UNIWERSYTET ŚLĄSKI
W KATOWICACH

University of Silesia in Katowice

Faculty of Natural Sciences

Institute of Biology, Biotechnology and Environmental Protection

Reneé Pérez Pérez

DOCTORAL THESIS

Protoplast-based approaches in *Fagopyrum esculentum* Moench. and *Fagopyrum tataricum* (L.) Gaertn: insights into growth regulation, regeneration efficiency and hybrid cell wall dynamics

Supervisor:

dr hab. Alexander Betekhtin, prof. UŚ

University of Silesia in Katowice

Co-supervisor:

dr Anna Milewska-Hendel

University of Silesia in Katowice

Katowice, 2025

Acknowledgements

I would like to express my gratitude to my supervisor, dr hab. Alexander Betekhtin, prof. UŚ, for giving me the opportunity to work with him, for his scientific expertise and for teaching me during these four years.

I would also like to thank my co-supervisor, dr Anna Milewska-Hendel, for her guidance, knowledge and support inside and outside the profession.

A very special thanks to dr Artur Piński for his great help in all scientific works and experiments.

Thanks to Magdalena Zaranek for her help in lab and to all co-authors who contributed to our publications.

A huge thank you to my lab bestie Pani Alicja, for her guidance, company, feeding me and especially for her emotional support during difficult times.

And the best thanks go to my parents, the ones truly responsible for this and every other professional goal I have achieved.

Content

1. List of publications included in the doctoral thesis

Publication 1 (P1):

Zaranek, M., **Pérez-Pérez, R.**, Milewska-Hendel, A., Betekhtin, A., Grzebelus, E.
Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn.

BMC Plant Biology **2023,** 385 (23), 743–757.

https://doi.org/10.1186/s12870-023-04402-9

IF$_{2023}$: 4.3

Ministry of Science and Higher Education points: 140

Publication 2 (P2):

Zaranek, M.*, **Pérez-Pérez, R.***, Milewska-Hendel, A., Grzebelus, E., Betekhtin, A.
Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench.

*equal contribution

Plant Cell, Tissue and Organ Culture (PCTOC), **2023,** 154, 673–687.

https://doi.org/10.1007/s11240-023-02542-2

IF$_{2023}$: 2.3

Ministry of Science and Higher Education points: 100

Publication 3 (P3):

Sala-Cholewa, K., Milewska-Hendel, A., **Pérez-Pérez, R.**, Grzebelus, E., Betekhtin, A.
Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells.

Plant Cell, Tissue and Organ Culture (PCTOC), **2024,** 157 (26).

https://doi.org/10.1007/s11240-024-02740-6

IF$_{2024}$: 2.3

Ministry of Science and Higher Education points: 100

Publication 4 (P4):

Pérez-Pérez, R.*, Piński, A.*, Zaranek, M., Beckmann, M., Mur, L., Nowak, K., Rojek-Jelonek, M., Kostecka-Gugała, A., Petryszak, P., Grzebelus, E., Betekhtin, A.
Effect of potent inhibitors of phenylalanine ammonia-lyase and PVP on *in vitro* morphogenesis of *Fagopyrum tataricum*.

*equal contribution

BMC Plant Biology **2025,** 25, 469

https://doi.org/10.1186/s12870-025-06440-x

IF$_{2023}$: 4.3

Ministry of Science and Higher Education points: 140

Total IF: 13.2

Total Ministry of Science and Higher Education points: 480

2. Fundings

The doctoral research was carried out as part of a project funded by the National Science Centre (NCN) entitled:

Genetic Analysis of the Formation of *Fagopyrum* Somatic Hybrids

Grant number: 2020/38/E/NZ9/00033

Project within the Sonata Bis 10 competition implemented in 2021-2025

Project investigator: Dr hab. Alexander Betekhtin, prof. UŚ

In the third year of PhD, I completed a two-month internship as part of the Erasmus+ programme at the Centre for Organismal Studies and the Electron Microscopy Core Facility (EMCF) of Heidelberg University, under the supervision of Dr Melanie Krebs and Dr Reza Shahidi, respectively.

3. List of abbreviations

2,4-D: 2,4-dichlorophenoxy acetic acid

***A*:** a gene in the *S*-locus that controls the anther height

AC: alternating current

AGPs: arabinogalactan proteins

AIP: 2-aminoindane-2-phosphonic acid

AOA: aminooxyacetic acid

AOPP: α-aminooxy-β-phenylpropionic acid

BAP: 6-benzylaminopurine

BM: basal medium

CM: callus multiplication medium

DAPI: 4′,6-diamidino-2-phenylindole fluorophore

DC: direct current

$DiOC_6$: 3,3′-dihexyloxacarbocyanine iodide

EC: embryogenic calli

FB28: fluorescent brightener 28 (calcofluor)

***Fe* (+) *Ft*:** hybrid cells between *F. esculentum* and *F. tataricum*

***G*:** gene in the *S*-locus that controls the style length

GlcA: glucuronic acid

I^P: gene in the *S*-locus that controls the pollen incompatibility

I^S: gene in the *S*-locus that controls the style incompatibility

KIN: kinetin

MC: morphogenic calli

OBHA: O-benzylhydroxylamine hydrochloride

***P*:** gene in the *S*-locus that controls the pollen size

PAL: phenylalanine ammonia-lyase

PBS: phosphate-buffered saline

PCA: principal components analysis

PECC: proembryogenic cell complex

PEG: polyethylene glycol

PSK: *a*-phytosulfokine

PVP: polyvinylpyrrolidone

***S-ELF3*:** *S-LOCUS EARLY FLOWERING 3*

***s-elf3-ψ*:** nonsense mutation in the *S-ELF3* in self-compatible species of *Fagopyrum*

TBO: toluidine blue

TDZ: thidiazuron

4. Streszczenie

Rodzaj *Fagopyrum* to grupa roślin dwuliściennych należąca do rodziny rdestowatych. Rodzaj obejmuje 22 gatunki, zarówno jednoroczne, jak i wieloletnie, które rosną głównie na wyżynach euroazjatyckich. Najważniejszymi uprawianymi gatunkami są gryka zwyczajna (*F. esculentum,* zwana również słodką gryką) i gryka tatarka (*F. tataricum*, zwana również gorzką gryką). Do głównych przyczyn ograniczonej powszechności uprawy gryki w Polsce i Europie należą niskie plony, różnice w nawykach żywieniowych oraz ograniczona świadomość producentów żywności i konsumentów dotycząca wartości odżywczej oraz innych korzystnych właściwości gryki. Biotechnologia oparta na protoplastach i systemy regeneracji roślin stanowią obiecujące podejście do przezwyciężenia tych ograniczeń, ułatwiając badanie przeprogramowania komórkowego, totipotencji i hybrydyzacji somatycznej w kontrolowanych warunkach. Niniejsza rozprawa doktorska koncentruje się na *F. tataricum* i *F. esculentum* jako systemach modelowych do badania rozwoju protoplastów, procesów morfogenezy oraz *de novo* rekonstrukcji ściany komórkowej komórek hybrydowych *F. esculentum* (+) *F. tataricum,* zajmując się krytycznymi lukami w tych procesach. Badania miały na celu optymalizację izolacji protoplastów i warunków hodowli poprzez ocenę materiału źródłowego, metod immobilizacji i dodatków do podłoży hodowlanych; zwiększenie regeneracji roślin poprzez morfogenezę za pośrednictwem cytokininy; scharakteryzowanie dynamiki rekonstrukcji ściany komórkowej *de novo* w komórkach rodzicielskich, a także w komórkach hybrydowych uzyskanych poprzez elektrofuzję protoplastów; oraz określenie roli regulatorów fenolowych w procesach rozwojowych.

Wyniki badań, szczegółowo opisane w czterech publikacjach naukowych, podkreśliły, w jaki sposób warunki hodowli bezpośrednio wpływają na zachowanie i regenerację komórek. Wykazano, że kalus morfogennny *F. tataricum* i kalus embriogenny *F. esculentum* stanowią lepsze źródła protoplastów w porównaniu z hipokotylami siewek, zapewniając wyższą wydajność izolacji. Unieruchomienie wyizolowanych protoplastów w matrycy agarozowej o niskiej temperaturze topnienia, hodowanych w podłożu uzupełnionym o α-fitosulfokinę, zwiększyło podział komórek, tworzenie kolonii i późniejszy rozwój mikrokalusów. Co najważniejsze, w fazie regeneracji typy i stężenia cytokinin były krytyczne dla osiągnięcia morfogenezy, przy czym thidiazuron przyspieszał somatyczną embriogenezę u *F. esculentum,* co prowadziło do regeneracji całej rośliny w ciągu dwóch miesięcy; podczas gdy połączenie benzyloaminopuryny i kinetyny promowało zarówno somatyczną embriogenezę, jak i organogenezę u *F. tataricum*, co dawało zregenerowane rośliny w ciągu trzech miesięcy.

Nowy protokół elektrofuzji skutecznie umożliwił tworzenie komórek hybrydowych *F. esculentum* (+) *F. tataricum*, które zostały zebrane zgodnie ze zoptymalizowaną metodą sortowania, umożliwiając analizę porównawczą regeneracji ściany komórkowej. Immunolokalizacja składników ściany komórkowej wykazała, że *de novo* rekonstrukcja ściany komórkowej w komórkach hybrydowych i rodzicielskich następuje w ciągu 48 godzin. Zaobserwowano również specyficzną dla gatunku dynamikę białek arabinogalaktanowych, ekstensyny, ksyloglukanu i pektyn, przy czym komórki hybrydowe wykazywały wzorce podobne do rodzicielskich, z wyjątkiem opóźnionego odkładania metyloestryfikowanego homogalakturonanu u *F. esculentum*, co sugeruje potencjalną rozbieżność w metabolizmie pektyny podczas wczesnego etapu rekonstrukcji ściany komórkowej.

Ostatnie badania wykazały że kontrolowanie zawartości fenoli i flawonoidów za pomocą inhibitorów amoniakoliazy L-fenyloalaniny (AIP, AOPP, OBHA) i adsorbentu (PVP) bezpośrednio wpływają na reakcje protoplastów oraz wzrost i regenerację kalusa *F. tataricum*. Niskie stężenia AIP i PVP sprzyjają tworzeniu kolonii komórkowych podczas hodowli protoplastów i regeneracji roślin diploidalnych, podczas gdy AOPP i OBHA dają zarówno rośliny diploidalne, jak i tetraploidalne. Z drugiej strony regeneracja roślin z proembriogennych kompleksów komórkowych o wyższych stężeniach AIP wykazała zmniejszony stres oksydacyjny, stosunek glutationu do utlenionego glutationu i zwiększoną ekspresję genów związanych z embriogenezą, podczas gdy PVP głównie adsorbował związki fenolowe, zmieniając dostępność składników odżywczych.

Poza rozwijaniem wiedzy teoretycznej w zakresie biotechnologii roślin, rozprawa dostarcza przełomowych spostrzeżeń na temat technologii protoplastów, łącząc warunki hodowli z odpowiedziami komórkowymi i efektami regeneracji. Ustanawia również pierwszy powtarzalny protokół dla produkcji komórek hybrydowych *Fagopyrum* i opisuje molekularne i metaboliczne podstawy ograniczeń regeneracji zależnej od związków fenolowych.

5. Summary

The genus *Fagopyrum* is a group of dicotyledonous plants belonging to the family *Polygonaceae*. The genus includes 22 species, both annual and perennial, which grow mainly in the Eurasian highlands. The most important cultivated species are common buckwheat (*F. esculentum*, also called sweet buckwheat) and Tartary buckwheat (*F. tataricum*, also called bitter buckwheat). The main reasons for the low prevalence of buckwheat cultivation in Poland and Europe are low yields, differences in dietary habits and low awareness of food producers and consumers about the nutritional value and other beneficial properties of buckwheat. Protoplast-based biotechnology and plant regeneration systems present a promising approach to overcome these limitations, facilitating the study of cellular reprogramming, totipotency, and somatic hybridisation under controlled conditions.

This doctoral thesis focuses on *F. tataricum* and *F. esculentum* as model systems to investigate protoplast development, morphogenesis processes, and *F. esculentum* (+) *F. tataricum* hybrid cells' *de novo* cell wall reconstruction, addressing critical gaps in these processes. The study aimed to optimise protoplast isolation and culture conditions by evaluating source material, immobilisation methods, and culture media additives; enhance plant regeneration via cytokinin-mediated morphogenesis; characterise *de novo* cell wall reconstruction dynamics in parental and hybrid cells obtained via protoplast electrofusion; and determine the role of phenolic regulators in developmental outcomes.

The findings, detailed across four scientific publications, highlighted how culture conditions directly influence cellular behaviour and regeneration. *F. tataricum* morphogenic callus and *F. esculentum* embryogenic callus were demonstrated to be better sources of protoplasts compared with seedlings hypocotyls, yielding higher isolation efficiency. The immobilisation of the isolated protoplasts in a low-melting-point agarose matrix, cultivated in medium supplemented with α-phytosulfokine, enhanced cell division, colony formation and the subsequent development of microcalli. Most notably, during the regeneration stage, cytokinin types and concentrations were critical to achieve morphogenesis, with thidiazuron accelerating somatic embryogenesis in *F. esculentum*, leading to whole plant regeneration within two months; while benzylaminopurine–kinetin combination promoted both somatic embryogenesis and organogenesis in *F. tataricum*, yielding regenerated plants within three months.

A novel electrofusion protocol successfully generated *F. esculentum* (+) *F. tataricum* hybrid cells, which were manually collected following an optimised sorting method, enabling a comparative analysis of cell wall regeneration. Immunolabelling of cell wall components revealed that *de novo* cell wall reconstruction in hybrid and parental cells occurs within 48 hours. It also showed species-specific dynamics in arabinogalactan proteins, extensin, xyloglucan, and pectins, with hybrid cells exhibiting parental-like patterns but a delayed methylesterified homogalacturonan deposition in *F. esculentum*, suggesting potential divergence in pectin metabolism during early wall assembly.

Finally, this study demonstrated that controlling phenolic and flavonoid contents using phenylalanine ammonia-lyase inhibitors (AIP, AOPP, OBHA) and adsorbent (PVP) directly impacts *F. tataricum* protoplast and callus responses during growth and regeneration. Low concentrations of AIP and PVP promote cell colony formation during protoplast culture and diploid plant regeneration, whereas AOPP and OBHA yield both diploid and tetraploid plants. On the other hand, plant regeneration from proembryogenic cell complexes with higher concentrations of AIP exhibited reduced oxidative stress, glutathione/oxidised glutathione ratio, and upregulated expression of embryogenesis-related genes, while PVP primarily adsorbed phenolics, altering nutrient availability.

Beyond advancing theoretical knowledge in plant biotechnology, the thesis provides transformative insights into protoplast technology, linking culture conditions to cellular responses and regeneration products. It also establishes the first reproducible protocol for *Fagopyrum* hybrid cell production and delineates the molecular and metabolic basis of phenolic-mediated regeneration bottlenecks.

6. Introduction

6.1. *Genus Fagopyrum, importance and breeding challenges*

The genus *Fagopyrum*, commonly known as buckwheat, is a member of the Polygonaceae family that includes around 22 species (Jha *et al.*, 2024), among which *Fagopyrum esculentum* (common buckwheat) and *Fagopyrum tataricum* (Tartary buckwheat) are the most widely cultivated. These species are valued for their agronomic potential, nutritional benefits, and health-promoting qualities. Buckwheat is a rich source of bioactive compounds, including flavonoids such as rutin, quercetin, and C-glycosylflavones, which have been shown to possess antioxidant, anti-inflammatory, and cardioprotective effects (Nalinkumar and Singh, 2020, Huda *et al.*, 2021). Additionally, buckwheat is gluten-free and contains high-quality proteins with essential amino acids, making it a valuable crop for addressing global food security and nutritional challenges (Krkošková and Mrázová, 2005, Woo *et al.*, 2010).

Despite its potential, buckwheat cultivation faces several challenges, including low yields and reproductive barriers that limit genetic improvements (Taylor and Obendorf, 2001, Cawoy *et al.*, 2009). *F. esculentum* has a broader distribution, especially in temperate and subtropical regions, due to its adaptability to poor-quality soils, low fertiliser requirements, and use as a cover crop and honey plant. It has a dioecious sexual system that produces two heterostylic floral morphs: short-styled flowers (Thrum) and long-styled flowers (Pin) (Image***** 1 A, B). This dimorphism results in self-incompatibility, which promotes genetic diversity in populations and reduces the risks of inbreeding depression. However, since fertilisation occurs between the flower morphs, there is a high dependence on pollinators for its success (Cardoso *et al.*, 2018). This phenomenon is genetically controlled by the *S* – Supergene Complex, also known as the *S*-locus (*sterility*), which directly influences floral morphology and self-incompatibility (Lewis and Jones, 1992). The *S*-locus includes five genes, two controlling the female function of the flower (*G* – style length and I^S – style incompatibility), also known as *S-ELF3*, and three genes controlling the male function (*P* – pollen size, I^P – pollen incompatibility and *A* – anther height) (Image 1) (Fawcett *et al.*, 2023). Thrum flowers contain the *S*-locus, in contrast to Pin flowers, which lack the *S*-locus entirely (Image 1 A', B') (Yasui *et al.*, 2012, Matsui and Yasui, 2020, Fawcett *et al.*, 2023).

On the other hand, *F. tataricum* characterises a higher resistance to environmental stresses and is frequently cultivated in mountainous and high-altitude regions, particularly in Asia and

***** To prevent any potential ambiguity or plagiarism concerns, pictures presented in the introduction of the thesis are labelled as ***"Image"*** while the ones showed on the scientific publications are called ***"Figure"***

parts of Eastern Europe (Bonafaccia *et al.*, 2003, Han *et al.*, 2011). It is a self-compatible species with one flower type with stamens and styles of equal size (homostyly, Image 1 C). This species also contains the *S*-locus, but the genes related to female function (*S-ELF3*) are silenced due to a nonsense mutation (*s-elf3-ψ*) that has occurred naturally during the evolutionary process (Image 1 C') (Fawcett *et al.*, 2023).

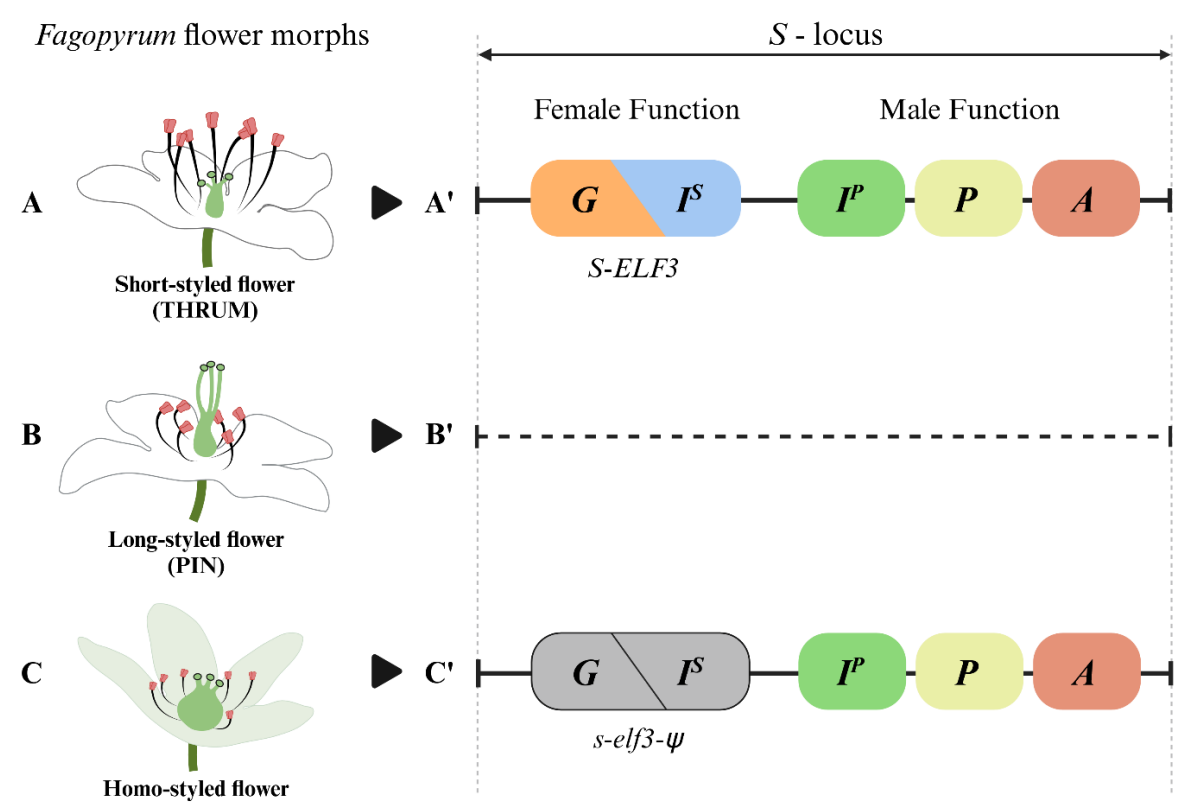


Image 1. Schematic representation of flower types in *Fagopyrum* and their respective set of genes of the *S* – locus. Heterostyly in *F. esculentum* flowers: (A) Thurm flower, style shorter than stamens and (A') complete *S* – locus with female and male function-related genes. (B) Pin flower, style higher than stamens and (B') complete absence of the *S* – locus. Fertilisation occurs between the pistil of Pin flowers and the stamens of Thrum flowers, and conversely (cross-pollination). (C) *F. tataricum* flower with homogeneous style and stamen, and (C') *S* – locus with the *s-elf3-ψ* nonsense mutation in the *S-ELF3* region. Fertilisation occurs between the pistil and stamens of the same flower (self-pollination). *G*: style length; I^S: style incompatibility; I^P: pollen incompatibility; *P*: pollen size; *A*: anther height. Adapted from (Fawcett *et al.*, 2023). Image made by Reneé Pérez-Pérez with BioRender.com.

In an attempt to overcome the reproductive barriers or the agronomic disadvantages that both species face, they have been cross-pollinated with each other, as well as with other wild species such as *F. cymosum* and *F. urophyllum* (heterostyly) or *F. homotropicum* (homostyly) using the traditional pollination methods (Yui *et al.*, 2004, Fesenko *et al.*, 2022). Only the crossing

of Pin flowers from *F. esculentum* and *F. homotropicum* has successfully eliminated the heterostyly trait and produced self-fertilised flowers (Fawcett *et al.*, 2023). However, the early seed-shattering trait of *F. homotropicum* remains in the progeny and also represents a significant disadvantage from an agricultural and economic point of view. Crossing with other species has been unsuccessful due to strong pre-zygotic barriers, such as temporal, behavioural, mechanical, and gametic isolation, which prevent mating. Additionally, post-zygotic barriers, including hybrid unviability, sterility or breakdown, prevent hybrid offspring from surviving or reproducing, ultimately maintaining species distinctness and genetic integrity (Woo *et al.*, 2001, Shaikh *et al.*, 2002).

