



Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench

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Abstract

In the present study, a high yield of isolated protoplasts from the agronomically important crop *Fagopyrum esculentum* was obtained by applying a mixture of cellulase, pectolyase, and driselase. We demonstrated that the yield of morphogenic callus-derived protoplasts was 1×10^6 protoplasts per g of fresh tissue. For hypocotyls used as the protoplast source, the number of released cells was twice lower. The protoplasts, embedded in an agarose matrix and cultured in a modified Kao and Michayluk media supplemented with phytosulfokine, re-enter the cell cycle and start to develop, forming microcalli. The plating efficiency was about 20% in the case of hypocotyl- and morphogenic callus-derived protoplasts. For plant regeneration, the medium was supplemented with different combinations of cytokinin. Somatic embryogenesis and organogenesis occur during the cultivation of the protoplast-derived tissues, depending on the applied protoplast source. For the first time, an effective protoplast-to-plant system for *F. esculentum* has been developed.

Key message

Morphogenic callus- and hypocotyl-derived protoplasts of buckwheat after embedding in agarose beads and culture in phytosulfokine enriched medium regenerated into plants.

Keywords Buckwheat · Growth regulators · Organogenesis · Plating efficiency · Somatic embryogenesis

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
BM	Basal medium
CM	Callus multiplication medium
CPPU	<i>N</i> -(2-chloro-4-pyridyl)- <i>N'</i> -phenylurea
FDA	Fluorescein diacetate
FW	Fresh weight
IBA	Indole-3-butyric acid
KIN	Kinetin
LMPA	Low melting point agarose
MC	Morphogenic callus
NAA	1-Naphthalenacetic acid
PGRs	Plant growth regulators
PSK	Phytosulfokine- α
PUT	Putrescine
PVP	Polyvinylpyrrolidone
RM	Regeneration medium
RT	Room temperature
TDZ	Thidiazuron

Introduction

The genus buckwheat (*Fagopyrum*) is a promising functional food source that contains various phenolic compounds, especially rutin, quercetin and C-glycosylflavones (orientin, isoorientin, vitexin), which have a positive therapeutic or dietary effect for promoting human health (Zhang et al. 2015). To date, 23 buckwheat species have been identified (Tomasiak et al. 2022). One of the most important species is *Fagopyrum esculentum* Moench. (common buckwheat), a multipurpose crop with a high nutritional value, mainly high-quality proteins with essential amino acids (Woo et al. 2010). Common buckwheat is also considered a nectariferous and pharmaceutical plant (Kwon et al. 2013). As it has the ability to do well on unproductive soils and does not require extensive fertilisation, common buckwheat is an attractive economic crop and low-cost supplement to cereal grains (Kumar and Saraswat 2018). The biggest problem with common buckwheat is the short life of its single flower (1 day) (Cawoy et al. 2009) and very short growing period (70–90 days). Moreover, the sensitive to ground frost, high temperatures and drought may cause strong flower and embryo abortions. So far, buckwheat *F. homotropicum* has been cross-pollinated with *F. tataricum* (Tartary buckwheat) or *F. tataricum* with *F. esculentum* in order to transfer genes with a greater resistance to frost and a higher seed yield. Because of the strong barriers that prevent cross-pollination between different species, these studies have been unsuccessful (Shaikh et al. 2001; Woo et al. 2001). Common buckwheat forms dimorphic plants with flowers whose pistils and stamens have different lengths (pin and thrum types), which results in self-incompatibility (Adachi 1990; Cawoy et al. 2009). Therefore, fertilisation occurs between both of these flower types after cross-pollination (Cawoy et al. 2006; Taylor and Obendorf 2001). Among the most important reasons for the low yield of common buckwheat are: (1) self-incompatibility; (2) insufficient fertilisation; (3) embryo abortion; (4) sensitivity to heat and drought stress; and (5) deficiency of the assimilates that occur in ageing plants (Slawinska and Obendorf 2001; Taylor and Obendorf 2001).

Plant biotechnology techniques, specifically tissue and cell cultures, represent a solution to overcome the reproductive barriers for this species. In previous studies related to Tartary and common buckwheat regeneration, the most widely used explants have been cotyledon and hypocotyl segments from seedlings (Adachi et al. 1989; Hou et al. 2015; Kwon et al. 2013; Lachmann 1990). Explants from mature plants, such as petioles, leaves and nodes, have also been used (Slawinska 2009; Woo et al. 2004). The successful regeneration of common buckwheat has been

previously reported via organogenesis or somatic embryogenesis using different variants of plant growth regulators (PGRs) (Kwon et al. 2013; Nešković et al. 1987). The application of protoplast cultures guarantees the unicellular origin of the somatic embryos; thus, the recovery of genotypes with novel traits would be favoured. The processes of protoplast isolation from common buckwheat plants have been improving for decades since the first attempt by Holländer-Czytko and Amrhein (1983). Adachi et al. (1989) reported for the first time about plant regeneration from hypocotyl-derived protoplasts of common buckwheat. However, they obtained low plating efficiency (approx. 1%) and abnormal morphology of the regenerated plants. Likewise, Gumerova (2004) achieved plant regeneration from hypocotyl-derived protoplast cultures, but the regenerative capacity of the protoplast-derived callus was low. On the other hand, Lachmann (1990) managed to obtain higher plating efficiency from hypocotyl-derived protoplasts of Tartary buckwheat, but plant regeneration was not induced. As it was mentioned by Woo et al. (1999), it is possible to isolate protoplasts from sperm cells of common buckwheat what can be useful in the case of protoplast fusion. Also hypocotyl-derived protoplasts were applied by Sakamoto et al. (2020) as a valuable tool for analysis of gene function.

As described above, hypocotyls have been commonly used as a source of protoplasts. However, common buckwheat's morphogenic callus (MC), due to its high regenerative potential, may be a desirable source of protoplasts (Takahata and Jumonji 1985; Yamane 1974). However, using the MC as a source of protoplasts has been little studied (Gumerova 2004). Therefore, in this work, we proposed an efficient protoplast-to-plant regeneration system of common buckwheat via callus formation starting with hypocotyls and the MC as the protoplast source.

Materials and methods

Plant materials for protoplast isolation

For MC induction and the development of etiolated hypocotyls, commercially available seeds of the Panda cultivar (the Malopolska Plant Breeding, Poland) were used. The callus lines (L1 and NL2) were obtained from immature zygotic embryos in the dark at 26 ± 1 °C on a RX medium as previously described (Betekhtin et al. 2019, 2017; Rumyantseva et al. 2005) and maintained with regular subcultures every 2–3 weeks on fresh RX medium composed of Gamborg B5 including vitamins (Gamborg et al. 1968), 2 g L⁻¹ N-Z-amine A, 2 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg L⁻¹ kinetin (KIN), 0.5 mg L⁻¹ 3-indoleacetic acid (Sigma-Aldrich, USA), 0.5 mg L⁻¹ 1-naphthaleneacetic acid

(NAA), 25 g L⁻¹ sucrose and 7 g L⁻¹ phyto agar (Duchefa, Netherland).

