene oxide-sterilized and γ-radiation-sterilized gel resulted in a non-uniform distribution of biomass. Microbial growth was higher in autoclaved alginate. Wong et al. (2001) investigated the factors influencing alginate properties with the aim of developing stable alginate/chondrocyte constructs for orthopedics; they studied the effect of biochemical composition (high, intermediate, low guluronic acid content) and sterilization method (0.2 μm syringe filter, autoclaving at 120°C for 20 min). In their study, alginate containing the highest percentage of guluronic acid was the most mechanically stable but often yielded lower matrix synthesis. Autoclaving caused a 50–60% decrease in the equilibrium modulus of the low-G alginates versus non-sterilized alginate, and a 20% decrease for the high-G alginate. Filter sterilization had no effect on the equilibrium modulus.

In this study we used low-viscosity sodium alginate. The strength of the gel varies with the amount of metal ions present in the solidifying medium (Draget et al., 2005). We applied 40 mM $CaCl_2$, enough for crosslinking and formation of a well-polymerized, stable gel. The autoclaved alginate solution was turbid and cloudy, and the filter-sterilized alginate solution was transparent. Filtration removes some contaminants such as proteins and polyphenols (Smidsrød and Skjåk-Bræk, 1990). The higher viability and increased division frequency we observed in protoplasts cultured on filter-sterilized alginate might be due to its higher purity.

Papers that specify the method of alginate sterilization mostly report the use of autoclaving. The results vary from species to species: for example, protoplast division frequency of 4–22% in *Nicotiana tabacum* (Adaoha Mbanaso and Roscoe, 1982), 11–75% in *Daucus carota* (Grzebelus et al., 2012), 17–92% in *Citrus sinensis* (Niedz, 1993) and 20–30% in *Beta vulgaris* (Hall et al., 1993). In reports specifying filter sterilization of alginate, plating efficiency was 1–30% in *Arabidopsis thaliana* (Dovzhenko et al., 2003), 6–78% in *Nicotiana tabacum* (Dovzhenko et al., 1998) and 0–35% in *Nicotiana sanderae* (Draget et al., 1988). It is difficult to compare the effects of alginate sterilization method on protoplast culture, since the cited studies use different species, embedding techniques (layers/beads) and culture media, all of which affect the results. Maćkowska et al. (2014) compared the two methods of alginate sterilization for protoplast embedding in cultivated and wild carrots. They showed a positive effect of alginate filter-sterilization on protoplast plating efficiency but the effect was also strongly genotype-dependant. Our results in *Brassica oleracea* cultivars are in line with their findings, unambiguously showing that alginate sterilization by filtration was superior to autoclaving, for both protoplast viability and division frequency.

Protoplast culture is usually most successful when the culture medium is changed (Glimelius, 1984; Pelletier et al., 1983). Protoplast culturing in the system presented here allows easy exchange of the medium. The medium was simply removed with a pipette without damaging the cultured protoplasts, and fresh medium was added. The transparency of the obtained alginate layers facilitated microscopy observations and permitted quick and unambiguous identification of contamination. Some authors recommend hardening the alginate gels on ice (Damm and Willmitzer, 1988) or at low temperature (4–10°C) (Asada and Shuler, 1989). In our study that was not necessary.

Colony formation is usually established after 20–30 days. Once solidified, the alginate gel can be easily reliquifided by helation of calcium ions. This allows recovery of colonies developing from the embedded protoplasts without exposing them to excessive stress (Draget et al., 1988; Soderquist and Lee, 2005). The mean regeneration frequencies reported for *Brassica* spp. are usually higher from mesophyll protoplasts (0–55%) than from hypocotyls (0–30%) (Kirti et al., 2001; Ren et al., 2000). In the present study there were no differences in regeneration between protoplast sources. The regenerated plants were normal. They rooted, showed no variegation, and were easy to acclimatize.

CONCLUSIONS

Alginate is considered highly sensitive to thermal degradation, which alters its molecular structure and affects its mechanical stability. The presented method of alginate solution preparation and polymerization, combined with filter-sterilization, is advantageous in several respects: it is simple to perform, does not cause heat shock to the cultured cells, does not alter the molecular structure of the alginate, and produces gels of high purity and good viscosity. Leaf- and hypocotyl-derived *Brassica oleracea* protoplasts were successfully immobilized in filter-sterilized alginate layers and showed higher viability and division frequencies than those cultured in autoclaved alginate. This method might be tried in protocols to stimulate protoplast development in recalcitrant genotypes (e.g., Leguminosaceae family, woody species), as it is easy to perform, nontoxic, and does not involve the risk of cytological instability of cultured single cells under the influence of unbalanced concentrations of plant growth regulators (Carimi et al., 2003; Kwasniewska et al., 2012).

AUTHORS' CONTRIBUTIONS

KA concept, design of the experiment, statistical analysis, writing and preparing the manuscript; AA interpretation of the results, critical reading of the manuscript.

The authors declare that they have no conflicts of interest.

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