6.2. *Tissue culture and protoplast technology*

The disadvantages described above have stimulated interest in exploring alternative approaches to improve *Fagopyrum* production and resilience. Tissue culture-based methods like indirect somatic embryogenesis (embryos are formed from somatic cells through an intermediate callus phase) and organogenesis (organs development either directly from the explant or indirectly via callus formation) have been widely studied in *Fagopyrum* to facilitate the regeneration of whole plants from different explants such as hypocotyls, mesophyll, petioles, leaves and nodes (Tomasiak *et al.*, 2022). Callus-based regeneration enables large-scale propagation of genetically identical plants. It ensures the preservation of desirable traits by maintaining genetic stability and avoiding the variability associated with traditional seed propagation (Long *et al.*, 2022). *F. esculentum* and *F. tataricum* develop calli with different morphological and histological characteristics. *F. esculentum* callus contains light colour (milky white, yellowish or white) embryogenic masses with a smooth and shiny surface and a white or light yellow mass of parenchymatous cells (Image 2 A). The embryogenic cells are weakly vacuolated and have dense cytoplasm and a centrally located nucleus (Image 2 B, B'), while the parenchymatous cells present a large vacuole and a peripherally positioned nucleus (Image 2 B, B''). Depending on the age and culture conditions, some phenolic-containing cells may appear on the surface of *F. esculentum* calli (Rumyantseva *et al.*, 2004, Rumyantseva *et al.*, 2005). In contrast, morphogenic calli of *F. tataricum* show a more complex morphology consisting of spherical white proembryogenic masses called proembryogenic cell complexes (PECCs) located on the surface of a "soft" callus (Image 2 C). PECCs are formed by different cell types, including parenchymatous cells with amyloplasts containing large starch grains, constituting the central and most prominent part of the structure. Next, layers of meristematic

cells, which are the source of the embryogenically determined cells that will give rise to embryoids or new PECCs; and finally, on the surface, abundant phenolics-containing cells, with large vacuoles that accumulate considerable amounts of phenolic compounds (Image 2 D, D'). During the cyclical development of the morphogenic callus, mature PECCs disintegrate, thus giving rise to young PECCs and "soft" callus cells. The "soft" callus is mainly formed by elongated cells that are highly vacuolated (Image 2 E), and its function is mainly to nurse the growth of the PECCs, providing them with nutrients. It is brown due to the accumulation of phenolic compounds and becomes darker as the culture progresses. *F. tataricum* morphogenic calli exhibit remarkable nuclear genomic stability and cell proliferation and regeneration capacity for up to ten years of culture (Betekhtin *et al.*, 2017, Zaranek *et al.*, 2025).

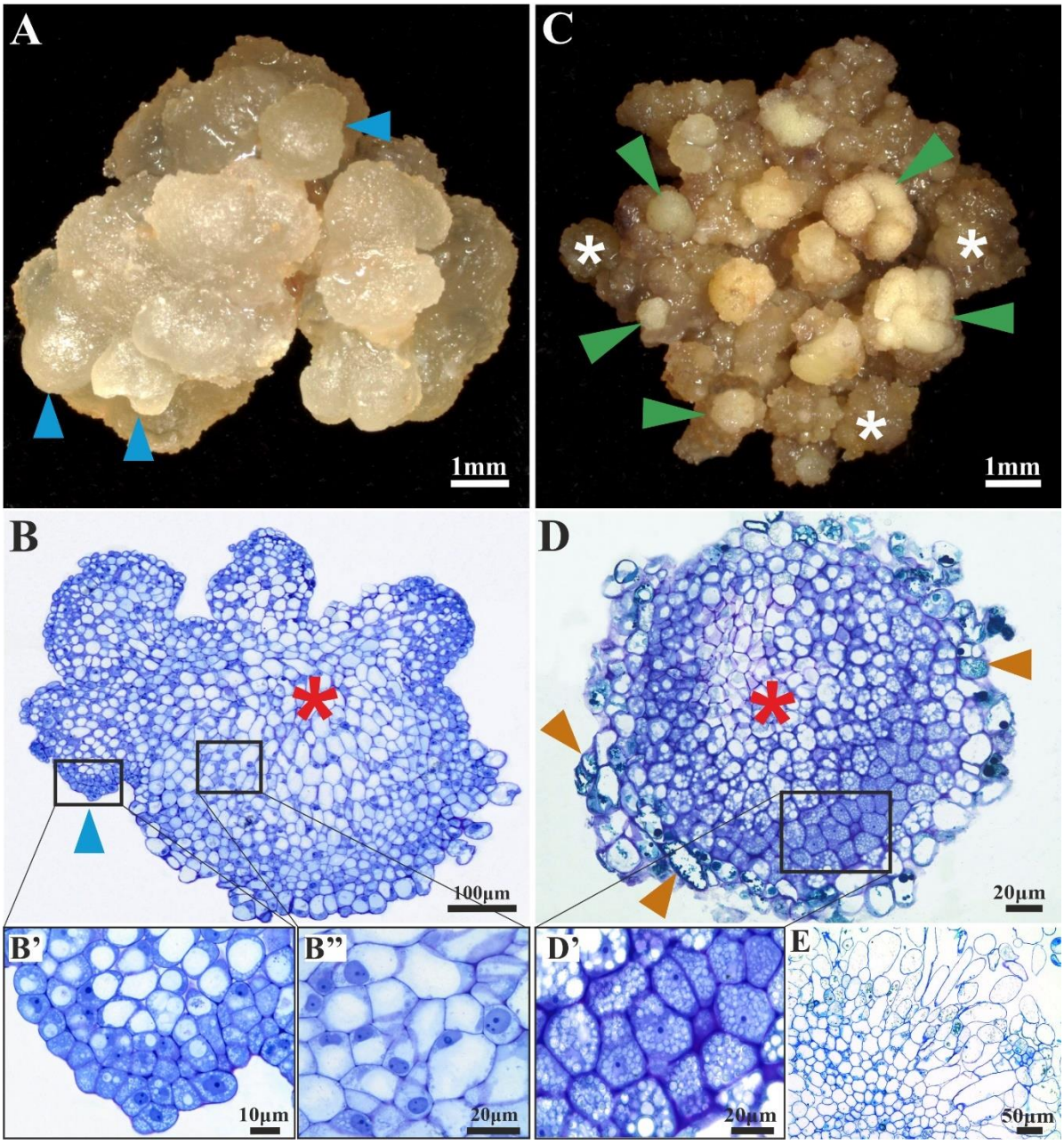


Image 2. (A) *F. esculentum* embryogenic callus and (B) histological section stained with toluidine blue (TBO) showing embryogenic cell masses (blue arrowheads, inset B') and parenchymatous cells (red asterisk, inset B''). (C) *F. tataricum* morphogenic callus with PECCs (green arrowheads) and 'soft callus' (white asterisk). (D) Histological sections stained with TBO of a PECC with parenchymatous cells in the centre of the structure (red asterisk) followed by embryogenic cells (inset D') and surficial phenolic-containing cells (orange arrowheads), and (E) soft callus formed by abundant enlarged parenchymatous cells.

Protoplast technology has also been prominent in crop improvement, offering a unique opportunity to introduce novel traits in crops of agricultural interest, avoiding the traditional limitations of cross-pollination (Adachi *et al.*, 1989, Lachmann and Adachi, 1990). This technique has been applied in *Fagopyrum*, with hypocotyls and mesophyll cells serving as the primary sources of protoplasts, leading to inconsistencies in callus formation and shoot regeneration (Adachi *et al.*, 1989, Tomasiak *et al.*, 2022). Early attempts at plant regeneration from protoplasts yielded limited success, with low plating efficiency and abnormal plant morphology being common issues (Holländer-Czytko and Amrhein, 1983, Gumerova *et al.*, 2003). Other studies, however, have demonstrated the potential of calli as an improved source of protoplasts due to their high regenerative capacity (Yamane, 1974, Takahata and Jumonji, 1985). The success of protoplast cultures depends not only on the protoplast source but also on the composition of the culture media and growth factors such as *a*-phytosulfokine (PSK) or putrescine which have been shown to enhance cell wall reconstruction, cell division, callus formation, and shoot regeneration in various species (Wang *et al.*, 2015, Vogrinčič *et al.*, 2024).

One of the major problems affecting *Fagopyrum* protoplast culture, especially in the early stages of cell colony** formation and during shoot regeneration, is the production and release of phenolic compounds into the medium (Gumerova *et al.*, 2015, Hou *et al.*, 2015). It occurs as the cells' natural defence response to their environment perturbations. Although *in vitro* culture aims to resemble ideal conditions as much as possible, it cannot completely imitate the natural environment from which the cultured cells come (Wijerathna-Yapa and Hiti-Bandaralage, 2023). It is known that the production of phenolic compounds in tissue cultures can increase up to tenfold in different species compared to plants grown under natural conditions (Rao and Ravishankar, 2002, Cai *et al.*, 2012). It can cause changes in gene expression, metabolic alterations and, in some cases, senescence or cell death (Smetanska, 2018). The most common strategy to avoid tissue damage by phenolic accumulation is frequent transfer of the explants to

** Structures resulting from mitotic divisions of a single cell. In all scientific publications shown in this thesis, the term ***cell aggregate*** has been used instead. However, the terminology was corrected to ***cell colony*** in one of the latest publications of our team (Zaranek et al., 2025) by the suggestion of its reviewers.

a fresh medium (Permadi *et al.*, 2024), but other approaches include adjusting growth conditions or supplementing the culture media with specific additives (Ngomuo *et al.*, 2014, Safwar *et al.*, 2015).

Most of the researched additives can be divided into three categories:
I) antioxidants, which are attributed to the capacity of the phenolics to inactivate free radicals (i.e., ascorbic acid, citric acid, and glutathione) (Ndakidemi *et al.*, 2014, Permadi *et al.*, 2024);

II) adsorbents, usually polymers that bind phenolics due to different kinds of interactions like hydrogen bonding and π-π interactions, as well as hydrophobic interactions (i.e., activated charcoal, polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone) (Thomas, 2008, Chen *et al.*, 2022);

III) inhibitors, which work by targeting and neutralising enzymes involved in the shikimate and phenylpropanoid pathways (i.e., aminooxyacetic acid (AOA) – pyridoxal phosphate-dependent enzymes and α-aminooxy-β-phenylpropionic acid (AOPP), O-benzylhydroxylamine hydrochloride (OBHA), 2-aminoindane-2-phosphonic acid (AIP) – phenylalanine ammonia-lyase (PAL) inhibitors) (Wanat *et al.*, 2018, Jiang and Penner, 2022).

Adsorbents and inhibitors are very popular among researchers (Amente and Chimdessa, 2021, Permadi *et al.*, 2024). They act through different mechanisms: inhibitors directly inhibit the phenolic synthesis pathway (phenylpropanoid pathway), especially its key enzyme PAL, increasing amino acid availability and reducing oxidative stress, while adsorbents target the phenolics already synthesised, decreasing oxidative stress but affecting nutrient availability. Despite the numerous studies on this topic (Urban and Hura, 2023, Permadi *et al.*, 2024), controlling the oxidative stress in *Fagopyrum* protoplast and tissue culture, especially *F. tataricum*, remains challenging.

6.3. *Cell wall reconstruction in protoplasts*

One of the first and most important stages of plant regeneration from protoplast cultures is undoubtedly *de novo* primary cell wall reconstruction. This is a critical step to ensure cell viability and normal development, as it gives way to the first cell divisions and defines the final cell shape (Yang *et al.*, 2008, Tagawa *et al.*, 2019). The primary cell wall is a dynamic structure composed mainly of cellulose microfibrils, matrix polysaccharides, polyphenolic compounds and structural proteins (Showalter, 1993). Cellulose is the major component of the wall,

comprised of hydrogen-bonded β-1,4-linked glucan chains that are synthesised at the plasma membrane by large cellulose synthase complexes. Its microfibrils wrap tightly around the cell and provide the major mechanical resistance to external stresses and internal osmotic pressures (Mutwil *et al.*, 2008). It can be synthesised even within the first few hours after the establishment of a protoplast culture and serve as a scaffold for other cell wall polymer depositions such as hemicelluloses and pectins (Pedersen *et al.*, 2023, Huh *et al.*, 2025). Hemicellulosic polysaccharides are complex molecules that associate with cellulose microfibrils, providing a cross-linked matrix. Xyloglucan, which contain a β-1,4-glucan backbone, heavily substituted with α-(1→6)-xylosyl residues in a regular pattern, is the major hemicellulose in the primary cell wall of dicotyledonous (Lerouxel *et al.*, 2006) and acts as a load-bearing structure and a spacer that prevents cellulose microfibrils from aggregating (Eckardt, 2008). On the other hand, pectins are complex heteropolymers comprised of three major domains, primarily homogalacturonan, rhamnogalacturonan I, and the substituted galacturonan, rhamnogalacturonan II. Due to its abundance in the middle lamellae, pectins were initially considered to function primarily in intercellular adhesion; however, as the cell wall regenerates, pectins are incorporated into the wall matrix, helping to structure and stabilise the new cell wall. They are also involved in a variety of cellular processes, including cell fate specification, morphogenesis, intercellular communication, and environmental sensing (Shin *et al.*, 2021). Other important cell wall components are arabinogalactan proteins (AGPs), characterised by a protein backbone with attached carbohydrate chains, particularly arabinose and galactose, involved in diverse developmental processes such as plant reproduction, adventitious root development, somatic and zygotic embryogenesis, and cell division, expansion and death (Ma and Johnson, 2023). Furthermore, extensins, hydroxyproline-rich glycoproteins, are directly related to cell wall reinforcement due to their ability to form cross-links within themselves (intra-molecular) and between different extensin molecules (inter-molecular), through specific enzymatic reactions, such as those catalysed by peroxidases (Castilleux *et al.*, 2021).

Previous studies have used, among others, immunocytochemical techniques to detect those specific cell wall components, which have allowed the analysis of the kinetics and timeline of their regeneration in various species. In *Beta vulgaris* L. tissues, regenerated from unfertilised ovules, immunolocalisation of pectin (recognised by JIM5, JIM7, LM5, LM6) and AGP (recognised by LM2, JIM13, JIM14) epitopes revealed their ubiquitous presence across different cell types. Pectin epitopes were consistently detected while specific AGP epitopes

(LM2, JIM13) were associated with morphogenetic processes, acting as developmental markers in callus, meristems, and somatic embryos, highlighting their selective roles in differentiation (Tomaszewska-Sowa, 2011). Similar analyses were performed by Wiśniewska and Majewska-Sawka (2008), where immunocytochemical analysis was used to explore variations in cell wall composition between intact leaves and regenerating protoplasts in sugar beet (*B. vulgaris*) and tobacco (*Nicotiana tabacum*). It was demonstrated that *B. vulgaris* (leaves and protoplasts) abundantly express AGP epitopes recognised by LM2 and MAC207 antibodies containing glucuronic acid (GlcA). In the case of the *N. tabacum* cells, they lack MAC207-binding epitopes and show only trace amounts of LM2-reactive AGPs. Authors highlighted that the presence of GlcA-rich AGPs in *B. vulgaris* may relate to its recalcitrant regeneration behaviour, while their absence in *N. tabacum* correlates with its high regeneration efficiency. Authors concluded that the differential expression of AGP epitopes (LM2/MAC207) and galactose-rich pectins between *B. vulgaris* and *N. tabacum* correlates with their distinct regenerative capacities, highlighting the importance of cell wall biochemistry in plant tissue culture. Godel-Jędrychowska *et al.* (2019), aiming to investigate the role of PSK during cell wall regeneration in *Daucus* spp. A spatio-temporal analysis of the distribution of pectin (recognised by LM19, LM20), AGP (recognised by JIM4, JIM8, JIM13), and extensin (recognised by JIM12) epitopes was performed. Results revealed that PSK accelerated *de novo* cell wall reconstruction, with LM20-pectin and JIM8/JIM13-AGPs appearing earliest after four days of culture. Moreover, methyl-esterified pectin's distribution varied depending on culture conditions and species, while AGPs expression and localisation were time- and tissue-specific, and extensin levels peaked during the mid-culture stage, particularly in PSK-treated cultures. Likely, in *Linum usitatissimum*, a comparison between pectin dynamics in hypocotyl-derived protoplasts embedded in agarose and alginate was performed using the 2F4 antibody specific for a calcium-induced supramolecular conformation of homopolygalacturonic acid. Immunolocalisation revealed that alginate stimulated higher methylesterified pectin secretion and subsequent de-esterification (3–8 days of culture), promoting cell wall differentiation; whereas agarose supported minimal pectin deposition, highlighting the matrix's role in regulating pectin metabolism (David *et al.*, 1995).

Protoplast culture is the only approach that could be used to directly observe cell wall regeneration from its initiation to completion (Shea *et al.*, 1989, Kuki *et al.*, 2020). The timing of cell wall reconstruction and the deposition of its components vary significantly across species and cell types, reflecting their unique structural and functional adaptation (Tagawa *et al.*, 2019).

For instance, dicots generally exhibit expedited cell wall reconstruction compared to monocots or woody plants (Kinnersley *et al.*, 1978). For example, *Nicotiana tabacum* protoplasts initiate cellulose synthesis within 5–24 hours, forming a functional cell wall in two or three days (Nagata and Takebe, 1970). In contrast, *Arabidopsis thaliana* protoplast requires 24–48 hours for initial cell wall assembly and 5–7 days to complete it (Yokoyama *et al.*, 2016, Kuki *et al.*, 2020, Huh *et al.*, 2025). In cereals protoplasts like *Oryza sativa,* cell wall regeneration initiates within two hours post-isolation, appearing as multiple fluorescent spots simultaneously across the cell surface, and finishes around 48 hours (Tan *et al.*, 2011). While *Zea mays* protoplasts show delayed kinetics, with cell wall regeneration observed after seven days of culture (Pilet *et al.*, 1984).

6.4. *Somatic hybridisation as an alternative to the cross-pollination barriers*

Developing efficient protoplast-to-plant regeneration systems is critical for applications such as somatic hybridisation, gene editing, and the production of genetically modified plants with improved agronomic traits (Sakamoto *et al.*, 2020). In *Fagopyrum*, somatic hybridisation between *F. esculentum* and *F. tataricum* protoplasts has been proposed as a means to overcome breeding barriers and introduce genes for stress tolerance and higher nutritional value (Lachmann and Adachi, 1990, Nešković *et al.*, 1995). The most widely used mechanism for this purpose is polyethene glycol (PEG)-mediated protoplast fusion, which has been successfully applied in different plant species such as *Glycine max, Helianthus annuus*, *Rubus* spp., *Jatropha curcas, Ricinus communis* and *Camellia oleifera* (Kao and Saleem, 1986, Landi *et al.*, 2001, Tudses *et al.*, 2015, Kativat and Tantasawat, 2017, He *et al.*, 2024). In *Fagopyrum*, there is only one report of this technique, from which putative hybrid protoplasts were obtained and developed into calli but failed to regenerate plants (Lachmann *et al.*, 1994).

Another technology that has gained interest is protoplast electrofusion (Image 3), which allows the creation of hybrid combinations of species that are sexually incompatible, thus facilitating the transfer of desirable genes among the related but sexually incompatible species without genetic transformation. This technology has also allowed not only intra-generic hybridisations, but the production of inter-generic hybrids as well (Varotto *et al.*, 2001, Zaman and Parihar, 2023). During this process, low-intensity electrical pulses are applied to bring neighbouring protoplasts closer together and then high voltages to induce the formation of pores in the cell membranes, allowing the cytoplasmic contents of adjacent protoplasts to merge (Image 3 A-C) (Wen *et al.*, 2020, Ranaware *et al.*, 2023). As a result, hybrid cells that contain

genomes and cytoplasm contents from both parental species (heterokaryon) are obtained (Image 3D). However, because it can have random effects, protoplasts from the same species may fuse and form hybrid cells with double or more copies of genetic material from the same parental material (homokaryon) (Image 3D). Unfused protoplasts and a high percentage of non-viable protoplasts can also emerge (Image 3D) (Al-Nema and Al-Mallah, 2020, Gieniec *et al.*, 2020).

In studies where a high percentage of viable hybrid protoplasts are required, such as *de novo* cell wall regeneration, it is essential to eliminate those undesired electrofusion products. To achieve this, protoplasts from both species are stained with contrasting coloured fluorophores such as rhodamine (red) or 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; green) during the isolation step (Image 3 B). Protoplasts that exhibit fluorescence for both fluorophores simultaneously can be manually sorted out using a single-cell micromanipulator (Mackowska *et al.*, 2023, Zaman and Parihar, 2023).

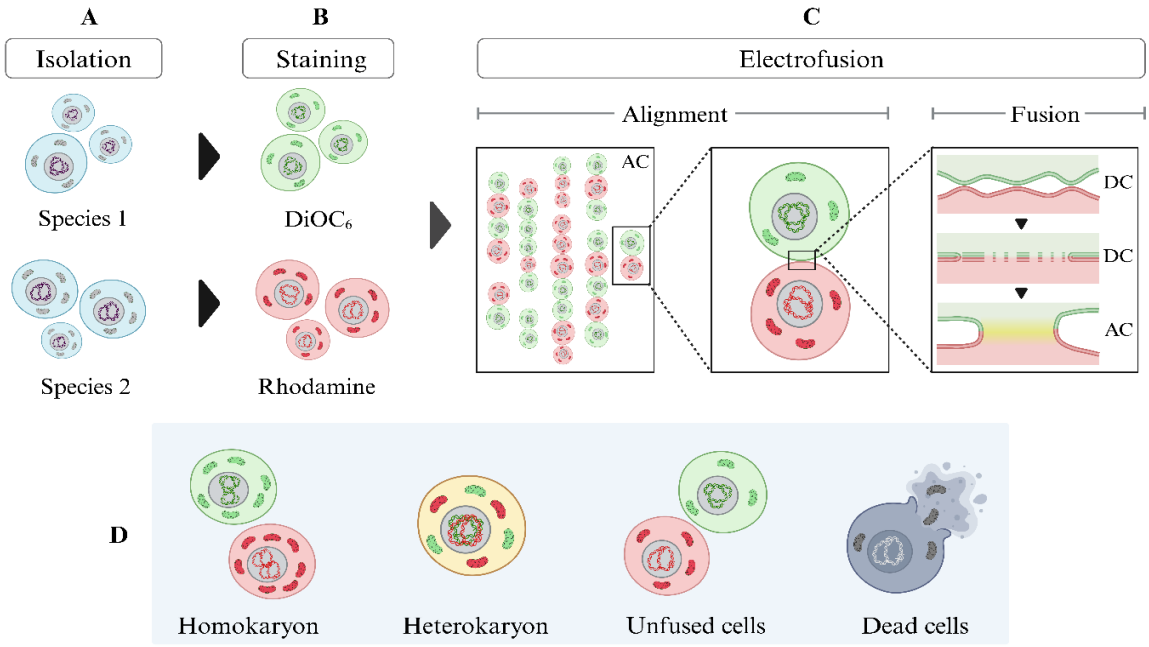


Image 3. Schematic representation of the protoplast electrofusion of two different species. After the protoplasts are isolated (A), each species is stained with contrasting fluorophores ($DiOC_6$ – green fluorescence and Rhodamine – red fluorescence) (B) and mixed in an equal-concentration solution. The protoplast electrofusion is performed in two main steps: 1) the unspecific alignment of neighbouring cells (same species protoplasts can align together as well as protoplasts from different species) by applying a low-intensity alternating current (AC), where the cell membranes come close enough forming parallel rows of protoplasts (pearls chain); and 2) the fusion itself, where short and consecutive pulses of high voltage direct current (DC) are used to disturb the continuity of the cell membranes opening small holes in them, which will stabilise in a single membrane after applying a slight AC again (C). As a result of the electrofusion, hybrid protoplasts with double or more genomic

and cytoplasmic content from the same species (homokaryon) or both species (heterokaryon, yellow protoplast) can be obtained, as well as unfused and non-viable protoplasts (D). Image made by Reneé Pérez-Pérez with BioRender.com.