For hypocotyl development, the seeds were kept in distilled water overnight, and the seed coat was then removed. The surface sterilisation of the seeds was carried out in a three-step protocol: (1) the seeds were soaked in 70% ethanol for 30 s and then shaken (160 rpm for 30 min) in a 0.2% (v/v) solution of Scorpion 325 SC fungicide (Sygenta, Switzerland) with a drop of Tween 20 (Duchefa); (2) the seeds were immersed in a 20% (w/v) solution of chloramin T (sodium N-chlorotoluene-4-sulphonamide; Chempur, Poland) with 3000 mg L⁻¹ cefotaxime disodium (Duchefa) and one drop of Tween 20 for 30 min; between each step, the seeds were dipped in 70% ethanol for 30–45 s; (3) the seeds were washed three times with sterile distilled water for 5 min each time and soaked overnight in sterile distilled water. The next day, the second and third steps were repeated. After two days of sterilisation, the seeds were air-dried on sterile filter paper and placed in polystyrene Petri dishes (Ø9 cm) with

a MS medium supplemented with vitamins (Murashige and Skoog 1962), 200 mg L⁻¹ cefotaxime disodium, 30 g L⁻¹ sucrose and 7 g L⁻¹ plant agar (Duchefa). The Petri dishes were sealed with parafilm and incubated in the dark at 26 ± 1 °C for ten days.

Protoplast isolation and culture

The protoplasts were isolated from two types of source materials including around 12-day-old MC and 10-day-old hypocotyls. First, a pre-plasmolysis step was performed. One gram of 8-day-old MC L1 line or 2 g of 12-day-old NL2 line were incubated with PSII/F solution (Table 1) in a glass Petri dish (Ø9 cm). For the hypocotyls, 1 g of plant material was cut into 1 cm long pieces, and these were cut longitudinally in PSII solution (Table 1). In both cases, the pre-plasmolysis step took place in the dark at room temperature (RT) for 1 h. After this, the solution was removed, and enzymatic maceration was carried out by adding the

Table 1 Solutions used for protoplast isolation from morphogenic callus and hypocotyls of *Fagopyrum esculentum*

Solution	Composition	Concentration	g L ⁻¹
PSII/F (pH 5.6)	Mannitol (Duchefa, Netherland)	0.60 M	109.30
	CaCl ₂ (Sigma-Aldrich, USA)	5 mM	0.55
PSII (pH 5.6)	Mannitol	0.50 M	91.20
Enzyme solution E1 (pH 5.6)	Mannitol	0.60 M	109.40
	MES ^a Buffer (Sigma-Aldrich)	20 mM	3.90
	MgCl ₂ × 6H ₂ O (Sigma-Aldrich)	5 mM	1.00
	Cellulase Onozuka R10 (Duchefa)	1%	10.00
	Pectolyase Y-23 (Duchefa)	0.10%	1.00
Enzyme solution E2 (pH 5.6)	Mannitol	0.60 M	109.40
	MES Buffer	20 mM	3.90
	MgCl ₂ × 6H ₂ O	5 mM	1.00
	Driselase (Sigma-Aldrich)	0.15%	1.50
	Cellulase Onozuka R10	1.50%	15.00
	Pectolyase Y-23	0.10%	1.00
Enzyme solution E3 (pH 5.6)	Mannitol	0.60 M	109.40
	MES Buffer	20 mM	3.90
	MgCl ₂ × 6H ₂ O	5 mM	1.00
	Driselase	0.25%	2.52
	Cellulase Onozuka R10	1%	10.00
	Macerozyme R10 (Duchefa)	0.60%	6.00
Suc/MES (pH 5.8)	Sucrose	0.50 M	171.20
	MES buffer	1 mM	0.20
Suc-2/MES (pH 5.8)	Sucrose	0.60 M	205.40
	MES buffer	1 mM	0.20
W5 (pH 5.8)	NaCl (Sigma-Aldrich)	154 mM	9.00
	CaCl ₂ × 2H ₂ O (Sigma-Aldrich)	125 mM	18.36
	KCl (Sigma-Aldrich)	5 mM	0.37
	Glucose (Sigma-Aldrich)	5 mM	0.90

^aMES 2-(N-Morpholino) ethanesulfonic acid hydrate

enzyme solution E1, E2 or E3 (Table 1) for the L1, NL2 and hypocotyls, respectively. This step was performed overnight for 16 h with gentle shaking (50 rpm at RT) in the dark. The quality of the released protoplasts was checked using an inverted microscope (Axiovert S100; Carl Zeiss, Germany). The suspension was filtered using nylon filters (mesh size 100 µm; Millipore, USA) and then centrifuged for 5 min (1000 rpm at RT). The pellet was re-suspended in a Suc/MES solution (Table 1) for the callus or a Suc-2/MES solution (Table 1) for the hypocotyls, then W5 solution (Table 1) was carefully overlaid and it was centrifuged for 10 min (1200 rpm at RT). The ring of viable protoplasts, formed at the interphase of these two solutions, was collected in a new centrifuge tube. The collected protoplasts

were dissolved in W5 solution and centrifuged for 5 min (1000 rpm at RT). Next, the supernatant was removed, and the culture medium added. The culture medium was based on CPP medium according to Dirks et al. (1996) and supplemented with 1.0 mg L⁻¹ 6-benzylaminopurine (BAP) and 2.0 mg L⁻¹ NAA, and then called the basal medium (BM; Table 2). The density of the protoplasts was determined using a Fuchs-Rosenthal haemocytometer (Heinz Herenz, Germany) and then adjusted with the BM to 8 × 10⁵ or 5 × 10⁵ cells per ml for the MC lines and hypocotyls, respectively. The protoplasts were embedded in a filter-sterilised solution of 1.2% (w/v) low melting point agarose (LMPA; Duchefa), according to Grzebelus et al. (2012b). The mixture of protoplasts and agarose was dropped at a rate of four

Table 2 Media used for protoplast cultures, callus proliferation, and plant regeneration of *Fagopyrum esculentum*

Solution	Composition	Concentration	Weight L ⁻¹
Liquid basal medium (BM) for protoplast cultures (pH 5.6)	KM ^a macro-, micro-elements (Duchefa)	–	3.60 g
	KM organic acids ^b	–	see below
	Myo-inositol (Duchefa)	5.50 M	0.10 g
	Thiamine (Sigma-Aldrich)	3.70 M	10.00 mg
	Pyridoxine (Sigma-Aldrich)	6 M	1.00 mg
	Nicotinic acid (Sigma-Aldrich)	8 M	1.00 mg
	Glucose (Sigma-Aldrich)	0.40 M	74.00 g
	N-Z-amine A (Sigma-Aldrich)	4.60 M	0.25 g
	BAP (Sigma-Aldrich)	4.40 M	1.00 mg
	NAA (Sigma-Aldrich)	1 M	2.00 mg
Callus multiplication medium (CM) (pH 5.8)	MS ^c including vitamins (Duchefa)	–	4.40 g
	N-Z-amine A	3 mM	2.00 g
	2,4-D (Sigma-Aldrich)	9 M	2.00 mg
	KIN (Sigma-Aldrich)	9 M	0.20 mg
	PSK (PeptaNova GmbH, Germany)	100 nM	–
	Sucrose (POCH, Poland)	87 mM	30.00 g
	Phytigel (Sigma-Aldrich)	–	3.00 g
Regeneration medium (RM_MS3) (pH 5.8)	MS basal salt mixture (Duchefa)	–	4.30 g
	Sucrose	87 mM	30.00 g
	BAP	1.30 M	3.00 mg
	TDZ (Sigma-Aldrich)	4.50 M	1.00 mg
	Phytigel	–	3.00 g
Regeneration medium (RM_MS4) (pH 5.8)	MS basal salt mixture	–	4.30 g
	Sucrose	87 mM	30.00 g
	BAP	8.8 M	2.00 mg
	KIN	4.6 M	1.00 mg
	PVP (MW 40,000; Sigma-Aldrich)	0.0025%	0.025 g
	Phytigel	–	3.00 g
Rooting Medium (pH 5.8)	MS including vitamins	–	4.40 g
	Sucrose	87 mM	30.00 g
	Phytigel	–	3.00 g