Research on *de novo* cell wall reconstruction in plant hybrid cells remains scarce and lacks details, considering the synthesis timing of different cell wall components. Kästner *et al.* (2017) reported cell wall regeneration and cell division between 3 – 15 days of culture in hybrid cells derived from *Hydrangea* spp. mesophyll protoplasts, obtained via PEG-mediated fusion. This study did not examine the deposition timing of key cell wall components. To date, successful somatic hybridisation in *Fagopyrum* via protoplast electrofusion has not been reported; thus, there is an absence of detailed analysis on *de novo* cell wall reconstruction in hybrid cells and any related data on somatic hybrid regeneration.

Despite the advances in *in vitro* culture of *Fagopyrum* spp., there are still several challenges in applying protoplast culture and somatic hybridisation in buckwheat. The recalcitrance of hybrid protoplasts to regeneration, the instability of callus cultures, and the limited understanding of the molecular mechanisms underlying cell wall reconstruction are key areas for future research (Wiśniewska and Majewska-Sawka, 2007). Developing standardised protocols for protoplast isolation, culture, and regeneration is essential for the widespread adoption of these techniques.

7. Objectives and hypotheses

Protoplast culture and plant regeneration from calli systems are fundamental tools in modern plant biotechnology, offering unique insights into cellular reprogramming and developmental processes. These techniques allow the study of cell wall regeneration, totipotency, somatic embryogenesis and organogenesis processes under controlled *in vitro* conditions. Buckwheat species, such as *F. esculentum* and *F. tataricum*, serve as excellent models for these studies due to their versatile protoplast systems and diverse regenerative capacities.

The following research objectives have been set in the doctoral thesis:

1. Identify the key factors that enhance *F. tataricum* and *F. esculentum* protoplast development, including the source material, cell wall-digestion enzymes, immobilisation method, hormonal and growth regulator balance in the culture medium and the effects of phenolic inhibitors and adsorbents on cell division (**P1, P2**);
2. Optimise cytokinin type and concentration in the regeneration medium to induce morphogenesis and regenerate whole plants from protoplast-derived microcalli of *F. tataricum* and *F. esculentum* (**P1, P2**);
3. Develop an effective protoplast electrofusion protocol to produce hybrid cells between *F. esculentum* and *F. tataricum*, including the establishment of a hybrid cells selection system (**P3**);
4. Analyse differential cell wall reconstruction patterns from 0h to 72h in protoplasts of *F. esculentum* and *F. tataricum* and their hybrid cells *Fe* (+) *Ft* through immunostaining of the main cell wall epitopes: arabinogalactan proteins (JIM13, JIM16), extensin (JIM20), xyloglucan (LM25) and pectins (LM20, LM5, LM6), as well as cellulose using fluorescent brightener 28 (FB28) (**P3**);
5. Evaluate the effect of direct PAL inhibitors (AOA, AOPP, OBHA, AIP) and phenolic adsorbent (PVP) on *F. tataricum* protoplasts plating efficiency and the following protoplast-derived plant regeneration (**P4**);
6. Identify the key structural, genetic, and metabolic changes induced by AIP and PVP as medium additives during plant regeneration from morphogenic calli in *F. tataricum* (**P4**).

The following research hypotheses were formulated in the doctoral thesis:

1. Protoplast development in *F. esculentum* and *F. tataricum*, including cell division, cell colony and callus formation, is significantly influenced by the protoplast source material, immobilisation method, hormonal and growth regulator balance, as well as phenolics adsorbent and inhibitors.
2. Supplementing culture medium with cytokinins stimulates plant regeneration via protoplast cultures in *F. esculentum* and *F. tataricum*.
3. Protoplast electrofusion is an efficient method to obtain hybrid cells between *F. esculentum* and *F. tataricum* protoplasts.
4. *De novo* cell wall reconstruction in *F. esculentum* and *F. tataricum* protoplasts, as well as their *Fe* (+) *Ft* hybrid cells, differ in time and deposition of key cell wall components, including arabinogalactan proteins, extensin, xyloglucan, pectins and cellulose.
5. The use of different inhibitors of phenolic synthesis pathways or phenolic adsorbents during *F. tataricum* protoplast culture and subsequent plant regeneration counteracts negative effects of phenolics accumulation, such as low cell viability and cell division, oxidative browning of the microcalli and reduction of the regenerative potential.
6. AIP and PVP modulate structural, genetic, and metabolic responses during the regeneration of *F. tataricum* morphogenic calli, reducing phenolic accumulation and promoting favourable gene expression patterns associated with morphogenesis.

8. Materials and methods

Plant materials for protoplast isolation

Two species of *Fagopyrum* were used in the research: *F. esculentum*, genotype Panda (seeds obtained from Małopolska Hodowla, Poland), and *F. tataricum*, genotype k-17 (seeds obtained from the N. I. Vavilov Institute of Plant Genetic Resources, Saint-Petersburg, Russia). Seeds were sown in pots filled with a mixture of soil and vermiculite (3:1 ratio) and then grown in a greenhouse under constant temperature conditions of 25 ± 1 °C and a photoperiod of 16 hours light and 8 hours dark. Plants were illuminated with white-light lamps with a photon flux of 90 μmol m^{-2} s^{-1}. The protoplasts were isolated from two sources: hypocotyls and calli induced from immature embryos.

The aseptic hypocotyls were obtained *in vitro* from seeds of both species. Seeds were disinfected and then placed in Ø9 cm Petri dishes with Murashige and Skoog medium with vitamins (MS, Duchefa) supplemented with 30 g L^{-1} of sucrose (POCH) and 7 g L^{-1} of plant agar (Duchefa). The plates were incubated at 26 ± 1 °C in the dark for 10 days.

Embryogenic and morphogenic calli lines were induced from immature zygotic embryos of *F. esculentum* and *F. tataricum*, respectively (Betekhtin *et al.*, 2017, Betekhtin *et al.*, 2019). Calli were maintained at 26 ± 1 °C on RX medium containing Gamborg's B5 medium (Duchefa), 2 g L^{-1} N-Z-amine A (Sigma), 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma), 0.5 mg L^{-1} indole-3-aceticacid (IAA; Sigma), 0.5 mg L^{-1} α-naphthaleneacetic acid (NAA; Sigma), 0.2 mg L^{-1} kinetin (KIN; Sigma), 25 g L^{-1} sucrose (POCH) and 7 g L^{-1} phyto agar (Duchefa). The lines were subcultured every two weeks in the same medium.

The following techniques were used in the research:

- Isolation and culture of protoplasts from hypocotyls and calli of *F. tataricum* and *F. esculentum* (**P1**, **P2**, **P3** – *F. esculentum* and *F. tataricum*, **P4** – *F. tataricum*).
- Regeneration of plants from calli derived from protoplasts of *F. tataricum* and *F. esculentum* in media supplemented with different combinations of growth regulators and additives (**P1**, **P2 –** *F. esculentum* and *F. tataricum*, **P4 –** *F. tataricum*).
- Histological analysis of calli derived from *F. tataricum* and *F. esculentum* protoplasts included fixation, dehydration and embedding of the material in LR-White resin, sectioning using EM UC6 ultramicrotome (Leica Biosystems, Germany) and subsequent TBO staining (**P1 –** *F. tataricum*, **P2 –** *F. esculentum*).

- Symmetric electrofusion of protoplasts derived from calli of *F. tataricum* and *F. esculentum* to obtain hybrid cells *Fe* (+) *Ft* using the Super Electro Cell Fusion Generator 21 (NEPAGEN, Japan) (**P3**).
- Selection of hybrid cells *Fe* (+) *Ft* using a stripper tip MXL3-75 (ORIGIO Inc, USA) and a micromanipulator TransferMan® 4r (Eppendorf, Germany) (**P3**).
- Immunostaining of hybrid cells *Fe* (+) *Ft* and parental protoplasts using a set of seven primary antibodies targeting cell wall epitopes and a secondary antibody conjugated with Alexa Fluor 488 (**P3**)
- Determination of relative DNA content in plants derived from *F. tataricum* protoplasts using a flow cytometer (CyFlow Space, Sysmex) equipped with 365 nm UV LED as the light source (**P4**).
- Analysis of total phenolic and total flavonoid contents in morphogenic calli of *F. tataricum* during the regeneration process using the Folin–Ciocâlteu and aluminium chloride colourimetric methods, respectively (**P4**).
- Identification of the phenolic compounds profile using a high-performance liquid chromatography (HPLC) (**P4**).
- Untargeted metabolomic analysis of plants regenerated from morphogenic calli of *F. tataricum* by flow infusion electrospray high-resolution mass spectrometry (FIE-HRMS) using a Q Exactive Plus Hybrid Quadrupole Orbitrap Mass Analyser with an Acella UHPLC system (Thermo Fisher Scientific) (**P4**).
- Expression analysis of genes related to somatic embryogenesis in morphogenic calli of *F. tataricum* during the regeneration process using RT-qPCR (**P4**).

Detailed descriptions of the materials and methods used in this research, the results obtained, and their discussion can be found in the publications in the doctoral thesis marked with the numbers **P1**, **P2**, **P3** and **P4**.

9. Description of the research results

9.1. Optimisation of *F. tataricum* protoplast isolation and protoplast-derived plant regeneration.

Publication P1: Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn.

Zaranek, M., **Pérez-Pérez, R.**, Milewska-Hendel, A., Betekhtin, A., Grzebelus, E.

BMC Plant Biology **2023,** 385 (23), 743–757

https://doi.org/10.1186/s12870-023-04402-9

IF$_{2023}$: 4.3

Ministry of Science and Higher Education points: 140

This research presents a comprehensive approach to protoplast culture and plant regeneration in *F. tataricum*, highlighting significant advances in protoplast efficiency and viability and their development and regenerative capacity.

Protoplasts were isolated from hypocotyls and four morphogenic callus (MC) lines (MC1, MC2, MC4 and NL2018) from *F. tataricum* (Figure 2), demonstrating notable differences in the yield and viability. MC showed a higher protoplast release capacity than hypocotyls during the isolation. Line NL2018 stood out for its high protoplast yield ($3.93 \pm 0.09 \times 10^6$) and 78% viability, outperforming other lines, such as MC1, which showed a lower yield ($2.30 \pm 0.38 \times 10^6$) (Table1). The optimisation of the protoplast isolation process included different concentrations of driselase (0.1 – 0.25 %), a mixture of cell wall-degrading enzymes with cellulase, endo-1,3-β-glucanase and xylanase activities (Grison *et al.*, 2024). Although no significant differences were observed in the yield of hypocotyl-derived protoplasts by varying the concentration of driselase, this yield was five times lower compared to protoplasts derived from MC (Table 2), suggesting that the explant is a critical factor in the efficiency of the process.

Regarding the development of protoplast cultures, immobilisation in low-melting-point agarose was observed to be more effective than in alginate, promoting an increase in protoplast size and enhancing cell wall reconstruction (Figure 4A, C). The hormonal composition of the culture medium also influenced the development of protoplasts. The combination of 1 mg ml^{-1}

6-benzylaminopurine (BAP) and 2 mg ml^{-1} NAA showed a beneficial effect on the protoplast mitotic activity (Figure 4B, C), especially in the NL2018 line, where cell division and cell colony formation were observed in 5- and 8-day-old cultures, respectively (Figure 3D).

The application of PSK also positively impacted the mitotic activity of cells derived from MC and hypocotyl protoplasts (Figures 5 and 6). The plating efficiency, measured as the percentage of formed cell colonies in the 10^{th} day of culture, varied between 14% to 18% for protoplasts derived from MC in basal medium (BM: Kao and Michayluk macro/micro-elements, Kao and Michayluk organic acids, 5.50 M myo-inositol, 3.70 M thiamine, 6 M pyridoxine, 8 M nicotinic acid, 0.40 M glucose, 4.60 M N-Z-amine A, 4.40 M BAP, 1 M NAA) supplemented with 100 nM PSK. Contrastingly, when 10 μM AIP was added alongside PSK, plating efficiency varied between 12% and 21% , and the differences between treatments were not statistically significant (Figure 5). Furthermore, plating efficiency in protoplasts derived from hypocotyls followed a similar trend, with no significant differences observed between media containing PSK alone and PSK combined with either 0.025% PVP, 0.05% PVP, or 10 μM AIP, maintaining a range of 25% to 41% (Figure 6). This efficiency was approximately 33% higher than that observed in MC-derived cultures (Figures 5 and 6). However, in the callus multiplication medium (CM: MS medium with vitamins, 2 g L^{-1} N-Z-Amine A, 2 mg L^{-1} 2,4-D, 0.2 mg L^{-1} KIN, 30 g L^{-1} sucrose, 3 g L^{-1} phytagel), both concentrations of PVP considerably reduce the browning of the calli compared with the medium without PVP. The histological observations revealed that calli derived from MC protoplasts presented a heterogeneous structure with PECCs and meristematic cells (Figure 7B-D). In contrast, calli derived from hypocotyls showed a mass of parenchymal cells with large vacuoles and peripheral nuclei, suggesting a lower regenerative capacity than MC (Figure 7E).

Following one month of culture on regeneration medium supplemented with 2 mg ml^{-1} BAP, 1 mg ml^{-1} KIN and 0.025% PVP, calli derived from MC protoplasts developed somatic embryos (Figure 3J) and shoots (Figure 3K). Finally, after three months, whole plants without morphological abnormalities regenerated (Figure 3L), confirming the viability of the protoplast-to-plant system in *F. tataricum* using MC as starting material.

Besides providing an efficient protocol for protoplast cultivation and plant regeneration, this study sets the basis for future applications in somatic hybridisation and genetic improvement of *F. tataricum.*

BMC Plant Biology

RESEARCH Open Access

Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn

Magdalena Zaranek[1], Reneé Pérez-Pérez[1], Anna Milewska-Hendel[1], Alexander Betekhtin[1*] and Ewa Grzebelus[2*]

Abstract

Background *Fagopyrum tataricum* (Tartary buckwheat) is a valuable crop of great nutritional importance due to its high level of bioactive compounds. Excellent opportunities to obtain plants with the high level or the desired profile of valuable metabolites may be provided by in vitro cultures. Among known in vitro techniques, protoplast technology is an exciting tool for genetic manipulation to improve crop traits. In that context, protoplast fusion may be applied to generate hybrid cells between different species of *Fagopyrum*. To apply protoplast cultures to the aforementioned approaches in this research, we established the protoplast-to-plant system in Tartary buckwheat.

Results In this work, cellulase and pectinase activity enabled protoplast isolation from non-morphogenic and morphogenic callus (MC), reaching, on average, 2.3×10^6 protoplasts per g of fresh weight. However, to release protoplasts from hypocotyls, the key step was the application of driselase in the enzyme mixture. We showed that colony formation could be induced after protoplast embedding in agarose compared to the alginate matrix. Protoplasts cultured in a medium based on Kao and Michayluk supplemented with phytosulfokine (PSK) rebuilt cell walls, underwent repeated mitotic division, formed aggregates, which consequently led to callus formation. Plating efficiency, expressing the number of cell aggregate formed, in 10-day-old protoplast cultures varied from 14% for morphogenic callus to 30% for hypocotyls used as a protoplast source. However plant regeneration via somatic embryogenesis and organogenesis occurred only during the cultivation of MC-derived protoplasts.

Conclusions This study demonstrated that the applied protoplast isolation approach facilitated the recovery of viable protoplasts. Moreover, the embedding of protoplasts in an agarose matrix and supplementation of a culture medium with PSK effectively stimulated cell division and further development of Tartary buckwheat protoplast cultures along with the plant regeneration. Together, these results provide the first evidence of developing a protoplast-to-plant system from the MC of *Fagopyrum tataricum* used as source material. These findings suggest that

*Correspondence:
Alexander Betekhtin
alexander.betekhtin@us.edu.pl
Ewa Grzebelus
ewa.grzebelus@urk.edu.pl

Full list of author information is available at the end of the article

Tartary buckwheat's protoplast cultures have potential implications for the species' somatic hybridization and genetic improvement.

Keywords 2-aminoondane-2-phosphonic acid (AIP), Agarose, Driselase, Hypocotyl, Morphogenic callus, Non-morphogenic callus, Phenolic compounds, Plating efficiency, Polyvinylpyrrolidone (PVP), Tartary buckwheat

Background

Fagopyrum tataricum (L.) Gaertn., known as Tartary buckwheat, is one of the two most widely cultivated buckwheat species belonging to the family *Polygonaceae*. This self-pollinating, annual and dicotyledonous crop is grown in difficult climatic conditions, mainly in the mountain regions of southwest China [1, 2]. It is an excellent natural source of biologically active substances containing many flavonoids and phenolic compounds, especially rutin, quercetin and C-glycosylflavones, which has been used primarily in herbal medicine and the pharmaceutical industry [3, 4]. Flavonoid compounds improve the elasticity of the veins and support the circulatory system, while rutin is used in treating postoperative scars or body burns due to X-rays radiation [5]. Moreover, buckwheat is a rich source of starch, high-quality proteins, antioxidants, dietary fibre, vitamins and trace elements [6, 7]. Likely to common buckwheat (*Fagopyrum esculentum* L.) Tartary buckwheat is a plant with a health-promoting effect on the human body [8, 9]. In addition, it was shown that in plants, rutin enhances the defence system against environmental stress factors like UV light, low temperature, and desiccation [10]. Likewise, the high concentration of rutin protects buckwheat plants against insect pests [11] and has an effect on deterring animals [12]. The relatively good fatty acid composition, high dietary fibre content, and high vitamin B level make this plant an excellent food material with potential medicinal and pharmaceutical applications [13]. The nutraceutical properties of Tartary and common buckwheat include anti-oxidant, anti-ageing, anti-neoplastic properties, and cardio-protective and hepato-protective properties [4].

So far, in vitro culture systems for callus induction, plant regeneration, and the synthesis of phenolic compounds have been studied for buckwheat [14]. Protoplast-based procedures are one of the new plant breeding technologies that may be promising for buckwheat crop improvement [15]. Nonetheless, the possibility of protoplast regeneration into plants is fundamental in the successful application of somatic hybridisation or protoplast transformation [16] for transferring significant agronomical traits (i.e. tolerance to biotic/abiotic stresses and higher content of beneficial compounds) from wild *Fagopyrum* species [17]. Additionally, the buckwheat protoplast-based techniques may help obtain gene-edited plants with improved agronomical features by applying protoplast transfection. Nowadays, applying biotechnology tools to Tartary buckwheat may attract scientists due to it producing metabolites essential for preserving human health, creating genetically transformed plants and generating somatic hybrids [2, 16] as well in developmental biology research to the subcellular localisation of proteins and the assessment of gene activity [18].

Using protoplast cultures as a routine research tool requires the examination of different cultivars, ecotypes, and plant tissues to choose those with the best developmental and regenerative response in protoplast cultures [19–21]. The next crucial step is selecting an appropriate protoplast culture technique among cultures in liquid, semi-solid or solid medium with agar, agarose or alginate. Additionally, protoplast development can be ensured by applying additional supplements, such as peptide growth factors, polyamines or inhibitors of phenolics compounds. An excellent example of peptide growth factors application is PSK - a sulphated pentapeptide that promotes cell growth and proliferation [22], enhances the growth of callus [23], roots [24], shoots [25], and buds formation [26] and can improve somatic embryogenesis [27, 28]. Other compounds, such as polyamines, impact the maintenance of protoplast viability, increase mitotic activity and shoot regeneration and decrease oxidative stress [29]. The oxidation of phenolics in tissue culture harms the growth of tissues in in vitro conditions and leads to the browning of tissues and the growth medium. As a result, it reduces tissue growth, decreases regeneration rates and leads to cell culture necrosis [30]. Therefore, to reduce tissue browning, some compounds can be applied. Polyvinylpyrrolidone (PVP) is used to absorb phenolics released during protoplast cultures [31–33] or the propagation of woody plant species [34]. Another is 2-aminoindane-2-phosphonic acid (AIP), a specific competitive phenylalanine ammonia-lyase (PAL) inhibitor [30, 35, 36]. It should be noted that the application of AIP reduced flavonoid content and increased protoplast isolation frequency, effected on cell wall reconstruction, cell division, and decreased browning of suspension and callus culture of the *Ulmus americana* L [30, 36]. An alternative approach is to use some antioxidants. Ascorbic acid, citric acid and activated charcoal eliminate phenolics and other substances secreted into the culture medium by explants [32, 37–40]. The addition of activated charcoal to the protoplast culture medium improved colony and microcalli formation in chrysanthemum-derived protoplast cultures [39] and overcame the problem of cell browning during protoplast cultures of

Eustoma grandiflorum [38], *Vitis vinifera* L [41]. or *Solanum tuberosum* L [40].

The literature data concerning protoplast cultures of the buckwheat species are limited. So far, only one successful plant regeneration from hypocotyl-derived protoplasts of common buckwheat has been published [42]. In the case of Tartary buckwheat, Lachman and Adachi [43] reported callus formation in hypocotyl-derived protoplast cultures. Therefore, the main objective of this study was to (1) identify some factors promoting protoplast development and (2) develop a protoplast-based system for plant regeneration in Tartary buckwheat.

Results

Comprehensive protoplast cultures and plant regeneration were carried out as presented in Fig. 1.

Morphology of callus used as protoplast source

Protoplasts were isolated from one line of the non-morphogenic callus (NC, Fig. 2a) and four lines of the morphogenic callus (MC1, MC2, MC4, NL2018, Fig. 2b-e) of *Fagopyrum tataricum.* The 7-year-old NC line was characterised by a fragile structure and rapid growth and was formed exclusively from parenchymatous-type cells, which emerged on the surface of the MC1 line after several years of culture. On the other hand, the MC lines were varied in age; they were 10-, 4- and 2-year-old for MC1 and MC2; NL2018; MC4, respectively. They consisted of proembryogenic cell complexes (PECCs) and a 'soft' callus that appears during the cyclical disintegration of PECCs. PECCs are white structures (nodules) on the callus surface that appear one week after transfer to fresh medium. Therefore, the protoplasts were isolated from a 1-2-week-old callus, counting from the previous passage. The three lines of MC were different in the size of PECCs. The MC1, MC2 and MC4 lines had similar PECCs (Fig. 2b-d, red arrows), in contrast to the line NL2018, characterised by very small PECCs (Fig. 2e, red arrow). Probably the softer structure of the line NL2018 effect the protoplast quality. The cells of NL2018 were

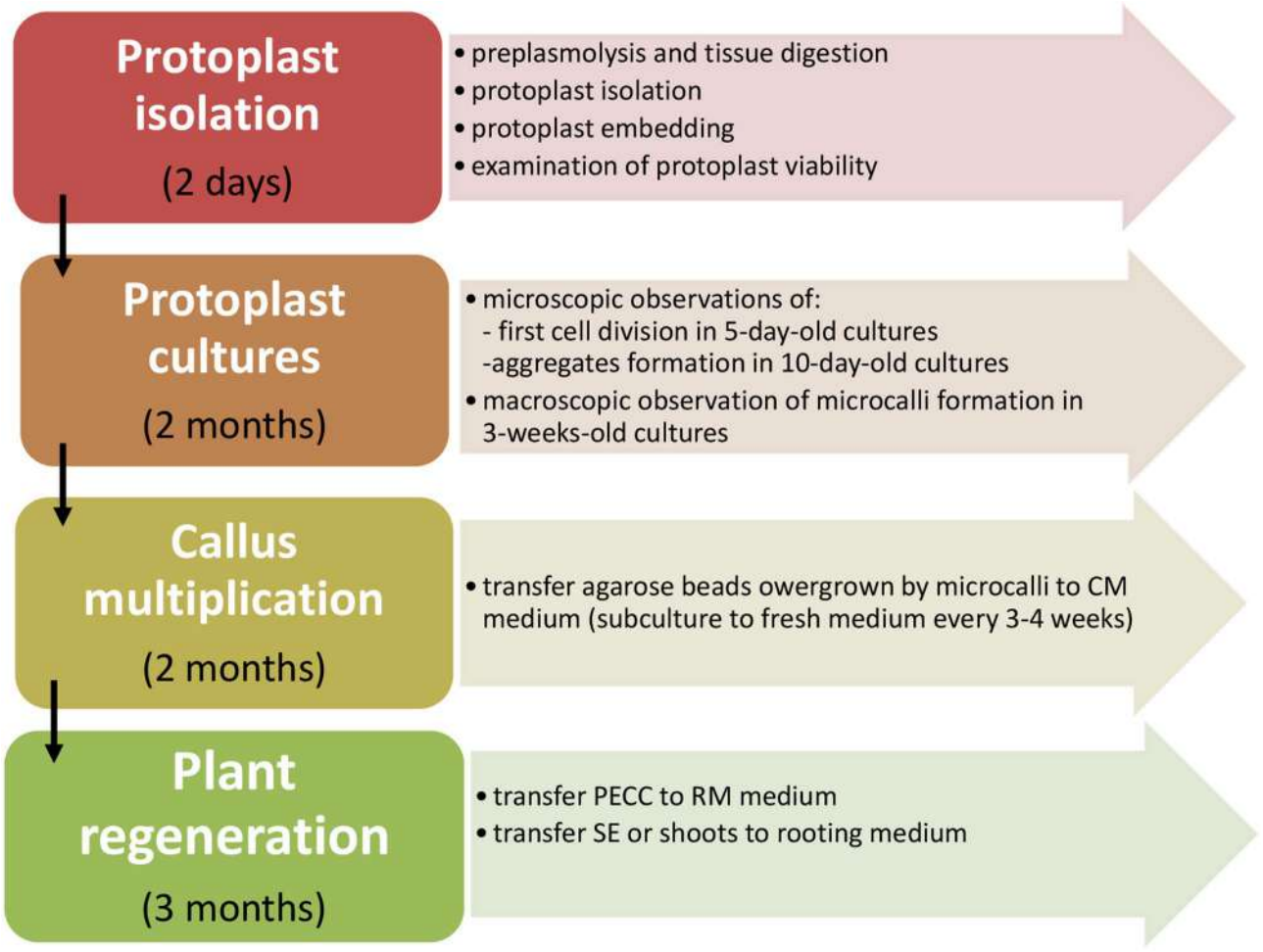


Fig. 1 Flow chart illustrating a step-by-step approach for plant regeneration via protoplast cultures of *Fagopyrum tataricum*. Details are described in the method section. *CM* callus multiplication medium; *PECC* pro-embryogenic cell complexes; *RM* regeneration medium; *SE* somatic embryos

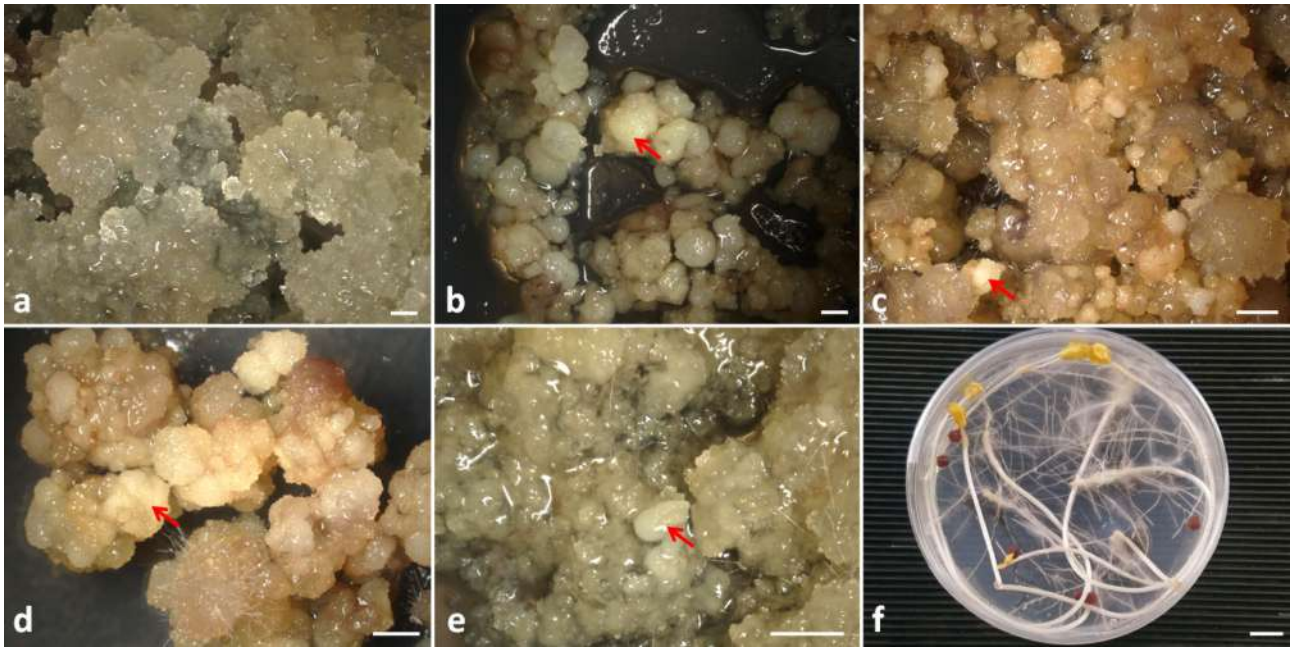


Fig. 2 Donor callus (**a-e**) and 10-day-old hypocotyls (**f**) of *Fagopyrum tataricum* used as source material for protoplast isolation. Morphology of 2-week-old callus lines: (**a**) non-morphogenic (NC) and morphogenic (MC) callus: (**b**) MC1, (**c**) MC2, (**d**) MC4, (**e**) NL2018. Arrows show proembryogenic cell complexes (PECCs) of MC. Scale bars: 1 mm (**a-e**), 1 cm (**f**)

not destroyed during protoplast cultures compared to the rest of the morphogenic lines.

Yield and viability of released protoplasts

Spherical protoplasts (Fig. 3a-c) were successfully isolated from NC, MC and hypocotyls (Fig. 2), and used as source material. The mean yield of NC protoplasts ($0.43 \pm 0.09 \times 10^6$) was six to nine times lower compared to MC protoplasts (Table 1). The highest protoplast yield from MC was noted for line NL2018 ($3.93 \pm 0.09 \times 10^6$), while the lowest was for the MC1 line ($2.30 \pm 0.38 \times 10^6$).

Different concentrations of driselase (a mix of several cell wall-degrading enzymes) to the enzyme mixture were applied to release protoplasts from the hypocotyl tissue and improve protoplast yield. The efficiency of protoplast yield reached, on average, 0.51×10^6 cells per g of tissue (Table 2). The mean number of released protoplasts varied from 0.39×10^6 after applying 0.25% driselase to 0.71×10^6 for 0.1% of driselase. However, differences observed in protoplast yield after applying different concentrations of driselase were insignificant. The average yield of hypocotyl-derived protoplasts was five-fold lower than from MC sources ($P \leq 0.01$).

Callus and hypocotyl-derived protoplasts, just after embedding in agarose beads, showed different viability as determined by fluorescein diacetate (FDA) staining (Tables 1 and 2). The viability of callus-derived protoplasts varied from 55% for NC to 78% for line NL2018; however, the observed differences were not significant (Table 1). Hypocotyl-derived protoplasts showed a different level of protoplast viability, depending on the driselase concentration during the maceration stage. The highest viability of hypocotyl protoplasts (81%) was obtained when digestion was performed using 0.25% driselase in the enzyme mixture.

FW fresh weight; *n* number of independent protoplast isolations. Means followed by the same letters within a column were not significantly different at $P \leq 0.05$.

FW fresh weight; *n* number of independent protoplast isolations. Means followed by the same letters within a column were not significantly different at $P \leq 0.05$.

Development of protoplast cultures

In preliminary experiments performed on NC protoplasts, (1) type of protoplast embedding matrix and (2) plant growth regulators (PGRs) composition in culture medium were examined. In 10-day-old cultures, positive symptom characteristics for the pre-mitotic period were observed, including: (1) cells enlargement in size, (2) change of the cell shape from spherical to oval, which was the morphological evidence of cell wall reconstruction and (3) reorganisation of the cytoplasm and cell organelles. Out of two applied embedding systems, immobilisation of protoplasts in SeaPlaque agarose better affected cell development. On average, twice as many pre-mitotic symptoms were observed in comparison to the alginate embedding system (Fig. 4a). Auxins and cytokinins used in various concentrations in culture medium also influenced the occurrence of pre-mitotic symptoms (Fig. 4b). The highest number (16%) of cells with positive

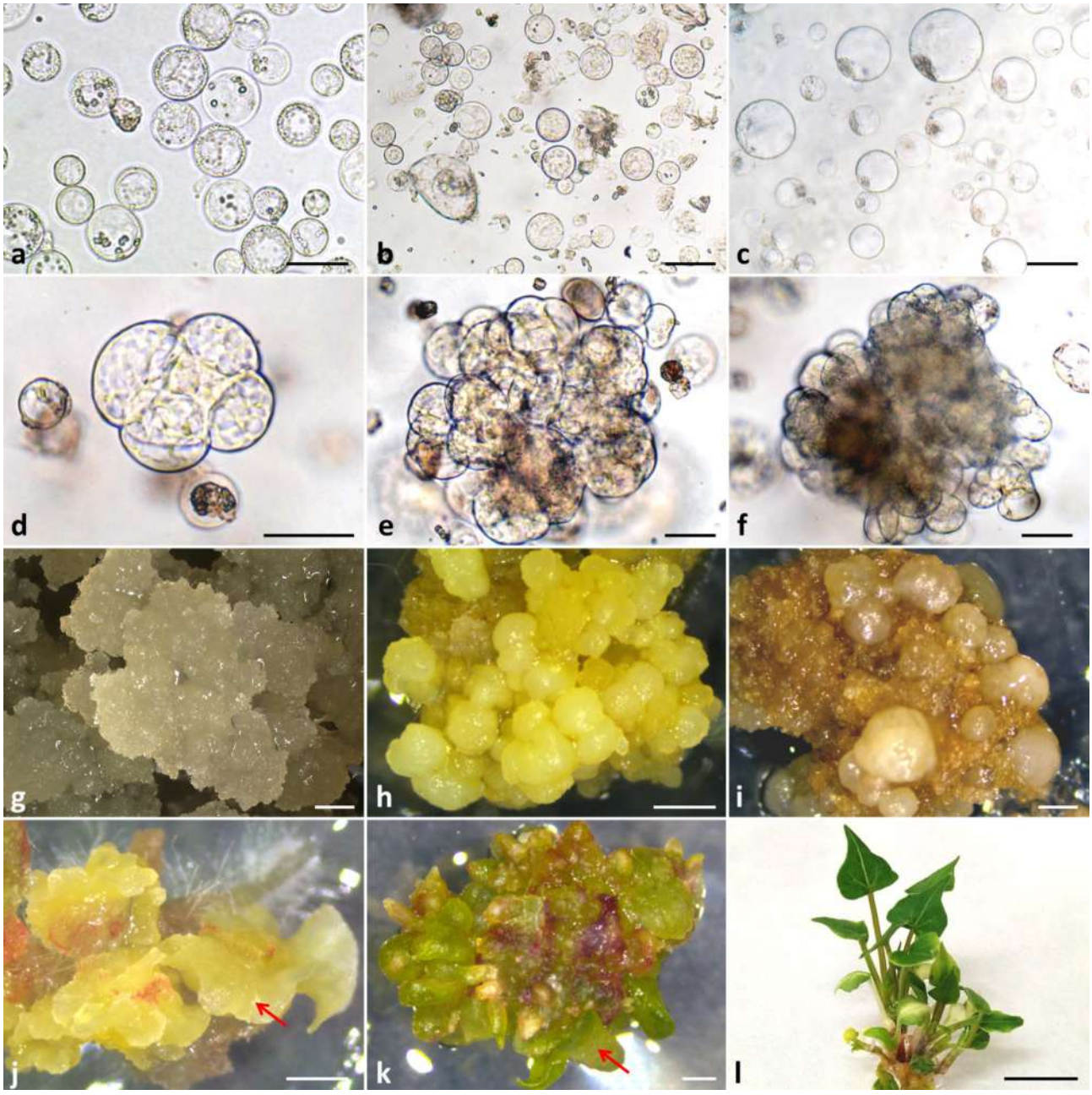


Fig. 3 Plant regeneration in protoplast cultures of *Fagopyrum tataricum*. Freshly isolated protoplasts from (**a**) non-morphogenic callus (NC), (**b**) morphogenic callus (MC) and (**c**) hypocotyls; multicellular aggregate in 8- (**d**), 10- (**e**), 20- (**f**) day-old protoplast cultures originating from MC; callus obtained from NC- (**g**), MC- (**h**) and hypocotyl- (**i**) derived protoplast cultures four months after protoplast isolation; subsequent stages of plant regeneration via somatic embryogenesis (**j**) and organogenesis (**k**) with - arrow indicating somatic embryo and shoot, respectively (after one month of regeneration); (**l**) plant of Tartary buckwheat regenerated from MC-derived protoplast cultures (after two month of regeneration). Scale bars: 50 µm (**a-f**), 1 mm (**g-k**), 1.5 cm (**l**)

symptoms was observed in culture variant medium III (supplemented with 0.2 mg L^{-1} kinetin (KIN) and 3.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D)), while the lowest (6.7%) was observed in medium IV (supplemented with 0.2 mg L^{-1} KIN and 2.0 mg L^{-1} 6-benzylaminopurine (BAP)), independent of the protoplast embedding system (Fig. 4c). In culture media variants I, II, V and VI the frequency of pre-mitotic symptoms was similar and reached, on average, 13% (Fig. 4b). Based on these results, in further experiments, protoplasts were embedded in agarose.

The MC1 line was used as a protoplast source in the preliminary experiments with morphogenic callus. Protoplasts embedded in agarose beads were cultured in the same six culture variants media as applied to NC-derived protoplasts (Fig. 4b). In 10-day-old cultures, mainly

Table 1 Isolation efficiency and viability of *Fagopyrum tataricum* callus-derived protoplasts

Protoplast source	Callus	Protoplast yield (× 10^6/g FW)		Protoplast viability (%)	
	line	n	Mean ± SE	n	Mean ± SE
Non-morphogenic callus	NC	3	0.43 ± 0.09^c	2	54.50 ± 5.50^a
Morphogenic callus	MC1	3	2.30 ± 0.38^a	3	66.67 ± 7.67^a
	MC2	2	2.44 ± 0.46^{ab}	2	68.75 ± 2.75^a
	MC4	2	2.40 ± 0.50^{ab}	2	64.00 ± 8.00^a
	NL2018	3	3.93 ± 0.09^b	2	77.93 ± 4.56^a
Mean/Total		13	2.28 ± 0.36	11	67.36 ± 3.23

Table 2 Effect of driselase concentration on yield and viability of protoplasts originating from *Fagopyrum tataricum* hypocotyls

Driselase	Protoplast yield (× 10^6/g FW)		Protoplast viability (%)	
concentration (%)	n	Mean ± SE	n	Mean ± SE
0.10	2	0.71 ± 0.06^a	2	72.00 ± 0^{ab}
0.15	2	0.43 ± 0.18^a	2	63.50 ± 0.50^a
0.25	2	0.39 ± 0.01^a	2	81.50 ± 3.50^b
Mean/Total	6	0.51 ± 0.08	6	72.33 ± 3.41

negative symptoms such as plasmolysis, broken cells or cells without developmental features were observed. However, in 2-month-old cultures in medium variant VI the microcalli was formed. Based on that observation, the medium variant VI was applied to the following protoplast cultures and named as basal medium (BM) for protoplast cultures. Among tested MC lines, only the NL2018 line revealed the ability to undergo cell divisions in protoplast cultures. Supplementation of the BM with PSK showed a beneficial effect on the mitotic activity of MC- and hypocotyls protoplast-derived cells (Figs. 5 and 6). Although first mitotic divisions were occasionally observed in the 5-day-old protoplast cultures of MC and hypocotyls, multicellular aggregates were already formed in 8-day-old cultures (Fig. 3d). As determined under the microscope, cells rich with dense cytoplasm in the aggregates were tightly packed, suggesting their embryogenic competence (Fig. 3e, f).

In 10-day-old protoplast cultures, the plating efficiency demonstrated by the number of cell aggregates formed was determined. For MC-derived protoplast cultures, this parameter ranged from 14–18% (Fig. 5) in control medium variants and from 12–21% for variants supplemented with AIP (Fig. 5). Nevertheless, differences in protoplast efficiency after the application of AIP were statistically insignificant. In culture two variant media (C and E) supplemented with PSK, putrescine (PUT) and N-(2-chloro-4-pyridyl)-N'-phenyl urea (CPPU), the highest number of cell aggregates (from 16 to 21%) was observed (Fig. 5).

In 10-day-old hypocotyl protoplast cultures, the number of cell aggregates varied, depending on the culture medium variant, from 25 to 41%, however, observed differences were statistically insignificant (Fig. 6). AIP and PVP applied additionally to the culture media to reduce the accumulation of phenolics and thus avoid culture browning did not influence the positive development of the culture. About twice the higher level of plating efficiency (33%) was observed in hypocotyl protoplast cultures compared to the MC protoplast cultures (15%).