^aKM (Kao and Michayluk 1975)

^bacc. to Kao and Michayluk (1975): sodium pyruvate 20 mg L⁻¹, citric acid 40 mg L⁻¹, malic acid 40 mg L⁻¹, fumaric acid 40 mg L⁻¹

^cMS (Murashige and Skoog 1962)

beads per Petri dish (\varnothing 6 cm). After solidification of the agarose beads, the BM was added to each dish and the medium was additionally supplemented with 100 nM phytosulfokine (PSK), 0.25–0.75 mg L⁻¹ chloropyridin phenylurea (CPPU; Sigma-Aldrich), 8 mg L⁻¹ putrescine (PUT; Sigma-Aldrich) or 0.025–0.05% polyvinylpyrrolidone (PVP, MW 40,000) in different combinations. To prevent bacterial contamination, the culture media of hypocotyl-derived protoplasts were supplemented with 200 mg L⁻¹ cefotaxime disodium. The protoplast cultures were incubated at 26 ± 1 °C in the dark for 60 days. The medium, with all supplements, was renewed on the 10th day of culture.

Plant regeneration

After two months of protoplast culture, agarose beads overgrown with the protoplast-derived callus was transferred to a callus multiplication medium (CM; Table 2). The cultures were incubated at 26 ± 1 °C in the dark and subcultured every three weeks on the same CM. Next, the callus was transferred to the regeneration medium (RM; Table 2) and maintained in a climate room at 28 ± 1 °C with a 16/8 h (light/dark) photoperiod (a light intensity of 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$; fluorescent lamps Sylvania Gro-lux T8, USA). The RM_MS3 regeneration medium was used for the protoplast-derived callus originating from the NL2 line, while the regeneration of the protoplast-derived callus originating from the L1 line and hypocotyls was carried out on the RM_MS4 medium (Table 2). Initially, the NL2 line was also tested on RM_MS4 medium; however, there was no evidence of regeneration after several months of subculture. The NL2 callus-derived somatic embryos were separated, transferred to the RM_MS3 medium and subcultured every two weeks in the same medium until shoots were obtained. The callus originating from the L1 line and hypocotyls were subcultured every three weeks on RM_MS4 medium. For rooting, shoots were transferred to sterile vessels (150 mm L \times 90 mm W) with rooting medium (Table 2) and maintained in a climate room at 25 ± 1 °C with a 16/8 h (light/dark) photoperiod (a light intensity of 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$; fluorescent lamps Sylvania Gro-lux T8, USA). When the roots had grown large enough, the plants were transferred to a moss-coconut fiber substrate (Ceres International Ltd., Pyzdry, Poland) and placed in greenhouse conditions at 25 ± 1 °C, 16/8 h (light/dark) photoperiod (light intensity 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Histological analysis

The fixation was carried out following the methodology proposed by Betekhtin et al. (2019). The calli derived from protoplasts were fixed in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in phosphate-buffered saline (PBS)

overnight at 4 ± 1 °C. The samples were washed with PBS, followed by a dehydration process in increasing ethanol concentrations. Next, the samples were embedded in LR White resin (London Resin, St. Louis, USA) and left to polymerise for 24–48 h at 58 ± 1 °C. The samples were then cut into 1.5 μm thick sections using an EM UC6 ultramicrotome (Leica Biosystems, Wetzlar, Germany) and placed on glass slides coated with poly-L-lysine. The slides were stained with 0.05% Toluidine Blue O (Sigma-Aldrich) for 5 min and washed twice with distilled water. The stained sections were examined under an Olympus BX43F microscope equipped with the Olympus XC50 digital camera.

Data collection and statistical analysis

The protoplast yield was presented as the protoplast number per gram of fresh weight (FW) in 1 ml of suspension. The viability of the protoplasts was assessed, immediately after embedding the cells in an agarose matrix, by staining with fluorescein diacetate (FDA; Sigma-Aldrich), according to Grzebelus et al. (2012a). The viability was expressed as a percentage of protoplasts with apple-green fluorescence out of the total observed cells. Plating efficiency was determined in 10-day-old cultures and expressed as a percentage of cell aggregates per total number of observed undivided cells and cell colonies. Microscopic observations were performed under an inverted Axiovert S100 microscope with a filter set appropriate for FDA visualisation ($\lambda_{\text{Ex}} = 485$ nm, $\lambda_{\text{Em}} = 515$ nm). Image acquisition was performed under an inverted Leica DMI8 microscope (Leica Microsystems, Germany) equipped with a Leica DFC 7000 T camera conjugated with LAS X Extended Depth of Field and Deconvolution Modules.

At least three independent protoplast isolation experiments with a single treatment represented by three to four Petri dishes were carried out as repetitions. Microscopic observations were carried out on 100 cells per Petri dish. The mean values and standard errors were calculated. The overall effect of treatments was determined using analysis of variance (ANOVA) in Statistica ver. 13 (TIBCO Software Inc., USA) at $P \leq 0.05$. Tukey's honestly significant difference test was used to determine significant differences between the means.

Results

Plant materials

For protoplast isolation, two lines of the MC (L1, NL2; Fig. 1a, b) and etiolated hypocotyls (Fig. 2a) were used. The calli lines differed in age (L1, two-years-old; NL2, one-year-old). A dense globular milky-white structure characterises