Treatment	Pre-mitotic symptoms (%)		
	n	Mean ± SE	
Embedding matrix			a)
SeaPlaque agarose	3	17.45±1.55^a	
alginate	3	7.81± 0.96^b	
PGRs composition (mg L^{-1})			b)
I. KIN 0.2 + 2,4-D 1.0	3	13.47 ± 1.99^{ab}	
II. KIN 0.2 + 2,4-D 2.0	3	12.34 ± 2.37^{ab}	
III. KIN 0.2 + 2,4-D 3.0	3	16.15 ± 2.91^b	
IV. KIN 0.2 + BAP 2.0	3	6.71 ± 2.83^a	
V. BAP 1.0 + 2,4-D 2.0	3	14.76 ± 3.61^{ab}	
VI. BAP 1.0 + NAA 2.0	3	12.36 ± 3.48^{ab}	

Data represent means from three independent experiments.
For each treatment means followed by the same letters were not significantly different at $P \leq 0.05$

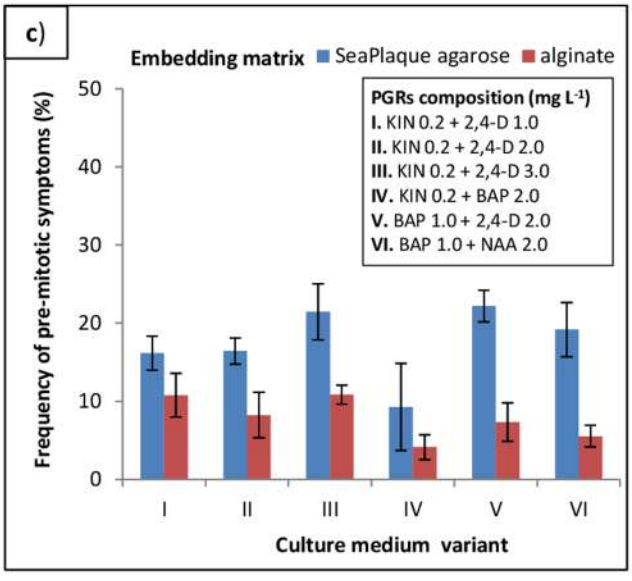


Fig. 4 Frequency of pre-mitotic symptoms in 10-day-old protoplast cultures originating from non-morphogenic callus of *Fagopyrum tataricum*. Effect of (**a**) embedding matrix, (**b**) plant growth regulators (PGRs) and (**c**) both treatments on culture development. *BAP* = 6-benzylaminopurine; *2,4-D* = 2,4 dichlorophenoxy acetic acid; *KIN* = kinetin; *NAA* = α-naphthalene acetic acid; *n* = number of independent protoplast isolations; *SE* = standard error. In chart bars represent means of three independent experiments ± SE

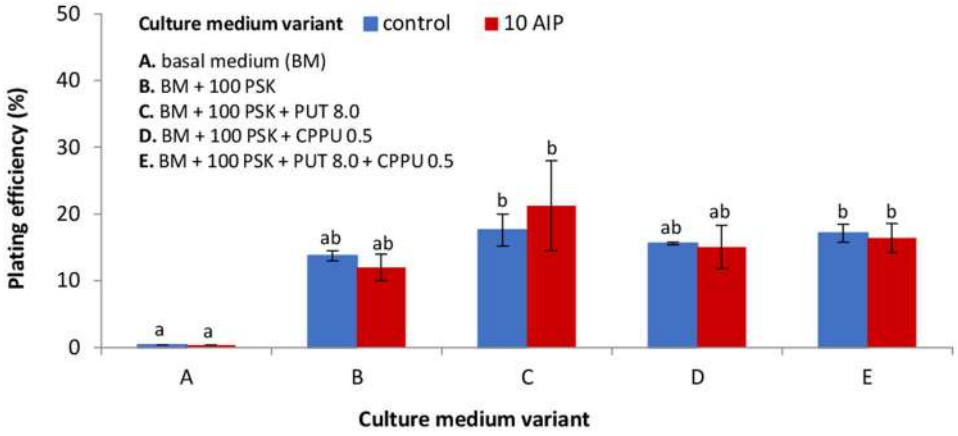


Fig. 5 Effect of plant growth regulators (PGRs) and AIP on plating efficiency in 10-day-old protoplast cultures originating from morphogenic callus (line NL2018) of *Fagopyrum tataricum*. PGRs composition in *BM* for protoplast cultures = 1.0 mg L^{-1} BAP (6-benzylaminopurine) + 2.0 mg L^{-1} NAA (α-naphthalene acetic acid); *AIP* = 10 µM 2-aminoindane-2-phosphonic acid; *100 PSK* = 100 nM phytosulfokine; *CPPU 0.5* = 0.5 mg L^{-1} N-(2-chloro-4-pyridyl)-N′-phenylurea; *PUT 8.0* = 8 mg L^{-1} putrescine. Bars represent means from two independent experiments ± SE (standard error). Means marked with the same letters were not significantly different at $P \leq 0.05$

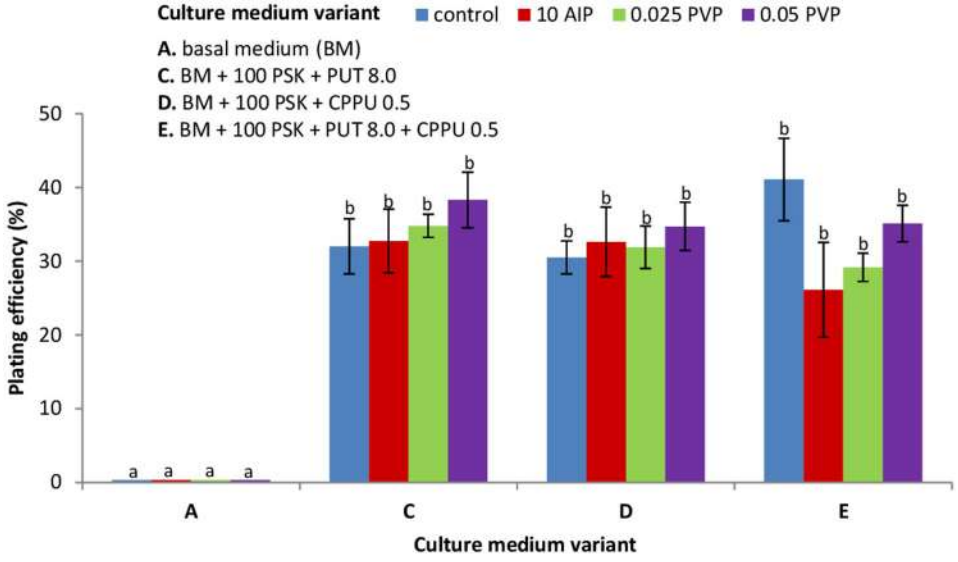


Fig. 6 Effect of plant growth regulators (PGRs) and compounds inhibiting (AIP) or absorbing (PVP) phenolics on plating efficiency in 10-day-old protoplast cultures originating from hypocotyls of *Fagopyrum tataricum*. PGRs composition in *BM* for protoplast cultures = 1.0 mg L^{-1} BAP (6-benzylaminopurine) + 2.0 mg L^{-1} NAA (α-naphthalene acetic acid); *100 PSK* = 100 nM phytosulfokine; *CPPU 0.5* = 0.5 mg L^{-1} N-(2-chloro-4-pyridyl)-N′-phenylurea; *PUT 8.0* = 8 mg L^{-1} putrescine; *10 AIP* = 10 µM 2-aminoindane-2-phosphonic acid; *0.025, 0.05 PVP* = 0.025% or 0.05% polyvinylpyrrolidone, respectively. Bars represent means from two to five independent experiments ± SE (standard error). Means marked with the same letters were not significantly different at $P \leq 0.05$

Independently on the protoplast source, multicellular aggregates continued to grow and become macroscopically visible after approximately three weeks of the culture. In the eighth week of culture, microcalli overgrew the agarose beads with different intensity, depending on the protoplast source. Medium development of microcalli was noted for NC- and MC-derived protoplast cultures. In the case of hypocotyl-derived protoplast cultures, the agarose beads were overgrown completely by microcalli. For NC-derived protoplast cultures the microcalli were observed for all medium variants except variant IV. For MC- and hypocotyl-derived protoplast cultures, microcalli developed regardless of the culture medium variant. Additionally, it was observed that the application of PVP to the culture reduced both the amount of floating metabolites in the protoplast medium and the browning of microcalli.

Histological observations of protoplast-derived callus

Histological observations revealed that callus developed from NC-derived protoplasts was composed of thin-walled parenchymatous cells, some of which were loosely

arranged (Fig. 7a). These cells varied in sizes, with a large vacuole and an irregular nucleus on the periphery of the cell protoplast (Fig. 7a inset). In the case of microcalli from morphogenic callus-derived protoplasts (line NL2018), histological analysis showed heterogenous callus with PECCs present, and thus several types of cells can be distinguished (Fig. 7b-d). The calli's surface noted some phenolic-containing cells (PCC) that had a large central vacuole in which phenolic compounds were accumulated (Fig. 7b and b inset, black arrows). Subsurficial tissue was composed of meristematic cells (Fig. 7b, red asterisk; Fig. 7c) that were characterised by the presence of several vacuoles, dense cytoplasm and round-shape nucleus with visible one or two nucleoli (Fig. 7c, red open arrow). The parenchymatous cells were present in the centre of PECCs (Fig. 7b, black asterisk). Histological observations confirmed the presence of embryogenic cells characterised by very dense cytoplasm, numerous small vacuoles and a large, round nucleus with one big nucleoli (Fig. 7d, red double arrows). Microcalli obtained

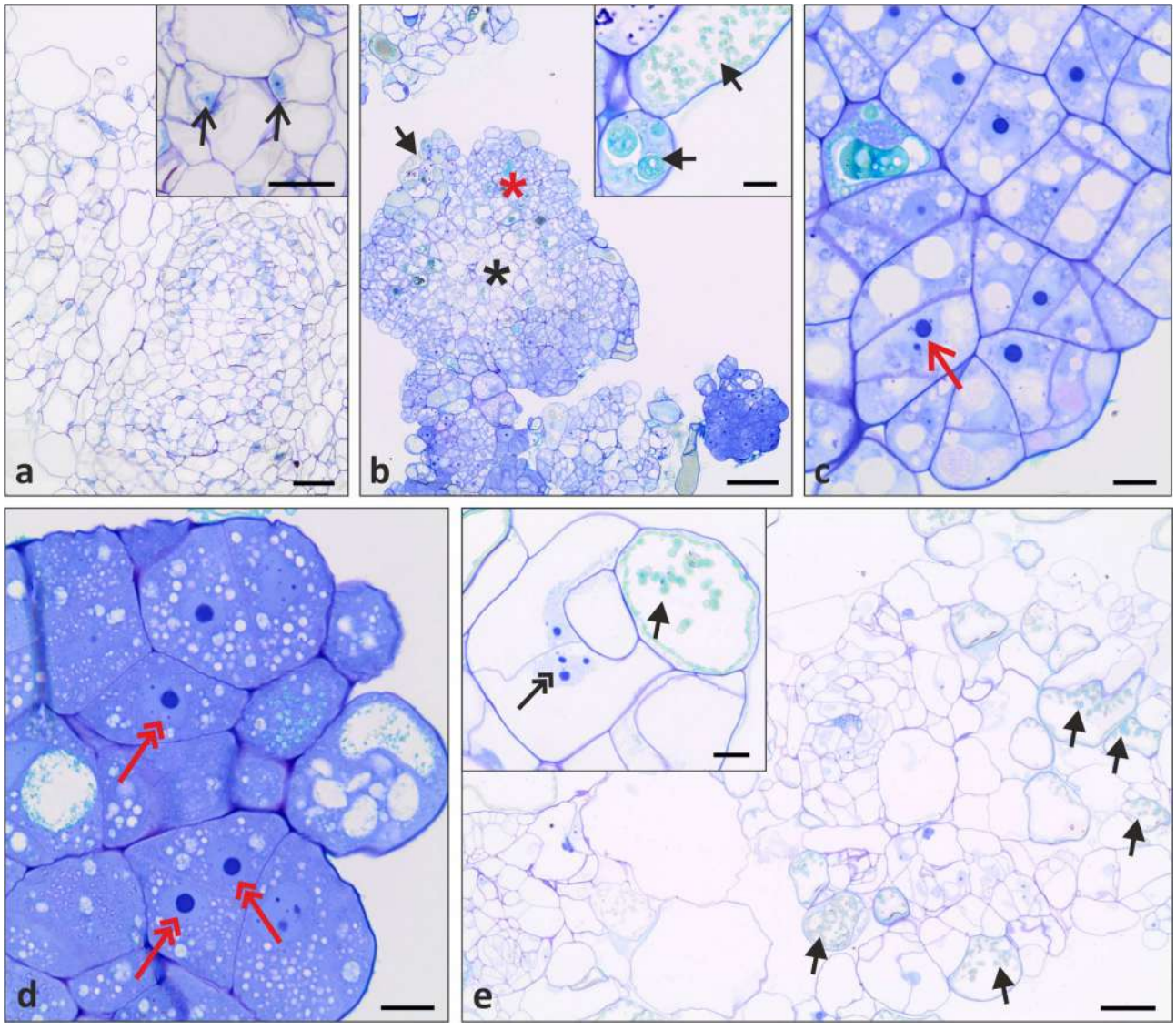


Fig. 7 Histological sections of protoplast-derived callus originating from: (**a**) non-morphogenic callus (NC), (**b-d**) morphogenic callus (MC; line NL2018) and (**e**) hypocotyls of *Fagopyrum tataricum*. Protoplast cultures from NC consisted of parenchymatous cells (**a**) with large vacuole and the nucleus in the periphery of the cell (**a** inset). Callus developed from MC-derived protoplasts (**b-d**) had morphogenic potential and the PECCs were observed (**b**). This callus consisted of phenolic-containing cells (**b** inset), meristematic cells (**c**) and embryogenic cells (**d**). Callus from hypocotyl-derived protoplast cultures was made of parenchymatous cells (**e**) and contained some cells with phenolic compounds (**e** inset) and some cells with nucleus with two or three nucleoli (**e** inset). Black open arrows show nucleus in the periphery of parenchymatous cells; black asterisk indicates parenchymatous cells of PECCs; red asterisk indicates meristematic cells of PECCs; black arrows indicate phenolic compounds; red open arrow shows nucleus with two nucleoli in meristematic cell; red double arrows indicate nucleus with large nucleoli in embryogenic cells; black double arrow shows nucleus with three nucleoli in parenchymatous cells. Scale bars: 10 µm (**b** inset, **c**, **d**, **e** inset), 50 µm (**a**, **a** inset, **e**), 100 µm (**b**)

from hypocotyl-derived protoplasts consisted of a mass of loosely arranged thin-walled parenchymatous cells (Fig. 7e). The vacuoles occupied almost the entire volume of the cells. As a result, the nucleus was located peripherally in the vicinity of the cell membrane (Fig. 7e inset). The nucleus was irregular in shape, and one to three nucleoli were observed (Fig. 7e inset, black open arrow). In some cells, the presence of phenolic compounds in the vacuole was detected (greenish colour after Toluidine Blue O staining; Fig. 7e and e inset, black arrows).

Plant regeneration from protoplast-derived tissue

Two-month-old protoplast-derived callus doubled its mass within the next two months on callus multiplication medium additionally enriched with PSK. Friable NC (Fig. 3g), soft callus with PECCs (Fig. 3h), and non-embryogenic callus (Fig. 3i) were observed in the cultures originating from NC, MC and hypocotyl protoplasts, respectively. After one month on the regeneration medium, the calli originating from MC protoplasts formed somatic embryos (Fig. 3j) and shoots (Fig. 3k). Finally, after about three months, plants without morphological abnormalities were produced (Fig. 3l).

Discussion

Plant protoplasts can dedifferentiate, re-enter the cell cycle, undergo repeated mitotic divisions, and develop into fertile plants [44, 45]. The protoplast technique has great potential for studying developmental biology [46], responses to stress conditions [25], in vitro selection or the production of useful secondary metabolites [47]. Especially the protoplast fusion and subsequent in vitro plant regeneration, as a tool of somatic hybridisation, offer opportunities for transferring entire genomes from one plant into another regardless of the interspecific crossing barriers [44].

Several source materials with different genotypes, cultivars, ages, and growth conditions of the source tissue are used by researchers for protoplast isolation [15]. In this research, protoplasts were isolated from callus (NC and MC) and hypocotyls to select material characterised by high regeneration capacity in protoplast cultures. In our study, a satisfactory number of protoplasts was achieved, reaching more than 2×10^6 protoplasts per g of callus and around 0.5×10^6 protoplasts per g of hypocotyls tissue. Similarly to our observations, a reduced number of hypocotyl-derived protoplasts in contrast to other source materials (e.g. leaves) was observed in studies on *Brassica oleracea* [48] and *Daucus carota* [21].

In order to improve the production of protoplasts from hypocotyl tissue, we applied driselase in the enzyme mixture. According to Thibault and Rouau [49], driselase is especially active towards carboxymethyl cellulose and hemicelluloses (xylan and laminarin). Those authors revealed that the application of driselase resulted in almost completely degraded polysaccharides (rhamnose, arabinose, galactose and glucuronic acid) in fibres from sugar beet pulp [49]. According to Lachmann and Adachi [43], it was possible to release protoplasts from 7-day-old hypocotyls of Tartary buckwheat without driselase. It seems that the genotype and the hypocotyl age might significantly influence the efficiency of protoplast isolation. Nevertheless, we noted satisfactory protoplast yield from the hypocotyls after the application of driselase. The activity of driselase may suggest that hypocotyl cell walls contain hemicelluloses such as laminarin and xylan, and therefore applying enzyme solution without driselase was unsuccessful. There is no literature data to confirm this suggestion, and this hypothesis will need further biochemical verification. Several authors have demonstrated that the addition of the driselase to the enzymatic mixture increased the protoplast yield isolated from *Kalanchoe blossfeldiana* [50], *Spathiphyllum wallisii, Anthurium scherzerianum* [51] and *Brassica oleracea* [52].

Different protoplast culture systems can be used, however, the immobilisation of protoplasts in a semi-solid medium ensures the physical separation of cells, decreases the production of polyphenols and prevents necrosis in the protoplast cultures [45, 53]. Interestingly, alginate is a common use alternative to agar or agarose. For *Daucus carota* [21, 54], *Brassica oleracea* [48] and *Beta vulgaris* [55], an increase in division frequency after protoplasts immobilisation in alginate was shown. In Tartary buckwheat, we did not observe such a positive effect of the alginate matrix on callus- and hypocotyl-derived protoplast cultures. However, the results of our study strongly demonstrated that the immobilisation of Tartary buckwheat protoplasts in agarose beads positively impacts their development. According to Brodelius and Nilsson [56], the production of secondary products from precursors and carbon sources was lower by the immobilised cells in agarose than for those embedded in alginate. Thus, we presume that immobilising Tartary buckwheat protoplasts in agarose might reduce the harmful secondary metabolites produced during protoplast cultures. Additionally, the applied SeaPlaque agarose is characterised by the reduction of helix structure and enables rapid delivery of gases and substances (hormones, signalling molecules, metabolites) to the embedded cells [57, 58]. Moreover, Shoichet et al. [59] demonstrated that the gel strength of cell-containing agarose, in contrast to alginate, is lower, which is connected with a reduction of cross-links between polymer chains of agarose. In the context of protoplast cultures, it makes it possible to increase in the space allowing the diffusion of the substances that were mentioned above. After applying the low melting point bead technique,

similar results were achieved in *Ulmus americana* protoplast cultures [60]. Also Pan et al. [61] reported that agarose was essential for cell division and colony formation for *Artemisia judaica* while alginate better affected the development of *Echinops spinosissimus* protoplasts.

Protoplast culture media, especially PGRs, are necessary for persistent mitotic divisions of protoplast-derived cells, aggregates formation, and their differentiation into plants [53]. According to Lachmann and Adachi [43], hypocotyl-derived protoplasts of Tartary buckwheat initiated cell division after three to five days after initiation of the culture. They formed cell aggregates in the medium enriched with BAP and naphthaleneacetic acid (NAA). In another research on common buckwheat protoplasts, Adachi et al. [42], after the application of different combinations of hormones, demonstrated the best response of protoplast development in a medium enriched with BAP and NAA. Our study demonstrated that only after applying PSK to BM medium supplemented with BAP and NAA, the first cell divisions took place in five-day-old cultures and the following development of protoplast cultures was observed. Thus, it seems that these hormones can be universal and used for both Tartary and common buckwheat.

A common way to support protoplast division and microcalli formation involves the application of additional supplements, such as peptide growth factors, polyamines, and compounds which can absorb or inhibit the production of phenolics. Our results demonstrated that supplementing the culture medium with PSK stimulated protoplast division and aggregates formation of hypocotyl- and MC-protoplast-derived cells. It should be noted that in PSK-free culture variant media, cell divisions were not observed. Also, applying PSK to callus multiplication medium enhanced the formation of embryogenic tissue. Similar stimulation of protoplast culture development as a result of PSK application was observed in *Beta vulgaris* [55], *Oryza sativa* [22], *Brassica oleracea* [19, 20], and *Daucus* ssp. [54]. Protoplast isolation is a stress-inducing procedure that can generate active oxygen species [44, 62]. Therefore, applying exogenous polyamines such as PUT seems to overcome this problem. Additionally, polyamines impact maintaining protoplast viability, increase mitotic activity and shoot regeneration [29]. Nevertheless, the application of PUT had no significant effect on the plating efficiency (number of cell aggregates formed) in MC- and hypocotyl-derived protoplast cultures of Tartary buckwheat. Comparable to our results, also in protoplast cultures of *Nigella damascena*, the application of PUT did not significantly affect plating efficiency [63]. We also implemented urea-type synthetic cytokinin (CPPU) that, according to the literature, participates in cell division and expansion [64]; induction of embryogenic callus [65] and shoot formation [66]. The supplementation of PSK-rich BM medium with PUT or CPPU or a combination of both enhanced the development of protoplast cultures and somatic embryos formation but did not increase the plating efficiency. This indicates that protoplast cultures of Tartary buckwheat are able to develop (i.e. to undergo the way from first mitotic to microcallus formation) only in the presence of PSK.

A common problem in protoplast and tissue cultures is oxidative browning of the culture media and tissue [30]. As mentioned in the background, phenolic compounds can block developmental processes in in vitro cultures. For our study, applying AIP (reversible inhibitor of PAL) in the MC- and hypocotyl-derived protoplast cultures did not prevent tissue browning or influence plating efficiency. In contrast to our results, *Ulmus americana*-derived protoplasts isolated from callus cultured in the presence of AIP were characterised by a higher rate of cell divisions and developed cell walls faster [36]. However, later studies showed, that AIP had no impact on the growth and development of protoplast-derived callus and shoots [60]. Another common compound applied to decrease tissue browning is PVP, which absorbs, among other compounds, phenolics [67]. Our study recorded visible reduction of tissue browning in protoplast-derived microcallus originating from hypocotyls. Nevertheless, the reduction of tissue browning was not associated with an increase in plating efficiency. Similarly to our observation in *Cyamopsis tetragonoloba* [31] and *Vitis* [32], the application of PVP did not prevent the browning of the culture media but reduced it to a low level.

So far, immature embryos, hypocotyls, and cotyledons of Tartary buckwheat were successfully applied to plant regeneration [9, 14]. According to Wang et al. [68], hypocotyl explants were better source material than cotyledons for Tartary buckwheat regeneration. Similarly, the regeneration of plants via somatic embryogenesis from hypocotyl explants was achieved by Han et al. [2]. In contrast to the presented examples, we did not observe plant regeneration in protoplast cultures originating from hypocotyls. Similarly to our results, Lachmann and Adachi [43] only reported about callus formation in hypocotyl-derived protoplast cultures. According to Pasternak et al. [69], the disadvantage of hypocotyls application as a source for protoplast isolation and cultures is rapidly increasing in cell ploidy level. For example, in *Cucumis sativus*, polysomaty was present in the hypocotyls and roots at the early stages of tissue differentiation. Moreover, the polysomatic nature of Tartary buckwheat plants [70] may explain the supposed polyploidisation of the tissue originating from hypocotyl protoplast cultures and lack of regeneration ability. Additionally, our histological observations revealed the presence of irregularity in shape nuclei and more than one nucleoli.

In non-morphogenic calli of *Beta vulgaris*, nuclei with irregular shapes and many nucleoli were observed, indicating polyploidy and aneuploidy [71]. A correlation between cell polyploidisation and instability of nuclei size and DNA content was found in the callus of *Allium fistulosum* [72]. Morphological characteristics of microcalli originating from hypocotyl protoplast cultures apparently explain this tissue's loss of regeneration capacity.