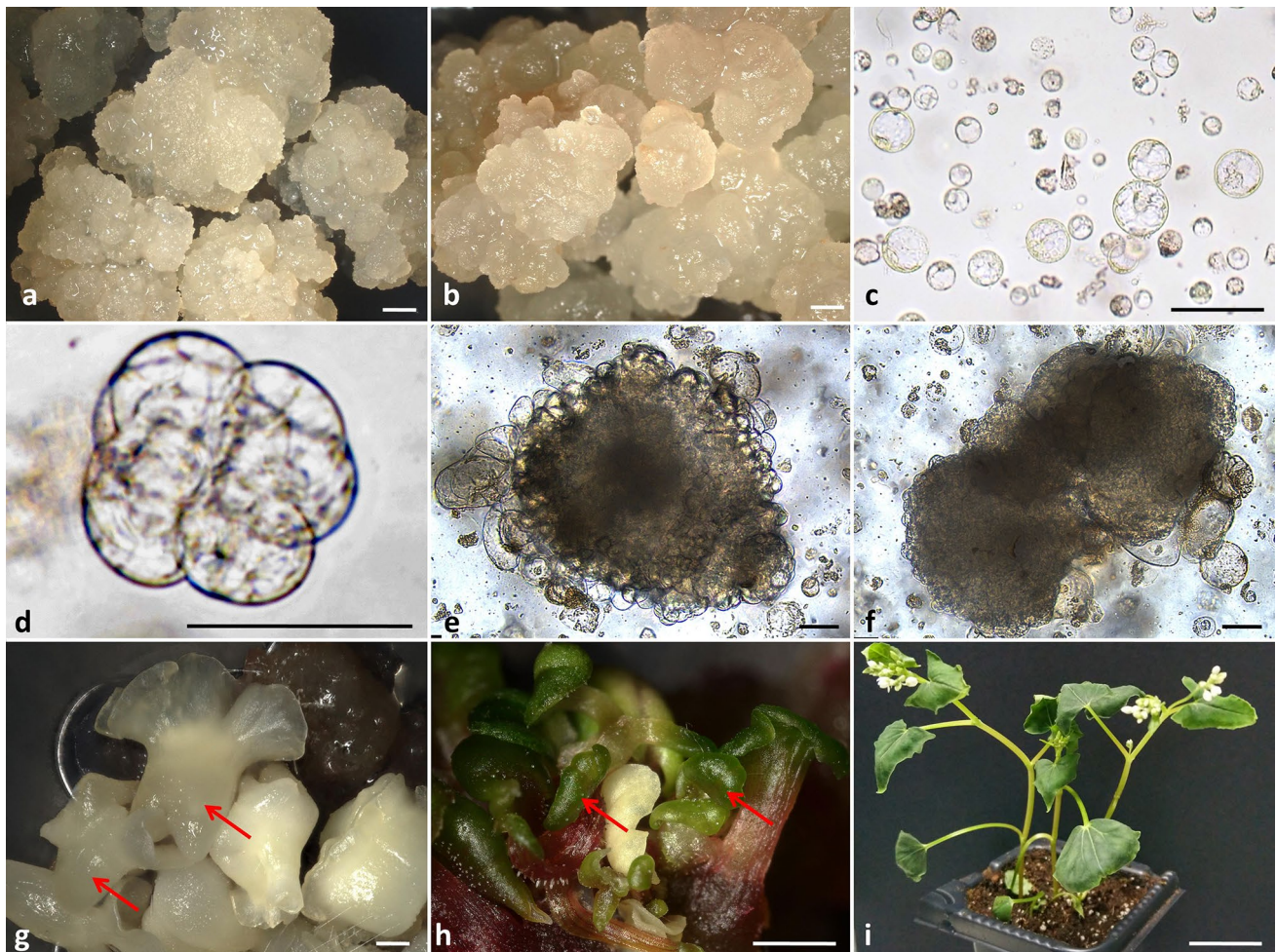


Fig. 1 Plant regeneration from morphogenic callus (MC)-derived protoplasts in *Fagopyrum esculentum*. Lines of MC used for protoplast isolation: **a** L1 and **b** NL2; **c** freshly MC-derived protoplasts; multicellular aggregate in **d** 6-, **e** 20- and **f** 30-day-old protoplast cultures; subsequent stages of plant regeneration from NL2 line-derived protoplast cultures on the regeneration medium: **g** formation

of somatic embryos (examples shown by arrows) in 6-day-old cultures and **h** somatic embryos converted into plants (examples shown by arrows) in 15-day-old cultures; **i** three-month-old protoplast-derived-flowering plant acclimatised to *ex vitro* conditions. Scale bars: 0.5 mm (**a–b**), 100 µm (**c**), 50 µm (**d–f**), 0.5 mm (**g**), 1 mm (**h**), 1.5 cm (**i**)

this type of calli due to the accumulation of starch grains (in the cytoplasm of storage cells). The surface of the calli is covered by an epidermal-like layer under which the meristematic cells and parenchymatous cells are located.

Protoplast isolation efficiency and viability

After overnight incubation in the enzyme solution, spherical protoplasts from both MC lines and hypocotyls were successfully released (Fig. 1c, 2b). The mean yield of MC-derived protoplasts varied from 0.83 to 1.54×10^6 (Table 3). The most efficient protoplast isolation was achieved for the NL2 line in the presence of E2 enzyme solution (1.54×10^6). The use of E1 enzyme solution reduced the number of released protoplasts by approximately half. The callus age

(between 8 and 12 days) had no effect on the protoplast isolation efficiency.

Although different enzyme solutions differing in enzyme activity and composition were used (Supplementary Table 1) in the preliminary experiments on protoplast isolation from hypocotyl tissue, the number of released cells was very low. Only after applying driselase to the enzyme solution was an adequate number of protoplasts recorded. Two concentrations of driselase for tissue digestion were tested, however, different numbers of released cells were not observed (Table 4). The average yield of hypocotyl-derived protoplasts was 0.44×10^6 per g FW. Nevertheless, the efficiency of protoplast isolation from hypocotyls was more than twice-fold lower than from MC sources ($P = 0.013$).

The quality of released MC-derived protoplasts assessed by FDA staining just after embedding in agarose varied

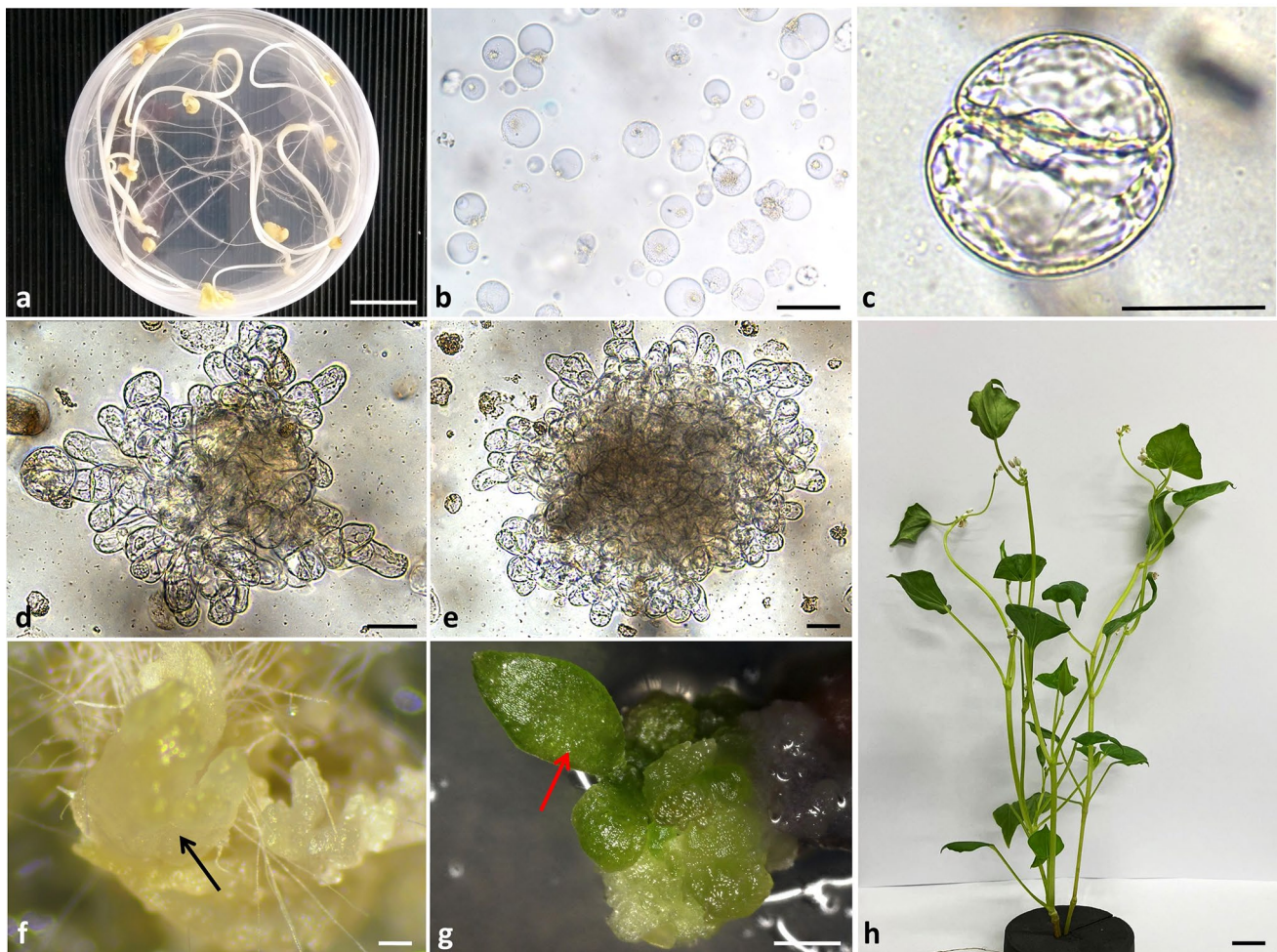


Fig. 2 Plant regeneration from hypocotyl-derived protoplasts of *Fagopyrum esculentum*. **a** 10-day-old etiolated hypocotyls used for protoplast isolation; **b** freshly hypocotyl-derived protoplasts; **c** 2-cell aggregate after the first mitotic division of the protoplast-derived cell; multicellular aggregate in **d** 10- and **e** 20-day-old protoplast cultures; **f** protoplast-derived microcalli with the somatic embryo (black arrow)

in two-month-old protoplast cultures; **g** shoot formation (red arrow) from the protoplast-derived callus after two weeks on the regeneration medium; **h** flowering protoplast-derived plant acclimatized to *ex vitro* conditions. Scale bars: 2 cm (**a**), 100 μ m (**b**), 50 μ m (**c–e**), 1 mm (**f–g**), 1 cm (**h**)

Table 3 Isolation efficiency and viability of protoplasts originating from morphogenic callus of *Fagopyrum esculentum*

Morphogenic callus line	Subculture intervals (days)	Age of donor material (days)	Enzyme solution	Protoplast yield ($\times 10^6$ /g FW)		Protoplast viability (%)	
				n	Mean \pm SE	n	Mean \pm SE
L1	21	10–12	E1	3	0.84 \pm 0.04 ^a	3	81.00 \pm 6.81 ^a
NL2	14	8	E1	3	0.83 \pm 0.25 ^a	3	73.90 \pm 6.61 ^a
NL2	14	12	E2	3	1.54 \pm 0.37 ^b	3	73.67 \pm 3.28 ^a
Mean/total				9	1.07 \pm 0.18	9	76.19 \pm 5.57

FW fresh weight, n number of independent protoplast isolations, SE standard error

E1: 1% Cellulase Onozuka R10 + 0.1% Pectolyase Y-23

E2: 1.5% Cellulase Onozuka R10 + 0.1% Pectolyase Y-23 + 0.15% Driselase

Means followed by the same letters within a column were not significantly different at $P \leq 0.05$

Table 4 Effect of driselase concentration on yield and viability of protoplasts originating from hypocotyls of *Fagopyrum esculentum*

Driselase concentration (%)	Protoplast yield ($\times 10^6$ /g FW)		Protoplast viability (%)	
	n	Mean \pm SE	n	Mean \pm SE
0.10	3	0.46 \pm 0.02 ^a	3	80.67 \pm 4.84 ^a
0.15	3	0.43 \pm 0.01 ^a	3	69.33 \pm 2.85 ^a
Mean/total	6	0.44 \pm 0.01	3	75.00 \pm 3.57

FW fresh weight, n number of independent protoplast isolations, SE standard error

Complete composition of enzymes in E3 enzyme solution used for hypocotyl digestion: 1% Cellulase Onozuka R10+0.6% Macerozyme R10+0.1–0.15% Driselase

Means followed by the same letters within a column were not significantly different at $P \leq 0.05$

from 73 to 81% (Table 3). Higher protoplast viability was recorded for the L1 line (81%) compared to NL2 line (74%). However, significant differences in protoplast viability were not observed after applying different enzyme mixtures and the callus lines used. The viability of hypocotyl-derived protoplasts reached an average of 75% (Table 4). The higher protoplast viability (80%) was noted after using 0.1% driselase in the enzyme solution. The higher concentration of driselase (0.15%) resulted in lower viability of hypocotyl-derived protoplasts (69%). Nevertheless, the observed differences were not statistically significant.

Development of protoplast cultures

Protoplasts from all source materials revealed the ability to undergo cell division after PSK was applied to the BM. The first division occurred between the third and seventh day of the culture (Fig. 2c), then the next ones took place resulting in the formation of multicellular aggregates (Fig. 1d, 2d–e) in around 10-day-old protoplast cultures. The plating efficiency of the MC L1 line depended on the culture medium variant used and was from 23 to 35% (Fig. 3a). However, no significant differences were observed. PVP was also applied to the culture media to absorb toxic metabolites and phenolic compounds and to support the development of the cells, but a clear positive effect of PVP on the development of protoplast cultures was not recorded.

In preliminary experiments with the NL2 line, the E1 enzyme solution was used to release the cells, which resulted in low efficiency (about 9%) of cell aggregate formation in culture medium variants I, II, IV, V, and VI as shown in Fig. 3. Based on these results, the E2 enzyme solution was applied in the following experiments. The plating efficiency in protoplast cultures originating from callus digestion in E2 solution was from 21 to 25% (Fig. 3b), but the differences were statistically not significant. For the hypocotyl-derived

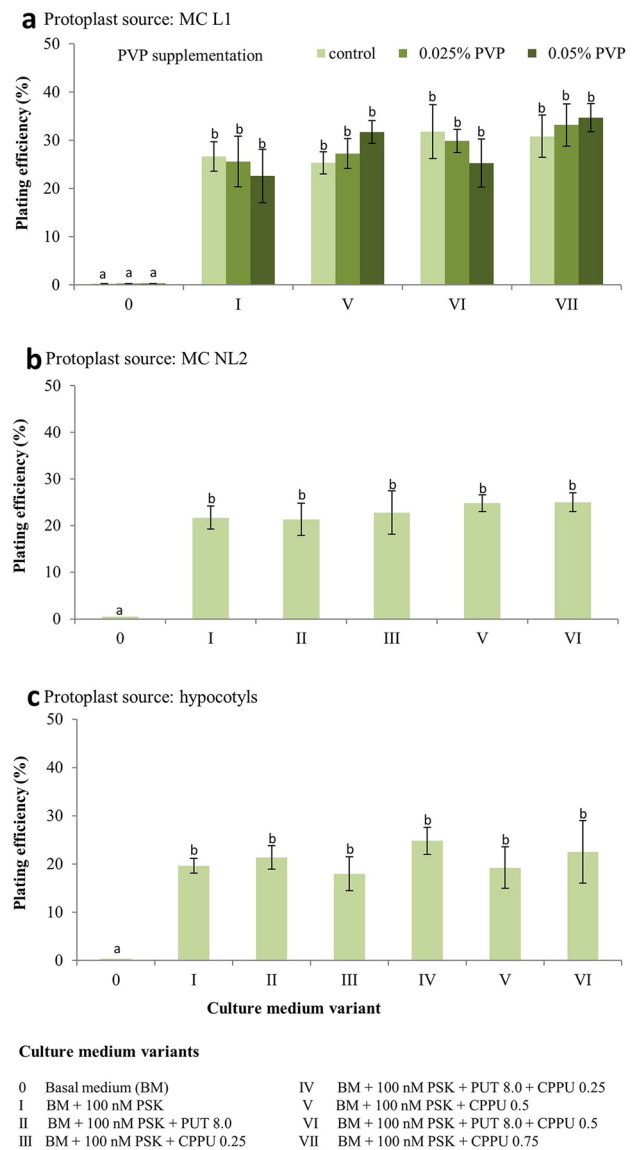


Fig. 3 Effect of different medium variants on plating efficiency in *Fagopyrum esculentum* 10-day-old protoplast cultures. Plant growth regulator composition in BM = BAP 1.0 mg L⁻¹ + NAA 2.0 mg L⁻¹; BAP = 6-Benzylaminopurine; NAA = naphthylacetic acid; 100 PSK = 100 nM phyto-sulfokine; CPPU 0.25, CPPU 0.5, CPPU 0.75 = 0.25, 0.5 or 0.75 mg L⁻¹ N-(2-chloro-4-pyridyl)-N'-phenylurea, respectively; PUT 8.0 = 8 mg L⁻¹ putrescine; 0.025, 0.05 PVP = 0.025% or 0.05% polyvinylpyrrolidone, respectively. Bars represent the means from three independent experiments \pm standard error. Means marked with the same letters were not significantly different at $P \leq 0.05$

protoplast cultures, the efficiency of cell aggregate formation was around 21% (Fig. 3c) in all culture medium variants used. A lower level of plating efficiency characterised the NL2 line and hypocotyl protoplast cultures compared to the L1 line ($P \leq 0.01$).