Due to the totipotency of plant cells, i.e. the possibility of their reprogramming from a differentiated state of a cell to a dedifferentiated state, plants are characterised by a high ability to regenerate, including when they are cultured in vitro [73]. Cellular reprogramming is associated with changes in transcriptome, which plays a significant role in the regulation of plant differentiation and plant development [74]. According to these views, we speculated that applying protoplast culture technology may result in the dedifferentiation of the NC cells of Tartary buckwheat, loss of their characteristic features, and reprogramming into embryogenically determined cells. The results demonstrate that the level of dedifferentiation of donor tissue during the removal of the cell wall and cell division is significant in protoplast regeneration. Yang et al. [75] hypothesised that non-embryogenic callus cells might have the ability to differentiate into embryogenic cells. Contrarily, Fehér [76] mentioned that protoplasts often retain the characteristic features of progenitor cells, which should be lost in the presence of hormones. Studies by Faraco et al. [77] showed that protoplasts retain their tissue- and cell-specific features during transient expression assay. These authors showed gene expression in protoplasts originating from the epiderma of petal and in the intact flower. Additionally, Sheen [78] pointed out that despite cell wall removal, protoplasts retain physiological responses and cellular activities as intact plants. Therefore, we may suppose that applied conditions and PGRs in protoplast cultures media were insufficient to complete cell dedifferentiation to embryogenically determined cells. As it was demonstrated by Betekhtin et al. [70], NC is composed mainly of parenchymatous cells, with inhibited capacity for morphogenesis. In our study, calli originating from NC-derived protoplast cultures consist of the same types and structures of the cells, characterised by friable structure, rapid growth, and lack of ability for regeneration. The irregular shaped nuclei of the protoplast-derived calli may indicate an increased amount of nuclear DNA. Similar observations were demonstrated for *Daucus carota* [79] and *Rosa hybrida* [89]. The authors noted a lack of regeneration after using as protoplast source non-embryogenic callus or non-embryogenic cell suspension cultures.

The cells of calli originating from MC-derived protoplast cultures were characterised by the abundance of embryogenic cells as described by Verdeil et al. [80]. The same features point out the ability to regenerate and strongly confirm the morphogenic character of the protoplast-derived tissue. According to Betekhtin et al. [70] MC is an excellent example of maintaining the regeneration potential due to genetic and cytogenetic stability in long-term cultivation. Transferring the calli originating from MC-derived protoplast cultures to a regeneration medium with BAP and KIN (supplemented with PVP) stimulates somatic embryogenesis and organogenesis with the following conversion into plants. In similar conditions, plant regeneration via somatic embryogenesis was demonstrated by Wang et al. [68] from hypocotyl explants. In summary, we suppose that the success of regeneration might depend on the genotype used in the study. The genotype-dependence in the development of protoplast cultures and their ability to regenerate was noted for *Brassica oleracea* [19, 25, 29, 48], *Daucus carota* [21, 54], *Beta vulgaris* [55] and *Musa ssp.* [81].

Conclusions

The present study demonstrated a successful approach for callus regeneration from hypocotyl- and, for the first time, plant regeneration from morphogenic callus-derived protoplasts of Tartary buckwheat. We demonstrated high cell colony and microcalli formation efficiency could be induced after protoplast embedding in agarose matrix and supplementing a culture medium with PSK. The presented protoplast-to-plant system enables using protoplasts as a model material for genetic engineering, i.e. genetic transformation of buckwheat to improve this agronomically important crop. This protocol can be helpful for precise genome editing using Cas9 ribonucleoprotein complexes. In addition, practical applications implemented for protoplast isolation, culture, and regeneration can be used in somatic hybridization between different *Fagopyrum* species.

Methods

Plant materials

As a protoplasts source, one line of the NC (Fig. 2a), four lines of the MC (MC1, MC2, MC4, NL2018; Fig. 2b-e) and etiolated hypocotyls of in vitro grown seedlings were used (Fig. 2f). The callus lines were obtained from the immature embryo of *F. tataricum* and maintained in the dark at 26±1°C on RX medium as described by Betekhtin et al. [70]. RX medium contained the mineral salts according to Gamborg's medium [82] (B5; Duchefa, The Netherlands), 2 g L^{-1} N-Z-amine A (Sigma-Aldrich, USA), 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (min. 98%) (2,4-D; Sigma-Aldrich), 0.5 mg L^{-1} indole-3-acetic acid (IAA; Sigma-Aldrich), 0.5 mg L^{-1} α-naphthalene acetic acid (NAA; Sigma-Aldrich), 0.2 mg L^{-1} kinetin (KIN; Sigma-Aldrich), 25 g L^{-1} sucrose (POCH, Poland) and 7 g L^{-1} phyto agar (Duchefa) [70]. The NC and MC

callus lines were subcultured every two weeks. Aseptic hypocotyls were produced in vitro from seeds (obtained from the collection of the N. I. Vavilov Institute of Plant Genetic Resources, Saint Petersburg, Russia) surface sterilised using a two-step procedure. First, seeds were dipped in 70% (*v/v*) ethanol for 30 s, then transferred to 0.1% (*v/v*) solution of fungicide Gwarant (Arysta, France) with one drop of Tween 20 (Duchefa) and placed on a gyratory shaker (160 rpm) and finally immersed in a 20% (*w/v*) solution of chloramin T (sodium N-chlorotoluene-4-sulphonamide; Chempur, Poland) with 800 mg L^{-1} cefotaxime disodium (Duchefa) and one drop of Tween 20 (30 min each step). After each step, the seeds were dipped in 70% ethanol for 30 s. Then the seeds were washed three times in sterile distilled water for 5 min each and left in the sterile distilled water overnight. On the next day, the washes in sterile water were repeated, the seeds were air-dried on a sterile filter paper and about eight seeds per Petri dish (Ø9 cm) were placed on solid Murashige and Skoog [83] medium with vitamins (MS; Duchefa) supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} plant agar (Duchefa) and maintained at 26±1°C in the dark for 10 days for seeds germination.

Protoplast isolation and culture

Protoplasts were isolated from 1-2-week-old callus and hypocotyls excised from 10-day-old seedlings, using the protocol of Grzebelus et al. [21] with some modifications. For protoplast isolation from callus 1 g of plant material was placed in a glass Petri dish (Ø9 cm) with preplasmolysis solution consisting of 0.6 M mannitol (Sigma-Aldrich) and 5 mM $CaCl_2$ (Sigma-Aldrich), cut into small pieces and then incubated for 1 h in the dark at 26±1°C. Release of protoplasts took place overnight (16 h) at 26±1°C, with gently shaking (30–40 rpm) in the enzyme mixture consisting of 1% (*w/v*) cellulase Onozuka R-10 (Duchefa), 0.1% pectolyase Y-23 (Duchefa), 20 mM 2-(N-Morpholino) ethanesulfonic acid (MES, Sigma-Aldrich), 5 mM $MgCl_2 \times 6H_2O$ (POCH), and 0.6 M mannitol, pH 5.6, filter-sterilised (0.22 µm; Millipore, Billerica, MA, USA). In the case of hypocotyls 1 g of tissue was cut into 1 cm pieces in length and then cut longitudinally in preplasmolysis solution (0.5 M mannitol). The tissue was macerated in the enzyme mixture containing of 1% cellulase Onozuka R10, 0.6% macerozyme R10 (Duchefa), 0.1–0.25% driselase® (Sigma-Aldrich), 20 mM MES, 5 mM $MgCl_2 \times 6H_2O$ and 0.6 M mannitol, pH 5.6, filter-sterilised (0.22 µm). The released protoplasts were separated from undigested tissue by filtration through a 100 µm nylon sieve (Millipore) and then centrifuged at 100 g for 5 min. Pellets were re-suspended in 0.5 M or 0.6 M sucrose with 1 mM MES for callus and hypocotyls, respectively, overlaid with W5 solution [84] and centrifuged at 145 g for 10 min. Protoplasts localised in the interphase between sucrose/MES and W5 solution were collected into a new tube and washed twice by centrifugation at 100 g for 5 min in W5 solution and then once in the culture medium. All protoplast culture media were based on the CPP medium according to Dirks et al. [85] and consisted of macro-, micro-elements and organic acids according to Kao and Mychayluk [86] (KM; Duchefa), vitamins according to B5 medium [82] (Duchefa), 74 g L^{-1} glucose (POCH) and 250 mg L^{-1} casein enzymatic hydrolysate (Sigma-Aldrich), pH=5.6, filter sterilised. After purification the protoplasts were suspended in 1 ml of the culture medium and their yield was determined using a Fuchs-Rosenthal haemocytometer (Heinz Herenz, Germany). The working density before cell embedding was adjusted to 8×10^5 or 5×10^5 cells per ml for callus- and hypocotyl-derived protoplasts, respectively. For protoplast embedding the filter-sterilised solution of 1.2% (*w/v*) SeaPlaque agarose (Duchefa) or filter-sterilised solution of 2.8% (*w/v*) alginic acid sodium salt (Sigma-Aldrich) were applied according to the protocol of Grzebelus et al. [55] and Grzebelus et al. [54], respectively. In the case of agarose embedding three to four 50 µl-aliquots of the protoplast/agarose mixture were dropped into a Petri dish (Ø 6 cm) and after solidification of the agarose beads (app. 15 min) 4 ml of the culture medium was added. For NC-derived protoplast cultures, the culture medium was supplemented with six different combinations of auxins and cytokinins, as shown in Fig. 4a. For MC- and hypocotyl-derived protoplast cultures the culture medium was supplemented with BAP 1.0 mg L^{-1} and NAA 2.0 mg L^{-1} and hereinafter referred to as basal medium (BM) for protoplast cultures. BM was additionally supplemented in different combinations with 100 nM phytosulfokine-α (PSK; PeptaNova GmbH, Germany), 8.0 mg L^{-1} Putrescine (PUT; Sigma-Aldrich), 0.5 mg L^{-1} N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU; Sigma-Aldrich), 0.025% or 0.05% polyvinylpyrrolidone (PVP, MW 40,000; Sigma-Aldrich) and 10 µM 2-aminoondane-2-phosphonic acid (AIP; Chemat, Poland) as shown in Figs. 5 and 6. To prevent endogenous bacterial contaminations, all protoplast culture media contained 300 mg L^{-1} ticarcillin disodium (Duchefa) or 200 mg L^{-1} cefotaxime disodium (Duchefa) in callus- or hypocotyl-derived protoplast cultures, respectively. Protoplast cultures were incubated at 26±1°C in the dark. After 10 days of culture, the medium with all supplements was replaced by a fresh one.

Histological analysis of protoplast-derived callus

Histological analyses were performed according to Betekhtin et al. [70] with minor modifications. Samples of microcalli obtained from two-month-old protoplast cultures were fixed in 4% paraformaldehyde (PFA, POCH) and 1% glutaraldehyde (GA, POCH) in 0.1 M phosphate

buffered saline (PBS, pH 7.2) overnight at 4°C. Subsequently, the samples were rinsed in PBS, dehydrated in increasing ethanol concentrations, and then embedded in LR White resin (Polysciences, PA). Samples were cut into 1.5 µm thick sections using a Leica EM UC6 ultramicrotome (Leica Biosystems, Germany), placed on glass slides coated with poly-L-lysine (Gerhard Menzel, Germany), stained with 0.05% Toluidine Blue O (Sigma-Aldrich) and mounted under a coverslip in Euparal medium (Sigma-Aldrich). The stained sections were examined under an Olympus BX43F microscope (Olympus LS, Tokyo, Japan) equipped with the Olympus XC50 digital camera.

Plant regeneration from protoplast-derived tissue

After about two months of protoplast culture, protoplast-derived callus in agarose beads were transferred to a callus multiplication medium (CM) consisting of macro-, micro-elements and vitamins according to MS medium [83], 2 g L^{-1} N-Z-amine A, 2.0 mg L^{-1} 2,4-D, 0.2 mg L^{-1} KIN, 100 nM PSK, 30 g L^{-1} sucrose and 3 g L^{-1} phytagel (Sigma-Aldrich). The cultures were maintained at 26±1°C in the dark and subcultured every three to four weeks. For plant regeneration, callus clumps or PECCs were transferred onto the regeneration medium (RM) containing macro- and micro-elements as in MS medium [83], 2.0 mg L^{-1} BAP, 1.0 mg L^{-1} KIN, 0.0025% PVP, 30 g L^{-1} sucrose, 3 g L^{-1} phytagel and cultured in a growth room at 28±2°C with a 16/8 h (light/dark) photoperiod, under light intensity of 55 µmol m^{-2} s^{-1}, and subcultured every three weeks. During three subcultures callus clumps and PECCs converted into bipolar and cotyledonary embryos, respectively. Small rooting shoots were transferred to a medium without PGRs containing macro-, micro-elements and vitamins according to MS [83], 30 g L^{-1} sucrose and 3 g L^{-1} phytagel and maintained in a growth room at 25±2°C with a 16/8 h (light/dark) photoperiod, under a light intensity of 55 µmol m^{-2} s^{-1}.

Numerical data collection and statistical analysis

The yield of protoplast isolation, protoplast viability and plating efficiency were determined. The protoplast yield was expressed as the number of protoplasts per gram of fresh weight of source material. Protoplast viability was assessed by staining the cells just after embedding in agarose beads with fluorescein diacetate (FDA; Sigma-Aldrich) according to Grzebelus et al. [21]. the viability of protoplasts was determined as a number of protoplasts with apple-green fluorescence per total number of observed cells (×100). Pre-mitotic symptoms in 10-day-old cultures of NC-derived protoplasts were expressed as the number of cells enlargement in size and with reorganized cytoplasm per total number of observed cells (×100). Plating efficiency was evaluated in 10-day-old cultures and expressed as the number of cell aggregates per total number of observed undivided cells and cell colonies (×100). Observations were performed using an Axiovert S100 inverted microscope (Carl Zeiss, Germany) equipped with a filter set appropriate for FDA detecting (λ_{Ex}=485 nm, λ_{Em}=515 nm).

At least two to five independent protoplast isolation experiments with a single treatment represented by three-four Petri dishes were carried out as biological repetitions. Microscopic observations were carried out on 100–200 cells per Petri dish. Means and the standard error of the means were calculated. Data were subjected to one-way analysis of variance (ANOVA) using Statistica 13 (TIBCO Software Inc., USA). Tukey's posthoc test was used to determine significant differences between the means.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
AIP	2-aminoondane-2-phosphonic acid
B5	Gamborg medium
BAP	6-benzylaminopurine
BM	basal medium
CM	callus multiplication medium
CPPU	N-(2-chloro-4-pyridyl)-N'-phenylurea
FDA	Fluorescein diacetate
GA	glutaraldehyde
KIN	kinetin
KM	Kao and Michayluk medium
MC	morphogenic callus
MS	Murashige and Skoog medium
NAA	α-naphthalene acetic acid
NC	non-morphogenic callus
PAL	phenylalanine ammonia-lyase
PBS	phosphate buffered saline
PCC	phenolic-containing cells
PECCs	proembryogenic cell complexes
PEMs	pro-embryogenic masses
PFA	paraformaldehyde
PGRs	plant growth regulators
PSK	phytosulfokine-α
PUT	putrescine
PVP	polyvinylpyrrolidone
RM	regeneration medium

Acknowledgements

The authors wish to thank Dorota Chachlowska and Adrianna Putowska for technical assistance with preliminary experiments with callus protoplast cultures.

Authors' contributions

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

Funding

This research was funded by the National Science Centre, Poland. Research project OPUS-19 (no. reg. 2020/37/B/NZ9/01499 awarded to AB).

Data Availability

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

Declarations

Ethics approval and consent to participate
The use of all plant materials in this study complies with relevant institutional, national, and international guidelines and legislation. Seeds of *F. tataricum* (sample k-17) are from the N. I. Vavilov Institute of Plant Genetic Resources collections, Saint Petersburg, Russia. Obtained seeds were multiplied in Plant Cytogenetic and Molecular Biology Group Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Poland. *F. tataricum* sample k-17 is a common cultivated landrace of *F. tataricum* and seeds are available on request from authors of the publication.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, 28 Jagiellonska st, Katowice 40-032, Poland
[2]Department of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, al. Mickiewicza 21, Krakow 31-120, Poland

Received: 30 January 2023 / Accepted: 4 August 2023
Published online: 10 August 2023

References

1. Bonafaccia G, Marocchini M, Kreft I. Composition and technological properties of the, flour and bran from common and tartary buckwheat. Food Chem. 2003;80(1):9–15.
2. Han M-H, Kamal A, Huh Y-S, A-Young J, Bae J, Chung K-Y, Lee M-S, Park SU, Jeong H, Woo S-H. Regeneration of plantlet via somatic embryogenesis from hypocotyls of tartary buckwheat. Aust J Crop Sci. 2011;5(7):865–9.
3. Zhang G, Xu Z, Gao Y, Huang X, Zou Y, Yang T. Effects of germination on the nutritional properties, phenolic profiles, and antioxidant activities of buckwheat. J Food Sci. 2015;80(5):H1111–1119.
4. Joshi DC, Zhang K, Wang C, Chandora R, Khurshid M, Li J, He M, Georgiev MI, Zhou M. Strategic enhancement of genetic gain for nutraceutical development in buckwheat: a genomics-driven perspective. Biotechnol Adv. 2020;39:107479.
5. Borkowska B, Robaszewska A. Zastosowanie ziarna gryki w różnych gałęziach przemysłu. Sci J Gdynia Maritime Univ 2012(73):43–55.
6. Krkošková B, Mrázová Z. Prophylactic components of buckwheat. Food Rer Int. 2005;38(5):561–8.
7. Li SQ, Zhang QH. Advances in the development of functional foods from buckwheat. Crit Rev Food Sci Nutr. 2001;41(6):451–64.
8. Farooq S, Rehman RU, Pirzadah TB, Malik B, Dar FA, Tahir I. Chapter twenty three - cultivation, agronomic practices, and growth performance of buckwheat. In: *Molecular Breeding and Nutritional Aspects of Buckwheat* Edited by Zhou M, Kreft I, Woo S-H, Chrungoo N, Wieslander G: Academic Press; 2016: 299–319.
9. Kumar M, Saraswat R. Plant regeneration and genetic transformation in buckwheat (*Fagopyrum* spp.), a multipurpose gluten free crop of high nutraceutical importance: a critical review. Ann of Plant Sci. 2018;7:1954–62.
10. Suzuki T, Morishita T, Kim S-J, Park S-U, Woo S-h, Noda T, Takigawa S. Physiological roles of rutin in the buckwheat plant. JARQ. 2015;49(1):37–43.
11. Stec K, Kordan B, Gabryś B. Quercetin and rutin as modifiers of aphid probing behavior. Molecules. 2021;26(12):3622.
12. Kreft I, Germ M, Golob A, Vombergar B, Bonafaccia F, Luthar Z. Impact of rutin and other phenolic substances on the digestibility of buckwheat grain metabolites. Int J Mol Sci. 2022;23(7):3923.
13. Bonafaccia G, Gambelli L, Fabjan N, Kreft I. Trace elements in flour and bran from common and tartary buckwheat. Food Chem. 2003;83(1):1–5.
14. Tomasiak A, Zhou M, Betekhtin A. Buckwheat in tissue culture research: current status and future perspectives. Int J Mol Sci. 2022;23(4):2298.
15. Reed KM, Bargmann BOR. Protoplast regeneration and its use in new plant breeding technologies. Front Genome Ed. 2021;3:734951.
16. Woo SH, Roy SK, Kwon SJ, Cho SW, Sarker K, Lee MS, Chung KY, Kim HH. Chapter three - Concepts, prospects, and potentiality in buckwheat (*Fagopyrum esculentum* Moench): A research perspective. In: *Molecular Breeding and Nutritional Aspects of Buckwheat* Edited by Zhou M, Kreft I, Woo S-H, Chrungoo N, Wieslander G: Academic Press; 2016: 21–49.
17. Mendler-Drienyovszki N, Cal AJ, Dobránszki J. Progress and prospects for interspecific hybridization in buckwheat and the genus *Fagopyrum*. Biotechnol Adv. 2013;31(8):1768–75.
18. Yoo S-D, Cho Y-H, Sheen J. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc. 2007;2(7):1565–72.
19. Kiełkowska A, Adamus A. Early studies on the effect of peptide growth factor phytosulfokine-α on *Brassica oleracea* var. capitata L. protoplasts. Acta Soc Bot Pol 2017, 86(8).
20. Kiełkowska A, Adamus A. Peptide growth factor phytosulfokine-α stimulates cell divisions and enhances regeneration from *B. oleracea* var. *capitata* L. protoplast culture. J Plant Growth Regul. 2019;38(3):931–44.
21. Grzebelus E, Szklarczyk M, Baranski R. An improved protocol for plant regeneration from leaf- and hypocotyl-derived protoplasts of carrot. Plant Cell Tiss Org. 2012;109(1):101–9.
22. Matsubayashi Y, Takagi L, Sakagami Y. Phytosulfokine-α, a sulfated pentapeptide, stimulates the proliferation of rice cells by means of specific high- and low-affinity binding sites. P Natal A Sci. 1997;94(24):13357–62.
23. Matsubayashi Y, Ogawa M, Kihara H, Niwa M, Sakagami Y. Disruption and overexpression of Arabidopsis phytosulfokine receptor gene affects cellular longevity and potential for growth. Plant Physiol. 2006;142(1):45–53.
24. Yamakawa S, Sakuta C, Matsubayashi Y, Sakagami Y, Kamada H, Satoh S. The promotive effects of a peptidyl plant growth factor, phytosulfokine-α, on the formation of adventitious roots and expression of a gene for a root-specific cystatin in cucumber hypocotyls. J Plant Res. 1998;111(3):453–8.
25. Kiełkowska A, Grzebelus E, Lis-Krzyścin A, Maćkowska K. Application of the salt stress to the protoplast cultures of the carrot (*Daucus carota* L.) and evaluation of the response of regenerants to soil salinity. Plant Cell Tiss Org. 2019;137(2):379–95.
26. Yang G, Shen S, Kobayashi T, Matsubayashi Y, Sakagami Y, Kamada H. Stimulatory effects of a novel peptidyl plant growth factor, phytosulfokine-α, on the adventitious bud formation from callus of *Antirrhinum majus*. Plant biotechnol. 1999;16(3):231–4.
27. Ochatt S, Conreux C, Moussa Mcolo R, Despierre G, Magnin-Robert J-B, Raffiot B. Phytosulfokine-alpha, an enhancer of in vitro regeneration competence in recalcitrant legumes. Plant Cell Tiss Org. 2018;135(2):189–201.
28. Gałuszka A, Gustab M, Tuleja M. *In vitro* morphogenetic responses from obligatory apomictic *Taraxacum belorussicum* val. N. Tikhom seedlings explants. Plant Cell Tiss Org. 2019;139(3):505–22.
29. Kiełkowska A, Adamus A. Exogenously applied polyamines reduce reactive oxygen species, enhancing cell division and the shoot regeneration from *Brassica oleracea* L. var. *capitata* protoplasts. Agronomy. 2021;11(4):735.
30. Jones AMP, Saxena PK. Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L.: a novel approach to reduce oxidative browning in plant tissue culture. PLoS ONE. 2013;8(10):e76802.
31. Saxena PK, Gill R. Removal of browning and growth enhancement by polyvinylpolypyrrolidone in protoplast cultures of *Cyamopsis tetragonoloba* L. Biol plant. 1986;28(4):313–5.
32. Reustle G, Natter I. Effect of polyvinylpyrrolidone and activated charcoal on formation of microcallus from grapevine protoplasts (*Vitis* sp). Vitis. 1994;33(3):117–21.
33. Prakash A, Rao KS, Kumar MU. Plant regeneration from protoplasts of *Capsicum annuum* L. cv. California wonder. J Biosci. 1997;22(3):339–44.
34. Lal M, Jamwal M, Bakshi P, Jasrotia A, Sharma N, Sharma M, Singh P, Sharma S, Kumar S. Influence of antioxidants on *in vitro* culture establishment of clonal apple rootstocks. Biol Forum-An Int J. 2021;13(2):381–5.
35. Peiser G, López-Gálvez G, Cantwell M, Saltveit ME. Phenylalanine ammonia lyase inhibitors control browning of cut lettuce. Postharvest Biol Tec. 1998;14(2):171–7.
36. Jones AMP, Chattopadhyay A, Shukla M, Zoń J, Saxena PK. Inhibition of phenylpropanoid biosynthesis increases cell wall digestibility, protoplast isolation, and facilitates sustained cell division in american elm (*Ulmus americana*). Bmc Plant Biol. 2012;12(1):75.