The continued growth of aggregates (Fig. 1e–f, 2e) resulted in the formation of microcalli for all protoplast

donor sources used. After 30 days of culture, microcalli originating from the NL2-derived protoplast cultures had overgrown the agarose beads and pro-embryogenic masses were observed. For the L1- and hypocotyl-derived protoplast cultures, agarose beads were overgrown by microcalli in 60-day-old cultures; moreover, somatic embryos were occasionally observed in the hypocotyl protoplast cultures (Fig. 2f).

Histological observations of callus-derived protoplast cultures

Histological observations revealed that the microcalli originating from the protoplasts of the L1 line were composed of parenchymatous cells with a big vacuole and an irregularly shaped nucleus with one or two nucleoli (Fig. 4a, inset 1). It should be noted that few phenolic-containing cells were detected on the surface of the callus (Fig. 4a, inset 2). Microcalli originating from protoplasts of the NL2 line were mainly composed of meristematic cells characterised by dense cytoplasm, small vacuoles and a round-shaped nucleus with one or two nucleoli (Fig. 4b, inset 1). Moreover, few phenolic-containing cells were observed on the surface of the microcalli (Fig. 4b, inset 2). Microcalli obtained from hypocotyl-derived protoplasts also contained phenolic-containing cells on the surface of the calli (Fig. 4c, inset 1). These microcalli were composed chiefly of parenchymatous cells with an irregularly shaped nucleus located near the cell wall (Fig. 4c, inset 2) and small regions of meristematic-like cells with two or three nucleoli and small vacuoles (Fig. 4c, inset 3).

Plant regeneration

Microcalli originating from all protoplast sources proliferated successfully on the CM medium. The L1 line was cultured on CM medium leading to the formation of dense globular calli that were transferred to the RM. After three months, yellow and brown calli were noted, and, sporadically, green or transparent-green shoot-like structures. After four months of regeneration, shoots started appearing, and plants developed. For the NL2 line, abundant growth of somatic embryos was observed in the first week of culture on the RM (Fig. 1g). After 15 days, green structures and some shoots (Fig. 1h) developed. To develop strong root system plants from all callus protoplast sources were kept in a rooting medium for 4 to 5 weeks and then successfully transferred to soil (Fig. 1i). The tissue derived from hypocotyl protoplast cultures doubled in mass after one month of cultivation on the CM medium. Plant regeneration occurred via somatic embryogenesis (Fig. 2f) or organogenesis (Fig. 2g). Scarce plant regeneration was noted after three months of regeneration (around nine plants from all the experiments undertaken) (Fig. 2h).

Discussion

Single cells like protoplasts may be applied in many fields, such as genetic manipulation, genome editing, the characterisation of plant genes, and somatic hybridisation (Grosser et al. 2010). Significantly, the last method may help overcome incompatibility and hybridisation barriers and develop

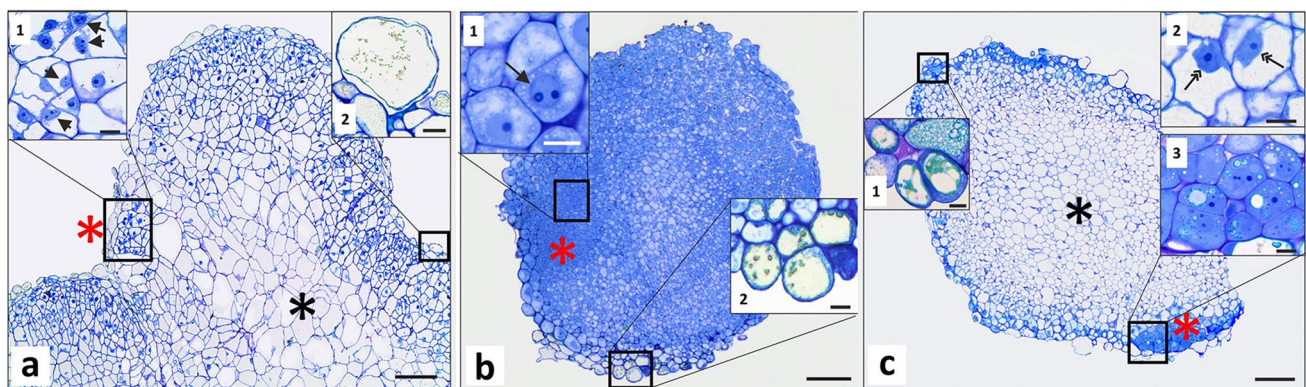


Fig. 4 Histological sections of protoplast-derived callus originating from morphogenic callus lines L1 (**a**), NL2 (**b**) and hypocotyls (**c**). **a** Calli originating from L1-derived protoplasts was composed chiefly of parenchymatous cells (black asterisk) with a large vacuole and an irregularly shaped nucleus with one or two nucleoli (red asterisk) with a nucleus with two or three nucleoli (inset 1); it also has a layer of phenolic-containing cells on the surface (inset 2). **b** Calli derived from protoplast cultures of the NL2 line presented abundant meristematic cells (red asterisk) with a nucleus with two nucleoli (inset 1)

and some phenolic-containing cells (inset 2). **c** Calli derived from the protoplasts of hypocotyls were characterised by the abundance of parenchymatous cells (black asterisk) with a large vacuole, a peripherally positioned nucleus (inset 2) and small regions of meristematic-like cells (red asterisk) with a nucleus with two or three nucleoli (inset 3). Black arrows indicate nuclei with two or three nucleoli; double black arrows indicate peripheral nuclei in parenchymatous cells. Scale bars: 10 μm (insets), 100 μm (**a**, **b**, **c**)

plant hybrids (Eeckhaut et al. 2013). As mentioned in the introduction, only a few attempts have been reported on the isolation and culture of protoplasts from common buckwheat (Adachi et al. 1989; Gumerova 2004; Rumyanzeva and Lozovaya 1988).