37. Jakhar ML, Verma R, Dixit DD. Effect of antioxidants on *in vitro* degree of browning and culture establishment of Guggul [*Commiphora wightii* (Arnott)]: A valuable desert medicinal plant. In: *Food Security through Agriculture & Allied Sciences: 2019*. 250–254.
38. Kunitake H, Nakashima T, Mori K, Tanaka M, Mii M. Plant regeneration from mesophyll protoplasts of lisianthus (*Eustoma grandiflorum*) by adding activated charcoal into protoplast culture medium. Plant Cell Tiss Org. 1995;43(1):59–65.
39. Adedeji OS, Naing AH, Kim CK. Protoplast isolation and shoot regeneration from protoplast-derived calli of *Chrysanthemum* cv. White ND. Plant Cell Tiss Org. 2020;141(3):571–81.
40. Carlberg I, Glimelius K, Eriksson T. Improved culture ability of potato protoplasts by use of activated charcoal. Plant Cell Rep. 1983;2(5):223–5.
41. Zhu Y-M, Hoshino Y, Nakano M, Takahashi E, Mii M. Highly efficient system of plant regeneration from protoplasts of grapevine (*Vitis vinifera* L.) through somatic embryogenesis by using embryogenic callus culture and activated charcoal. Plant Sci. 1997;123(1):151–7.
42. Adachi T, Yamaguchi A, Miike Y, Hoffmann F. Plant regeneration from protoplasts of common buckwheat (*Fagopyrum esculentum*). Plant Cell Rep. 1989;8(4):247–50.
43. Lachmann S, Adachi T. Callus regeneration from hypocotyl protoplast of tartary buckwheat (*Fagopyrum tataricum* Gaertn). Fagopyrum. 1990;10:62–4.
44. Eeckhaut T, Lakshmanan PS, Deryckere D, Van Bockstaele E, Van Huylenbroeck J. Progress in plant protoplast research. Planta. 2013;238(6):991–1003.
45. Davey MR, Anthony P, Power JB, Lowe KC. Plant protoplast technology: current status. Acta Physiol Plant. 2005;27(1):117–30.
46. Jiang F, Zhu J, Liu H-L. Protoplasts: a useful research system for plant cell biology, especially dedifferentiation. Protoplasma. 2013;250(6):1231–8.
47. Aoyagi H. Application of plant protoplasts for the production of useful metabolites. Biochem Eng J. 2011;56(1):1–8.
48. Kiełkowska A, Adamus A. An alginate-layer technique for culture of *Brassica oleracea* L. protoplasts. In Vitro Cell Dev Biol Plant. 2012;48(2):265–73.
49. Thibault J-F, Rouau X. Studies on enzymic hydrolysis of polysaccharides in sugar beet pulp. Carbohyd polym. 1990;13(1):1–16.
50. Castelblanque L, García-Sogo B, Pineda B, Moreno V. Efficient plant regeneration from protoplasts of *Kalanchoe blossfeldiana* via organogenesis. Plant Cell Tiss Org. 2009;100(1):107.
51. Duquenne B, Eeckhaut T, Werbrouck S, Van Huylenbroeck J. Effect of enzyme concentrations on protoplast isolation and protoplast culture of *Spathiphyllum* and *Anthurium*. Plant Cell Tiss Org. 2007;91(2):165–73.
52. Robertson D, Earle ED. Plant regeneration from leaf protoplasts of *Brassica oleracea* var. *italica* CV Green Comet broccoli. Plant Cell Rep. 1986;5(1):61–4.
53. Davey MR, Anthony P, Power JB, Lowe KC. Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv. 2005;23(2):131–71.
54. Mackowska K, Jarosz A, Grzebelus E. Plant regeneration from leaf-derived protoplasts within the *Daucus* genus: effect of different conditions in alginate embedding and phytosulfokine application. Plant Cell Tiss Org. 2014;117(2):241–52.
55. Grzebelus E, Szklarczyk M, Gren J, Sniegowska K, Jopek M, Kacinska I, Mrozek K. Phytosulfokine stimulates cell divisions in sugar beet (*Beta vulgaris* L.) mesophyll protoplast cultures. Plant Growth Regul. 2012;67(1):93–100.
56. Brodelius P, Nilsson K. Entrapment of plant cells in different matrices: a comparative study. Febs Lett. 1980;122(2):312–6.
57. Guastaferro M, Reverchon E, Baldino L. Agarose, alginate and chitosan nanostructured aerogels for pharmaceutical applications: a short review. Front Bioeng Biotechnol 2021, 9.
58. Forget A, Pique RA, Ahmadi V, Lüdeke S, Shastri VP. Mechanically tailored agarose hydrogels through molecular alloying with β-sheet polysaccharides. Macromol Rapid Comm. 2015;36(2):196–203.
59. Shoichet MS, Li RH, White ML, Winn SR. Stability of hydrogels used in cell encapsulation: an *in vitro* comparison of alginate and agarose. Biotechnol Bioeng. 1996;50(4):374–81.
60. Jones AMP, Shukla MR, Biswas GCG, Saxena PK. Protoplast-to-plant regeneration of american elm (*Ulmus americana*). Protoplasma. 2015;252(3):925–31.
61. Pan ZG, Liu CZ, Murch SJ, El-Demerdash M, Saxena PK. Plant regeneration from mesophyll protoplasts of the egyptian medicinal plants *Artemisia judaica* L. and *Echinops spinosissimus* Turra. Plant Sci. 2003;165(4):681–7.
62. Papadakis AK, Roubelakis-Angelakis KA. Oxidative stress could be responsible for the recalcitrance of plant protoplasts. Plant Physiol Bioch. 2002;40(6):549–59.
63. Klimek-Chodacka M, Kadluczka D, Lukasiewicz A, Malec-Pala A, Baranski R, Grzebelus E. Effective callus induction and plant regeneration in callus and protoplast cultures of *Nigella damascena* L. Plant Cell Tiss Org. 2020;143(3):693–707.
64. Li J, Xu J, Guo Q-W, Wu Z, Zhang T, Zhang K-J, Cheng C-y, Zhu P-y, Lou Q-F, Chen J-F. Proteomic insight into fruit set of cucumber (*Cucumis sativus* L.) suggests the cues of hormone-independent parthenocarpy. BMC Genomics. 2017;18(1):896.
65. Nakajima I, Kobayashi S, Nakamura Y. Embryogenic callus induction and plant regeneration from unfertilized ovule of 'Kyoho'grape. J Jpn Soc Hortic Sci. 2000;69(2):186–8.
66. Tsuro M, Koda M, Inoue M. Comparative effect of different types of cytokinin for shoot formation and plant regeneration in leaf-derived callus of lavender (*Lavandula vera* DC). Sci Hortic-Amsterdam. 1999;81(3):331–6.
67. Gray JC. Absorption of polyphenols by polyvinylpyrrolidone and polystyrene resins. Phytochemistry. 1978;17(3):495–7.
68. Wang CL, Dong XN, Ding MQ, Tang YX, Zhu XM, Wu YM, Zhou ML, Shao JR. Plantlet regeneration of Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) In vitro tissue cultures. Protein Pept Lett. 2016;23(5):468–77.
69. Pasternak T, Lystvan K, Betekhtin A, Hasterok R. From single cell to plants: mesophyll protoplasts as a versatile system for investigating plant cell reprogramming. Int J Mol Sci. 2020;21(12):4195.
70. Betekhtin A, Rojek M, Jaskowiak J, Milewska-Hendel A, Kwasniewska J, Kostyukova Y, Kurczynska E, Rumyantseva N, Hasterok R. Nuclear genome stability in long-term cultivated callus lines of *Fagopyrum tataricum* (L.) Gaertn. PLoS ONE 2017, 12(3).
71. Hagège D, Catania R, Micalef H, Gaspar T. Nuclear shape and DNA content of fully habituated nonorganogenic sugarbeet cells. Protoplasma. 1992;166(1):49–54.
72. Joachimiak A, Ilnicki T. Nuclear morphology, polyploidy, and chromatin elimination in tissue culture of *Allium fistulosum* L. Acta Soc Bot Pol 2003, 72(1).
73. Fehér A. Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? Front Plant Sci. 2019;10:536.
74. Gruel J, Deichmann J, Landrein B, Hitchcock T, Jönsson H. The interaction of transcription factors controls the spatial layout of plant aerial stem cell niches. npj Syst Biol Appl. 2018;4(1):36.
75. Yang X, Zhang X. Regulation of somatic embryogenesis in higher plants. Crit Rev Plant Sci. 2010;29(1):36–57.
76. Fehér A. Somatic embryogenesis — stress-induced remodeling of plant cell fate. BBA - Gene Regul Mech. 2015;1849(4):385–402.
77. Faraco M, Di Sansebastiano GP, Spelt K, Koes RE, Quattrocchio FM. One protoplast is not the other! Plant Physiol. 2011;156(2):474–8.
78. Sheen J. Signal transduction in maize and arabidopsis mesophyll protoplasts. Plant Physiol. 2001;127(4):1466–75.
79. Han J-E, Lee H-S, Lee H, Cho H, Park S-Y. Embryogenic stem cell identity after protoplast isolation from *Daucus carot*a and recovery of regeneration ability through protoplast culture. Int J Mol Sci. 2022;23(19):11556.
80. Verdeil JL, Alemanno L, Niemenak N, Tranbarger TJ. Pluripotent versus totipotent plant stem cells: dependence versus autonomy? Trends Plant Sci. 2007;12(6):245–52.
81. Assani A, Häicour R, Wenzel G, Foroughi-Wehr B, Bakry F, Côte F-X, Ducreux G, Ambroise A, Grapin A. Influence of donor material and genotype on protoplast regeneration in banana and plantain cultivars (*Musa* spp). Plant Sci. 2002;162(3):355–62.
82. Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res. 1968;50(1):151–8.
83. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15(3):473–97.
84. Menczel L, Nagy F, Kiss ZR, Maliga P. Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. Theor Appl Genet. 1981;59(3):191–5.
85. Dirks R, Sidorov V, Tulmans C. A new protoplast culture system in *Daucus carota* L. and its applications for mutant selection and transformation. Theor Appl Genet. 1996;93(5–6):809–15.
86. Kao KN, Michayluk MR. Nutritional requirements for growth of *Vicia Hajastana* cells and protoplasts at a very low population density in liquid media. Planta. 1975;126(2):105–10.

Publisher's Note

9.2. Optimisation of *F. esculentum* protoplast isolation and protoplast-derived plant regeneration.

Publication P2:

Zaranek, A.*, **Pérez-Pérez, R.***, Milewska-Hendel, A., Grzebelus, E., Betekhtin, A.

equal contribution

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

Plant Cell, Tissue and Organ Culture (PCTOC), 2023**,** 154, 673–687

https://doi.org/10.1007/s11240-023-02542-2

IF$_{2023}$: 2.3

Ministry of Science and Higher Education points: 100

This research focused on the isolation, cultivation and regeneration of protoplasts from two embryogenic callus (EC)******* lines (L1 and NL2) and hypocotyls of *F. esculentum* (Figures 1A, B and 2A, respectively).

Several enzyme combinations were tested for cell wall digestion, with variations in concentration and composition, including cellulase R10, cellulase RS, pectolyase Y-23, macerozyme R10, and driselase (Table 1). The yield of EC-derived protoplasts ranged from 0.83 to 1.54 × 10^6 cells per gram of tissue, with NL2 showing the highest efficiency when using E2 enzyme solution (0.15% driselase, 1.5% cellulase R10 and 0.1% pectolyase Y-23) (Table 3). In the case of hypocotyls, the addition of driselase to the enzyme solution was crucial to obtain an acceptable number of protoplasts, with an average yield of 0.44 × 10^6 cells per gram of fresh weight (Table 4), which still represents less than twice the EC-derived protoplasts efficiency. The viability, assessed by fluorescein diacetate staining, ranged from 73% to 81% in EC-derived protoplasts (Table 3) and from 75% to 80% in hypocotyl-derived protoplasts when driselase was used during maceration (Table 4). Although different concentrations of driselase were tested, no significant differences in the viability of hypocotyl-derived protoplasts were observed (Table 4). Adding 100 nM PSK to BM during protoplast culture enhanced cell division, which was observed between the third and seventh day of culture in protoplasts derived from all tested sources (Figure 3). Plating efficiency ranged between 21% and 35% for

*******While the P2 publication originally designated the *F. esculentum* callus as **morphogenic callus** (MC), further analysis of its developmental pathway revealed somatic embryogenesis as the primary regeneration mechanism, leading to the more accurate classification as **embryogenic callus** (EC).

the L1 line, while for the NL2 line and hypocotyls, it was around 21% (Table 5). Although 0.025% and 0.05% PVP was applied to BM to adsorb toxic and phenolic metabolites, no apparent impact on culture development was observed (Figure 3).

At 30 days of protoplast culture, microcalli from NL2 protoplasts were already overgrowing on the agarose beads and embryogenic masses were observed (Figure 1F). Abundant somatic embryos grew in the first week on MS4 regeneration medium supplemented with 1 mg L^{-1} thidiazuron (TDZ) and 3 mg L^{-1} BAP (Table 2, Figure 1G), followed by green structures and shoots after 15 days (Figure 1H). Finally, complete plants with strong root systems were obtained within two months (Figure 1I). On the other hand, microcalli derived from the L1 line completely covered the agarose beads after 60 days of culture. However, following three months on MS3 regeneration medium supplemented with 2 mg L^{-1} BAP and 1 mg L^{-1} KIN (Table 2), yellow and brown calli and, sporadically, shoot-like structures were observed. Finally, in the fourth month, shoots appeared and developed into whole plants. Similarly, the overgrowth of microcalli derived from hypocotyl protoplast on agarose beads was observed after 60 days of culture, occasionally showing somatic embryos (Figure 2F). A scarce plant regeneration occurred through somatic embryogenesis and organogenesis (Figure 2G) after three months on MS3 regeneration medium (Figure 3H). The regeneration timing achieved in this research (two to five months) considerably differs from previous studies in *F. esculentum*, where the plant regeneration from microcalli derived from hypocotyl protoplasts took between nine and eighteen months (Table 5).

The histological analysis of the microcalli supported these results, revealing that L1 and hypocotyl-derived callus shared similar characteristics, consisting primarily of parenchymatous cells with a big vacuole and irregular, peripherally located nuclei, along with sparse phenolic cells on their surfaces and small regions of meristematic-like cells (Figure 4A, C). In contrast, NL2-derived microcalli were predominantly composed of meristematic cells, characterised by dense cytoplasm, small vacuoles, and a central round-shaped nucleus with one or two nucleoli (Figure 4B). While all calli occasionally contained meristematic or meristematic-like cell clusters, NL2 uniquely maintained this organisation throughout, suggesting higher regenerative potential.

In summary, this study demonstrates the viability of the protoplast-to-plant system in *F. esculentum*, highlighting the importance of selecting an appropriate enzymatic solution depending on the protoplast source and the PSK supplementation. The research provides an

efficient and fast protocol for protoplast culture and development into whole plants, opening new possibilities for genetic improvement and somatic hybridisation in this species.

Supplementary material available online

Supplementary Table_1 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-023-02542-2/MediaObjects/11240_2023_2542_MOESM1_ESM.docx

Plant Cell, Tissue and Organ Culture (PCTOC) (2023) 154:673–687
https://doi.org/10.1007/s11240-023-02542-2

ORIGINAL ARTICLE

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

Magdalena Zaranek[1] · Reneé Pérez-Pérez[1] · Anna Milewska-Hendel[1] · Ewa Grzebelus[2] · Alexander Betekhtin[1]

Received: 20 March 2023 / Accepted: 30 May 2023 / Published online: 13 June 2023

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

Communicated by Wagner Campos Otoni.

Magdalena Zaranek and Reneé Pérez-Pérez have contributed equally to this work.

✉ Ewa Grzebelus
ewa.grzebelus@urk.edu.pl

✉ Alexander Betekhtin
alexander.betekhtin@us.edu.pl

Magdalena Zaranek
magdalena.zaranek@us.edu.pl

Reneé Pérez-Pérez
rene.perez-perez@us.edu.pl

Anna Milewska-Hendel
anna.milewska@us.edu.pl

[1] Faculty of Natural Sciences, Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice, Jagiellonska 28, 40-032 Katowice, Poland

[2] Department of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Avenue 29 Listopada 54, 31-120 Cracow, Poland

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
BM	Basal medium
CM	Callus multiplication medium
CPPU	*N*-(2-chloro-4-pyridyl)-*N*′-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 form 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
	$MgCl_2 \times 6H_2O$ (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
	$MgCl_2 \times 6H_2O$	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
	$MgCl_2 \times 6H_2O$	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
	$CaCl_2 \times 2H_2O$ (Sigma-Aldrich)	125 mM	18.36
	KCl (Sigma-Aldrich)	5 mM	0.37
	Glucose (Sigma-Aldrich)	5 mM	0.90

[a]*MES* 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 8×10^5 or 5×10^5 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
	Phytagel (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
	Phytagel	–	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
	Phytagel	–	3.00 g
Rooting Medium (pH 5.8)	MS including vitamins	–	4.40 g
	Sucrose	87 mM	30.00 g
	Phytagel	–	3.00 g

[a] *KM* (Kao and Michayluk 1975)

[b] acc. 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}

[c] *MS* (Murashige and Skoog 1962)

beads per Petri dish (Ø6 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 µmol m^{-2} 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 × 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 µmol m^{-2} 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 µmol m^{-2} 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 (λ_{Ex} = 485 nm, λ_{Em} = 515 nm). Image acquisition was performed under an inverted Lecia 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

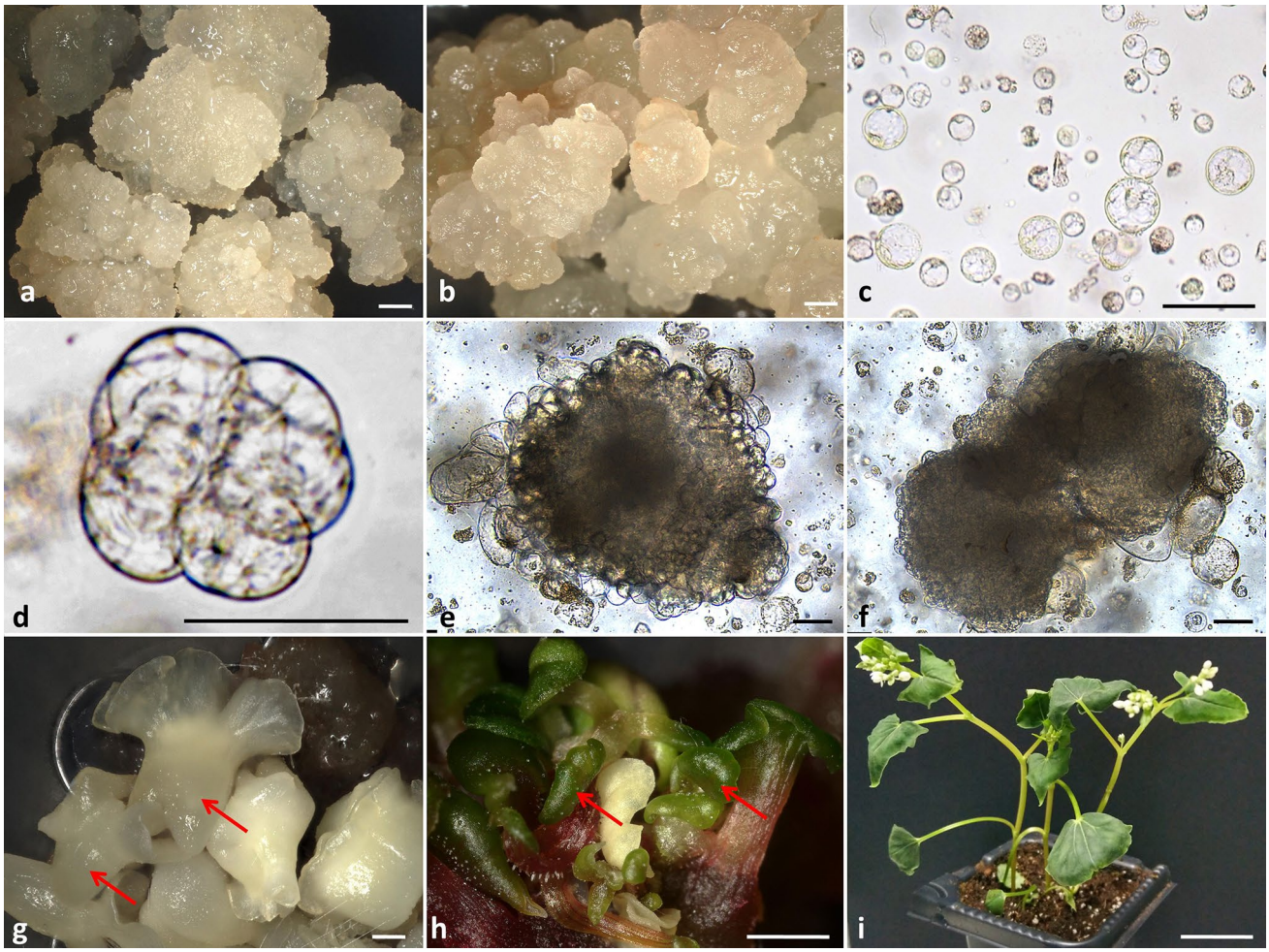


Fig. 1 Plant regeneration from morphogenic callus (MC)-derived protoplasts in *Fagopyrum esculentum*. Lines of MC used for protoplast isolation: **a** L1 and **b** NL2; **c** freshly MC-derived protoplasts; multicellular aggregate in **d** 6-, **e** 20- and **f** 30-day-old protoplast cultures; subsequent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