The establishment of a protoplast-to-plant system is affected by many factors, such as the protoplast isolation procedure, yield and quality of protoplasts, culture system, and medium composition (Rahmani et al. 2016). Several factors determine the satisfactory release of protoplasts from the source tissue. Protoplast source tissue, pre-treatment of the tissue before enzymatic maceration, composition of the enzyme solution, tissue digestion conditions, and the protoplast isolation method significantly affected the yield and viability of protoplasts. In the presented investigation, two lines of MC and hypocotyls of common buckwheat were used as the source material for protoplast isolation. The mean yield was around 1×10^6 or 0.44×10^6 protoplasts per g of FW released from the MC and hypocotyls, respectively. Adachi et al. (1989) mentioned releasing 5×10^6 protoplasts per 30 hypocotyls. Also, other materials such as the callus (Gumerova 2004; Rumyanzeva and Lozovaya 1988), mesophyll tissue (Lachmann 1994) and sperm cells (Woo et al. 1999) of common buckwheat were used for protoplast isolation. It has been noted that the authors did not mention the protoplast yield. We presumed that the differences in protoplast yield in our study might result from the different source tissue applied, the growth conditions, and the consequent differences in cell wall composition. Maceration of plant tissue and digestion of cell walls is connected with pectinase and cellulase activity (Noguchi et al. 1978) which characterise, for example, driselase (Kawai et al. 1979). Moreover, driselase had a better enzymolysis effect on the cell wall containing xylan, laminarin and cellulose (Ning et al. 2022). In our research, driselase was applied to improve cell wall digestion and increase tissue maceration. For the L1 line, the application of driselase was not necessary because of the lack of undigested tissue. However, for the NL2 line the beneficial effect of driselase application on the digestion activity and amount of released protoplasts was noted. Additionally, from the amount of enzyme mixture used to release protoplasts from hypocotyl tissue, driselase application resulted in a satisfactory number of obtained protoplasts. Similarly, in our previous work, protoplast isolation from the hypocotyls of Tartary buckwheat was possible after driselase treatment (personal communication). According to Kawai et al. (1979), driselase partially injures the cell wall of *Irpex lacteus* cotyledons and allows other enzymes to digest the source material. The application of driselase increases the protoplast yield of *Brassica oleracea* (Robertson and Earle 1986), *Spathiphyllum wallisii*, *Anthurium scherzerianum* (Duquenne et al. 2007) and *Kalanchoe blossfeldiana* (Castelblanque et al. 2009).

The establishment of an appropriate protoplast regeneration protocol is based on optimisation of the different culture conditions, such as the protoplast plating density, the type of cell culture system (e.g., embedding the cells in different gel matrixes) and the composition of the culture media (Davey et al. 2005b; Fehér and Dudits 1994). The overall protoplast density is crucial for cell wall regeneration and daughter cell formation (Davey et al. 2005b). According to Davey et al. (2005b), the typical range of protoplast density in the culture varied from 5×10^4 to 1×10^6 protoplasts per ml. The cell density applied for common buckwheat was from 10^4 to 10^5 or 5×10^4 cells per ml (Adachi et al. 1989; Rumyanzeva and Lozovaya 1988). In our study, a higher culture density (2.5×10^5 and 4×10^5 per ml) was applied than was optimal for protoplast development. In *Petunia hybrida* protoplast cultures (Kang et al. 2020), a plating density of 10×10^4 protoplasts per ml, in contrast to 5×10^4 , resulted in a higher frequency of division and the number of calli formed. Similar sightings were recorded by Adedeji et al. (2020) in *Chrysanthemum* cv. 'White ND' protoplast cultures. Furthermore, cultured protoplasts released growth factors that can stimulate the mitotic divisions of neighbouring cells (Davey et al. 2005b). Nevertheless, too high a cell density may result in the accumulation of phenolic compounds in the culture media leading to development of the culture stopping (Adedeji et al. 2020; Kang et al. 2020).

The protoplast embedding technique is the second factor that may significantly affect protoplast culture development. Embedding protoplasts in a semi-solid medium, such as agarose, enables the avoidance of cell agglutination that causes the accumulation of toxic substances, such as polyphenols, that may inhibit cell growth (Davey et al. 2005a; Deryckere et al. 2012). The gel matrix may affect membrane stabilisation by inhibiting lipid peroxidation and reducing metabolites and the diffusion of molecules essential for cell wall synthesis and protoplast division (Eeckhaut et al. 2013; Fehér and Dudits 1994). Furthermore, Deryckere et al. (2012) mentioned that the exchange of nutrients and gases may be more accessible due to the decreased concentration of the LMPA. The culture of Tartary buckwheat protoplasts in agarose beads in comparison to alginate layers had a positive impact on their development (personal communication). Following these results, an agarose embedding matrix was applied in the present study. LMPA beads were noted as a standard method for developing a protoplast-to-plant system in *Cichorium*. The authors noted that a solid or liquid medium was not optimal for protoplast cultures of the *Cichorium* genotypes used as the protoplasts burst and died. For the first time, this technique enables plant regeneration from protoplasts of *Cichorium endivia* genotypes (Deryckere et al. 2012). Also, *Ulmus americana* protoplasts did not survive in liquid or alginate bead culture systems compared to LMPA beads (Jones et al. 2015).

The optimal protoplast culture media may depend on the species, genotype, and source tissue used (Davey et al. 2005b). The appropriate nutrients, supplements, and PGRs are essential in protoplast cultures. Auxins and cytokinins are necessary for protoplast development (Davey et al. 2005b; Reed and Bargmann 2021). Most media are based on MS or B5 (Gamborg et al. 1968) compounds (Davey et al. 2005b); however, the type of PGRs and ratio may vary. In our study, the medium for protoplast cultures based on a Kao and Michaluk composition was applied and supplemented with NAA and BAP. For the development of protoplasts originating from the callus of common buckwheat, Gamborg's B5 mineral salts were added (Rumyanzeva and Lozovaya 1988). The authors reported the first mitotic divisions of protoplast-derived cells on days 6–7 of culture but further development of the culture was not observed. Adachi et al. (1989) applied MS salts to hypocotyl-derived protoplast cultures of common buckwheat and tested ten different compositions of PGRs and detected cell divisions after four days. In our investigation, a rich mineral-organic KM medium was applied, and the first cell divisions occurred after five days only after additional supplementation with PSK. We suggest the time differences for the first cell divisions might be due to the genotype used, tissue age, and composition of the culture medium. Applying mineral- and organic-rich media based on the KM formula even affected maintenance of the higher viability rate of *Beta vulgaris* protoplasts in contrast to the MS salt-based media (Grzebelus et al. 2012b). For *Kalanchoe*, the protoplast divisions were noted only for the KM medium; for the MS medium, the authors did not observe cell division (Cui et al. 2019).

A widespread way to enhance the mitotic divisions in protoplast cultures involves supplementing the culture medium with surfactants, polyamines, or artificial gases. This study shows the stimulating effect of the peptidyl growth factors, that is, PSK, on protoplast plating efficiency. For the first time, the positive effect of PSK was reported on *Asparagus officinalis* cell proliferation (Matsubayashi and Sakagami 1996). This sulphated peptide has been found to be effective for promoting cell division in suspension cultures of *Oryza sativa* (Matsubayashi et al. 1997) and protoplast cultures of *Beta vulgaris* (Grzebelus et al. 2012b), *Daucus* species (Mackowska et al. 2014), *Brassica oleracea* (Kiełkowska and Adamus 2017, 2019) and *Fagopyrum tataricum* (personal communication). Moreover, Grzebelus et al. (2012b) noted that PSK is able to reverse the recalcitrant behaviour of mesophyll protoplasts originating from *Beta vulgaris*. Apart from PSK, the polyamine PUT was tested. Polyamines regulate DNA replication, transcription, and translation, affecting cell division and differentiation (Davey et al. 2005b). They protect cells from the oxidative stresses generated during protoplast isolation and culture (Kiełkowska and Adamus 2021; Mackowska et al. 2014). However, in this study,

applying PUT was not found to have a significant effect on protoplast plating efficiency. Papadakis and Roubelakis-Angelakis (2005) noted PUT improves cell viability and plating efficiency and prevents the programmed cell death of protoplasts by decreasing the accumulation of superoxide. Huhtinen et al. (1982) demonstrated that the protoplast cultures of *Alnus glutinosa* and *A. incana* supported cell division and cell colony formation after the application of ornithine and PUT. Also, Kiełkowska and Adamus (2021) noted the increase in mitotic activity and shoot regeneration in protoplast cultures of *Brassica oleracea*. Similar to PUT, the application of CPPU, a urea-type synthetic cytokinin, did not increase the number of cell aggregates formed. It was noted that CPPU stimulates cell expansion and division during the development of the fruits of *Cucumis sativus* (Li et al. 2017) and *Actinidia arguta* (Kim et al. 2006). Moreover, the application of CPPU affects direct and secondary somatic embryogenesis (Bogdanovic et al. 2021; Murthy and Saxena 1994; Zhang et al. 2005). As phenolic compounds may negatively affect protoplast development, PVP was applied. However, no effect of PVP on the plating efficiency was observed in the present studies. Similar results were noted by Saxena and Gill (1986) and Reustle and Natter (1994). They did not see the apparent effect of PVP on guar and grapevine protoplast plating efficiency. To summarise, our data indicate that PSK is a powerful additional supplement enabling the development of common buckwheat protoplasts.

This study achieved the regeneration of common buckwheat from protoplasts isolated from different donor materials (MC and hypocotyls). Adachi et al. (1989) first attempted to isolate protoplasts from the hypocotyls of common buckwheat and reported abnormal regenerated plants after 18 months of callus culture. Likewise, Gumerova (2004) used the same protoplast source material and noted poor plant regeneration after nine months of culture. In both studies, regeneration was successful, but the yield was low, and the callus obtained from the protoplasts had a low regenerative ability. Compared with those research results, the procedures applied in this study resulted in faster plant regeneration since it took only three to five months. Like Adachi et al. (1989) and Gumerova (2004), we performed the protoplast-derived callus multiplication step on a medium supplemented with auxin and cytokinin. Such a combination has also been well studied for callus induction in other species, such as *Lycopersicon esculentum*, *Nigella damascena* and *Salvia moorcroftiana* (Bano et al. 2022; Chaudhry et al. 2007; Klimek-Chodacka et al. 2020). We followed the same scheme and used a medium supplemented with 2,4-D and KIN. It should be noted that, in our case, the callus multiplication medium (CM_MS1) was additionally supplemented with PSK. Undoubtedly, this medium stimulated callus growth for plant materials originating from all the protoplast sources tested. Although, in the case of the

Table 5 Comparison of protoplast culture development and protoplast-to-plant regeneration in *Fagopyrum esculentum*

Protoplast source	Protoplast yield (no. protoplasts per g of FW)	First division (days) ^a	Plating efficiency ^b	Plant regeneration ^c	References
cotyledon-derived callus	–	6–7	–	–	Rumyanzeva and Lozovaya (1988)
hypocotyls	5 × 10 ⁶ per 30 hypocotyls	4	1% (estimated after 4 weeks)	1.5 year	Adachi et al. (1989)
hypocotyls	8 × 10 ⁵	5	–	9 months	Gumerova (2004)
callus	5.8–6.9 × 10 ⁵	–	–	–	
hypocotyls	0.44 × 10 ⁶	5	21%	3 months	This research
embryo-derived callus L1 ^d	0.84 × 10 ⁶	6	29%	5 months	
embryo-derived callus NL2 ^d	0.83–1.54 × 10 ⁶	4	23%	2 months	

FW fresh weight

^aNumber of days after protoplast isolation

^bIn present research estimated in 10-day-old protoplast cultures

^cTime after transfer to regeneration medium

^dMorphogenic callus lines derived from immature zygotic embryo

– No information included in the publication

callus originating from hypocotyl- and L1-derived protoplasts, the histological sections revealed the abundance of parenchymatous cells and lack of or small presence of meristematic-like cells (Fig. 4b), which explains the long lasting and poor regeneration rate compared to the NL2 line.

Besides the characteristics of the source material, the culture medium's composition directly affects tissue regeneration (Adedeji et al. 2020). We used two variants of the MS regeneration medium: RM_MS3 and RM_MS4, which differed in cytokinin composition. The substitution of KIN for TDZ drastically changed the panorama of the experiment, showing an abundant growth of somatic embryos and rapid development of shoots (Fig. 1g). It is typical to use different combinations of PGRs during common buckwheat regeneration, especially cytokinins such as BAP + KIN (Woo et al. 2000), or auxins such as 2,4-D + NAA and IAA + IBA (Kumar and Saraswat 2018). Moreover, auxins, especially 2,4-D, promote the induction of common buckwheat somatic embryogenesis (Gumerova et al. 2001; Gumerova et al. 2003). However, Yang et al. (2012) state that using phenylurea derivatives, especially TDZ, in the RM, stimulates the development of embryogenic cells and, therefore, somatic embryogenesis. This indicates that TDZ may have an auxin effect. Besides, it has been shown that the use of TDZ in in vitro cultures of common buckwheat is more effective for shoot regeneration than traditional purine-type cytokinins (Guo et al. 1992). Berbec and Doroszevska (1999) noted similar results when testing different combinations of growth regulators during regeneration of two common buckwheat diploid (Kora and Hruszowska) and tetraploid cultivars (Emka). According to these authors, the frequency of shoot regeneration was higher after applying IAA + TDZ, even

after using TDZ as the only phytohormone in the medium. In our study, the histological sections of the protoplast-derived callus originating from the NL2 line revealed a high presence of meristematic cells, which could be directly influenced by the composition of the culture medium and could explain the short time needed for induction of somatic embryos and regeneration.

To sum up, compared with earlier works related to the regeneration of plants from common buckwheat via protoplast cultures, the protoplast yield and the time to the first division of protoplast-derived cells do not differ much (Table 5). The plating efficiency was considerably higher than in previous research, especially for protoplasts isolated from the L1 line. However, our tremendous success was the time to achieve plant regeneration. Complete regenerated plants were obtained in a maximum of five months, four times faster than Adachi et al. (1989) reported.

Conclusions

The potential of the protoplast-to-plant system for the regeneration of common buckwheat plants using MC-derived from immature embryos as the protoplast source has been confirmed. The use of PSK during protoplast culture and hormonal supplementation (TDZ + KIN and BAP + KIN) during plant regeneration played a critical role. It was also verified that TDZ is efficient for stimulating somatic embryogenesis. This study showed a rapid and potential technique for common buckwheat propagation using in vitro cultures. It is also the basis for future research related to buckwheat

crop improvement through genetic engineering or somatic hybridisation.

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Author contributions Conceptualisation: AB, EG; Methodology: MZ, RP-P, AM-H, AB, EG; Formal analysis: MZ, RP-P, AM-H, AB, EG; Investigation: MZ, RP-P, AM-H, AB; Resources: AB, EG; Writing—original draft: MZ, RP-P, AB; Writing—review and editing: MZ, RP-P, AM-H, AB, EG; Visualisation: MZ, RP-P; Supervision: AB, EG; Project administration: AB, EG; Founding acquisition: AB. All authors have read and approved the final manuscript.

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The use of all plant materials in this study complies with relevant institutional, national, and international guidelines and legislation. Seeds of *F. esculentum* cultivar Panda are commercially available and were purchased from the Malopolska Plant Breeding company (Poland).

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