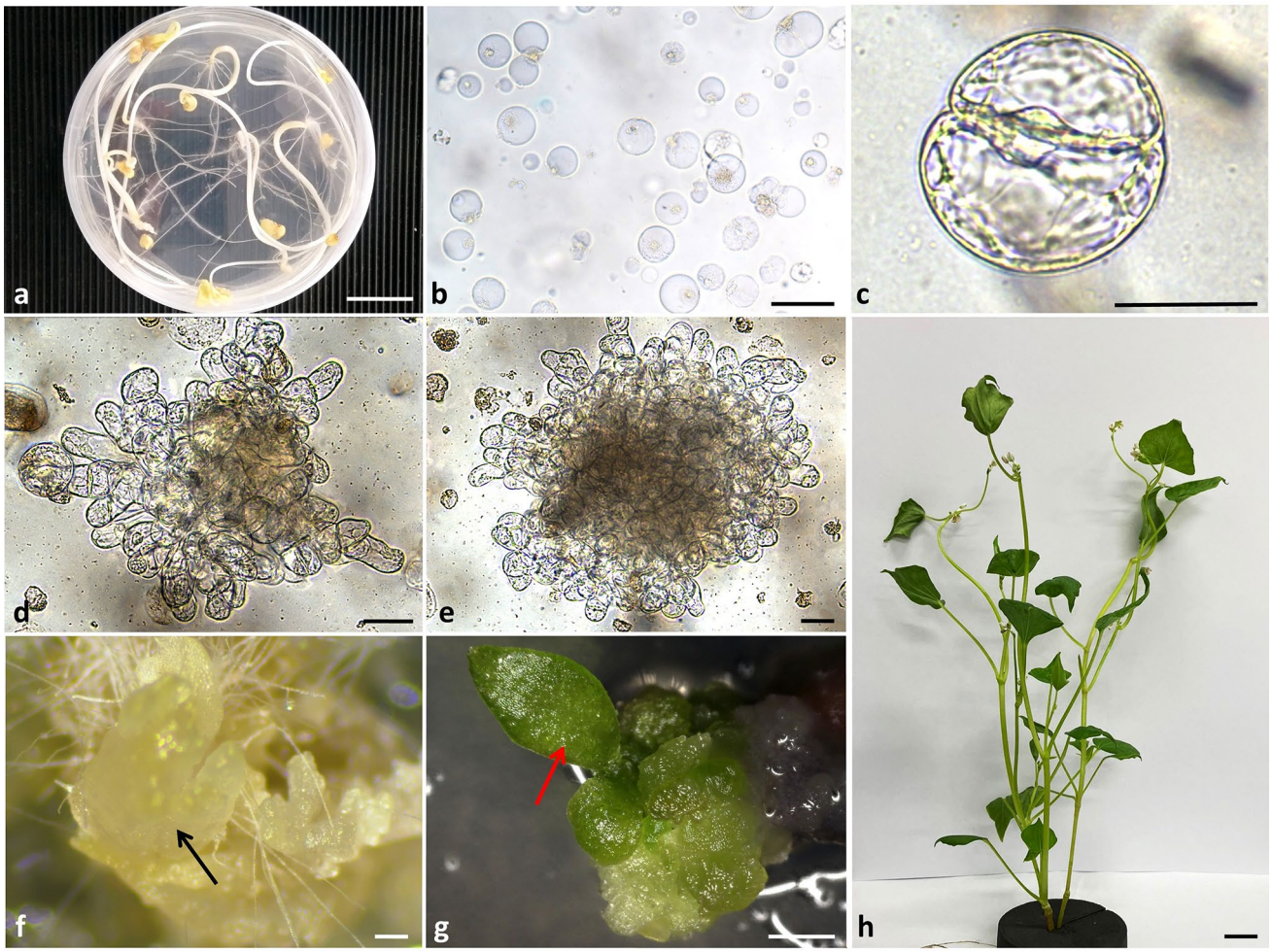


Fig. 2 Plant regeneration from hypocotyl-derived protoplasts of *Fagopyrum esculentum*. **a** 10-day-old etiolated hypocotyls used for protoplast isolation; **b** freshly hypocotyl-derived protoplasts; **c** 2-cell aggregate after the first mitotic division of the protoplast-derived cell; multicellular 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 µm (**b**), 50 µm (**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 ± SE	n	Mean ± SE
L1	21	10–12	E1	3	0.84 ± 0.04^a	3	81.00 ± 6.81^a
NL2	14	8	E1	3	0.83 ± 0.25^a	3	73.90 ± 6.61^a
NL2	14	12	E2	3	1.54 ± 0.37^b	3	73.67 ± 3.28^a
Mean/total				9	1.07 ± 0.18	9	76.19 ± 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 ± SE	n	Mean ± SE
0.10	3	0.46 ± 0.02[a]	3	80.67 ± 4.84[a]
0.15	3	0.43 ± 0.01[a]	3	69.33 ± 2.85[a]
Mean/total	6	0.44 ± 0.01	3	75.00 ± 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

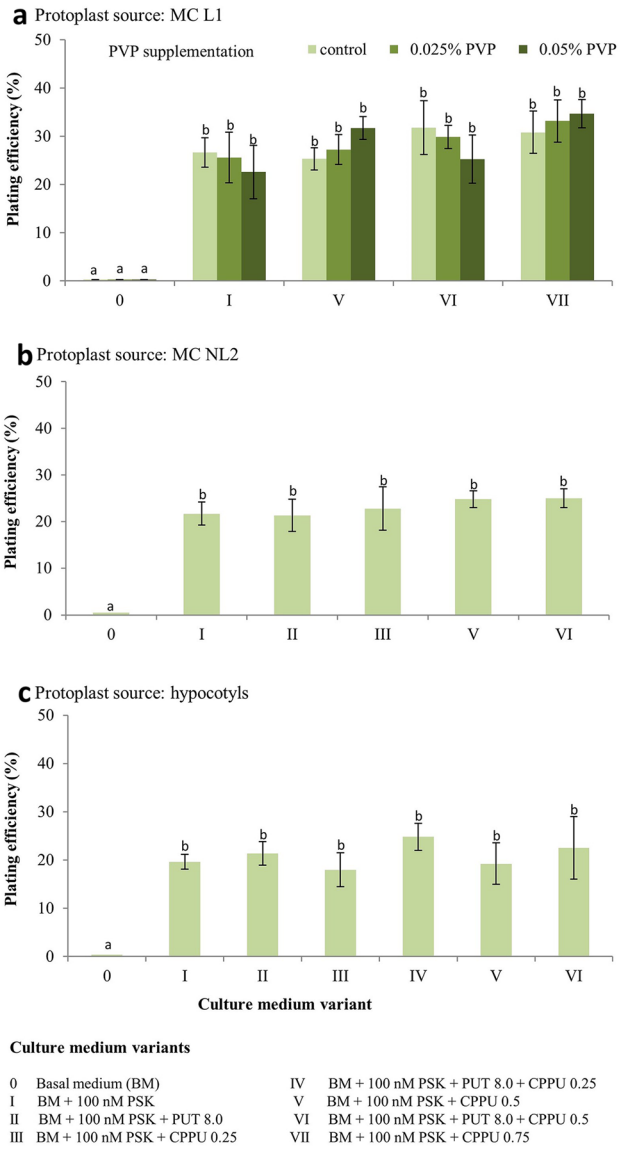


Fig. 3 Effect of different medium variants on plating efficiency in *Fagopyrum esculentum* 10-day-old protoplast cultures. Plant growth regulator composition in *BM* = BAP 1.0 mg L^{-1} + NAA 2.0 mg L^{-1}; *BAP* = 6-Benzylaminopurine; *NAA* = naphthylacetic acid; *100 PSK* = 100 nM phytosulfokine; *CPPU 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 ± 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 phenol-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

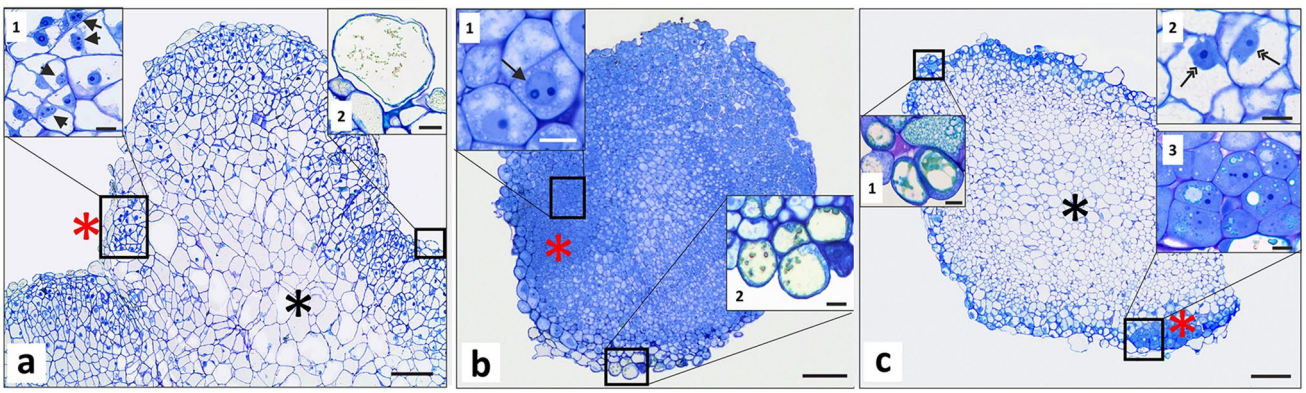


Fig. 4 Histological sections of protoplast-derived callus originating from morphogenic callus lines L1 (**a**), NL2 (**b**) and hypocotyls (**c**). **a** Calli originating from L1-derived protoplasts was composed chiefly of parenchymatous cells (black asterisk) with a big vacuole and an irregularly shaped 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 (insert 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 *Kalanchöe,* 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	$5.8—6.9\times10^5$	–	–	–	
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]	$0.83—1.54\times10^6$	4	23%	2 months	

FW fresh weight

[a]Number of days after protoplast isolation

[b]In present research estimated in 10-day-old protoplast cultures

[c]Time after transfer to regeneration medium

[d]Morphogenic 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 Doroszewska (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.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-023-02542-2.

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.

Funding This research was funded by the National Science Centre, Poland. Research project OPUS-19 (No. Reg. 2020/37/B/NZ9/01499 awarded to AB).

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).

References

Adachi T (1990) How to combine the reproductive system with biotechnology in order to overcome the breeding barrier in buckwheat. Fagopyrum 10(1):7–11

Adachi T, Yamaguchi A, Miike Y, Hoffmann F (1989) Plant regeneration from protoplasts of common buckwheat (*Fagopyrum esculentum*). Plant Cell Rep 8(4):247–250. https://doi.org/10.1007/bf00778544

Adedeji OS, Naing AH, Kim CK (2020) Protoplast isolation and shoot regeneration from protoplast-derived calli of *Chrysanthemum* cv. White ND Plant Cell Tiss Org 141(3):571–581. https://doi.org/10.1007/s11240-020-01816-3

Bano AS, Khattak AM, Basit A, Alam M, Shah ST, Ahmad N, Gilani SAQ, Ullah I, Anwar S & Mohamed HI (2022) Callus induction, proliferation, enhanced secondary metabolites production and antioxidants activity of *Salvia moorcroftiana* L. as influenced by combinations of auxin, cytokinin and melatonin. Braz Arch Biol Techn 65

Berbec A, Doroszewska T (1999) Regeneration *in vitro* of three cultivars of buckwheat (*Fagopyrum esculentum* Moench.) as affected by medium composition. Fagopyrum 16:49–52

Betekhtin A, Rojek M, Jaskowiak J, Milewska-Hendel A, Kwasniewska J, Kostyukova Y, Kurczynska E, Rumyantseva N & Hasterok R (2017) Nuclear genome stability in long-term cultivated callus lines of *Fagopyrum tataricum* (L.) Gaertn. PLoS ONE. https://doi.org/10.1371/journal.pone.0173537

Betekhtin A, Pinski A, Milewska-Hendel A, Kurczynska E, Hasterok R (2019) Stability and instability processes in the calli of *Fagopyrum tataricum* that have different morphogenic potentials. Plant Cell Tiss Org 137(2):343–357. https://doi.org/10.1007/s11240-019-01575-w

Bogdanovic MD, Cukovic KB, Subotic AR, Dragicevic MB, Simonovic AD, Filipovic BK, Todorovic SI (2021) Secondary somatic embryogenesis in *Centaurium erythraea* Rafn. Plants 10(2):199. https://doi.org/10.3390/plants10020199

Castelblanque L, García-Sogo B, Pineda B, Moreno V (2009) Efficient plant regeneration from protoplasts of *Kalanchoe blossfeldiana* via organogenesis. Plant Cell Tiss Org 100(1):107. https://doi.org/10.1007/s11240-009-9617-8

Cawoy V, Lutts S, Kinet JM (2006) Osmotic stress at seedling stage impairs reproductive development in buckwheat (*Fagopyrum esculentum*). Physiol Plantarum 128(4):689–700. https://doi.org/10.1111/j.1399-3054.2006.00801.x

Cawoy V, Ledent J-F, Kinet J-M, Jacquemart A-L (2009) Floral biology of common buckwheat (*Fagopyrum esculentum* Moench). Eur J Plant Sci Biotechnol 3(1):1–9

Chaudhry Z, Afroz A, Rashid H (2007) Effect of variety and plant growth regulators on callus proliferation and regeneration response of three tomato cultivars (*Lycopersicon esculentum*). Pak J Bot 39(3):857–869

Cui J, Kuligowska Mackenzie K, Eeckhaut T, Müller R, Lütken H (2019) Protoplast isolation and culture from *Kalanchoë* species: optimization of plant growth regulator concentration for efficient callus production. Plant Cell Tiss Org 138(2):287–297. https://doi.org/10.1007/s11240-019-01624-4

Davey MR, Anthony P, Power JB, Lowe KC (2005a) Plant protoplast technology: current status. Acta Physiol Plant 27(1):117–130. https://doi.org/10.1007/s11738-005-0044-0

Davey MR, Anthony P, Power JB, Lowe KC (2005b) Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv 23(2):131–171. https://doi.org/10.1016/j.biotechadv.2004.09.008

Deryckere D, Eeckhaut T, Van Huylenbroeck J, Van Bockstaele E (2012) Low melting point agarose beads as a standard method for plantlet regeneration from protoplasts within the Cichorium genus. Plant Cell Rep 31(12):2261–2269. https://doi.org/10.1007/s00299-012-1335-8

Dirks R, Sidorov V, Tulmans C (1996) A new protoplast culture system in *Daucus carota* L. and its applications for mutant selection and transformation. Theor Appl Genet 93(5–6):809–815. https://doi.org/10.1007/BF00224080

Duquenne B, Eeckhaut T, Werbrouck S, Van Huylenbroeck J (2007) Effect of enzyme concentrations on protoplast isolation and protoplast culture of *Spathiphyllum* and *Anthurium*. Plant Cell Tiss Org 91(2):165–173. https://doi.org/10.1007/s11240-007-9226-3

Eeckhaut T, Lakshmanan PS, Deryckere D, Van Bockstaele E, Van Huylenbroeck J (2013) Progress in plant protoplast research. Planta 238(6):991–1003. https://doi.org/10.1007/s00425-013-1936-7

Fehér A, Dudits D (1994) Plant protoplasts for cell fusion and direct DNA uptake: culture and regeneration system. In: Vasil IK, Thorpe TA (eds) Plant cell and tissue culture. Springer, Netherlands, Dordrecht, pp 71–118

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50(1):151–158. https://doi.org/10.1016/0014-4827(68)90403-5

Grosser JW, Calovic M & Louzada ES (2010) Protoplast fusion technology—somatic hybridization and cybridization. Plant Cell Culture 175–198

Grzebelus E, Szklarczyk M, Baranski R (2012a) An improved protocol for plant regeneration from leaf- and hypocotyl-derived protoplasts of carrot. Plant Cell Tiss Org 109(1):101–109. https://doi.org/10.1007/s11240-011-0078-5

Grzebelus E, Szklarczyk M, Gren J, Sniegowska K, Jopek M, Kacinska I & Mrozek K (2012b) Phytosulfokine stimulates cell divisions in sugar beet (*Beta vulgaris* L.) mesophyll protoplast cultures. Plant Growth Regul 67(1):93–100. https://doi.org/10.1007/s10725-011-9654-2

Gumerova EA (2004) Realisation of the morphogenic potential of common buckwheat (*Fagopyrum esculentum* Moench.) hypocotyls depending on the method of regeneration KIBB KSC Russian Academy of Science

Gumerova E, Gatina E, Chuenkova S, Rumyantseva N (2001) Somatic embryogenesis in common buckwheat *Fagopyrum esculentum* Moench. In: Proceedings of the 8th international symposium on Buckwheat, Chunchon, Korea, Citeseer, pp 377–381

Gumerova EA, Galeeva EI, Chuyenkova SA, Rumyantseva NI (2003) Somatic embryogenesis and bud formation on cultured *Fagopyrum esculentum* hypocotyls. Russ J Plant Physl+ 50(5):640–645. https://doi.org/10.1023/A:1025640107932

Guo F, Zhou J, Luo X, Ma H (1992) Plant regeneration of tetraploid plants of *Fagopyrum esculentum* Moench in tissue culture. In: Proceedings of the 5th international symposium on Buckwheat, pp 309–314

Holländer-Czytko H, Amrhein N (1983) Subcellular compartment of shikimic acid and phenylalanine in buckwheat cell suspension cultures grown in the presence of shikimate pathway inhibitors. Plant Sci Lett 29(1):89–96. https://doi.org/10.1016/0304-4211(83)90027-5

Hou SY, Sun ZX, Bin LH, Wang YG, Huang KS, Xu DM, Han YH (2015) Regeneration of buckwheat plantlets from hypocotyl and the influence of exogenous hormones on rutin content and rutin biosynthetic gene expression *in vitro*. Plant Cell Tiss Org 120(3):1159–1167. https://doi.org/10.1007/s11240-014-0671-5

Huhtinen O, Honkanen J, Simola L (1982) Ornithine- and putrescine-supported divisions and cell colony formation in leaf protoplasts of Alders (*Alnus Glutinosa* and *A. Incana*). Plant Sci Lett 28(1):3–9. https://doi.org/10.1016/S0304-4211(82)80003-5

Jones AMP, Shukla MR, Biswas GCG, Saxena PK (2015) Protoplast-to-plant regeneration of American elm (*Ulmus americana*). Protoplasma 252(3):925–931. https://doi.org/10.1007/s00709-014-0724-y

Kang HH, Naing AH, Kim CK (2020) Protoplast isolation and shoot regeneration from protoplast-derived callus of *Petunia hybrida* Cv. Mirage Rose Biol 9(8):228. https://doi.org/10.3390/biology9080228

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia Hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126(2):105–110. https://doi.org/10.1007/Bf00380613

Kawai M, Katsumata R, Tsuruta T, Shimura G, Suga Y, Samejima H (1979) The disintegration of soybean by a cellulase preparation from *Irpex lacteus* Fr. and the enzyme relating to it. Agr Biol Chem Tokyo 43(9):1855–1862. https://doi.org/10.1080/00021369.1979.10863737

Kiełkowska A, Adamus A (2017) Early studies on the effect of peptide growth factor phytosulfokine-α on *Brassica oleracea* var. capitata L. protoplasts. Acta Soc Bot Pol.https://doi.org/10.5586/asbp.3558

Kiełkowska A, Adamus A (2019) Peptide growth factor phytosulfokine-α stimulates cell divisions and enhances regeneration from *B. oleracea* var. *capitata* L. protoplast culture. J Plant Growth Regul 38(3):931–944. https://doi.org/10.1007/s00344-018-9903-y

Kiełkowska A, Adamus A (2021) Exogenously applied polyamines reduce reactive oxygen species, enhancing cell division and the shoot regeneration from *Brassica oleracea* L. var. *capitata* protoplasts. Agronomy 11(4):735. https://doi.org/10.3390/agronomy11040735

Kim JG, Takami Y, Mizugami T, Beppu K, Fukuda T, Kataoka I (2006) CPPU application on size and quality of hardy kiwifruit. Sci Hortic 110(2):219–222. https://doi.org/10.1016/j.scienta.2006.06.017

Klimek-Chodacka M, Kadluczka D, Lukasiewicz A, Malec-Pala A, Baranski R, Grzebelus E (2020) Effective callus induction and plant regeneration in callus and protoplast cultures of *Nigella damascena* L. Plant Cell Tiss Org 143(3):693–707. https://doi.org/10.1007/s11240-020-01953-9

Kumar M, Saraswat R (2018) Plant regeneration and genetic transformation in buckwheat (*Fagopyrum* spp.), a multipurpose gluten free crop of high nutraceutical importance: a critical review. Ann Plant Sci 7:1954–1962. https://doi.org/10.21746/aps.2018.7.1.7

Kwon S-J, Han M-H, Huh Y-S, Roy SK, Lee C-W, Woo S-H (2013) Plantlet regeneration via somatic embryogenesis from hypocotyls of common buckwheat (*Fagopyrum esculentum* Moench.). Korean J Crop Sci 58(4):331–335

Lachmann SA (1990) Callus regeneration from hypocotyl protoplast of tartary buckwheat (*Fagopyrum tataricum* Gaertn.). Fagopyrum 10:62–64

Lachmann S, Kishima Y, Adachi T (1994) Protoplast fusion in buckwheat: preliminary results on somatic hybridization. Fagopyrum 14:7–12

Li J, Xu J, Guo Q-W, Wu Z, Zhang T, Zhang K-J, Cheng C-y, Zhu P-y, Lou Q-F, Chen J-F (2017) Proteomic insight into fruit set of cucumber (*Cucumis sativus* L.) suggests the cues of hormone-independent parthenocarpy. Bmc Genomics 18(1):896. https://doi.org/10.1186/s12864-017-4290-5

Mackowska K, Jarosz A, Grzebelus E (2014) Plant regeneration from leaf-derived protoplasts within the *Daucus* genus: effect of different conditions in alginate embedding and phytosulfokine application. Plant Cell Tiss Org 117(2):241–252. https://doi.org/10.1007/s11240-014-0436-1

Matsubayashi Y, Sakagami Y (1996) Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. Proc Natl Acad Sci USA 93(15):7623–7627. https://doi.org/10.1073/pnas.93.15.7623

Matsubayashi Y, Takagi L, Sakagami Y (1997) Phytosulfokine-α, a sulfated pentapeptide, stimulates the proliferation of rice cells by means of specific high- and low-affinity binding sites. Proc Natl Acad Sci USA 94(24):13357–13362. https://doi.org/10.1073/pnas.94.24.13357

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15(3):473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Murthy BNS, Saxena PK (1994) Somatic embryogenesis in peanut (*Arachis hypogaea* L.): stimulation of direct differentiation of somatic embryos by forchlorfenuron (CPPU). Plant Cell Rep 14(2):145–150 doi:https://doi.org/10.1007/BF00233779

Nešković M, Vujičić R, Budimir S (1987) Somatic embryogenesis and bud formation from immature embryos of buckwheat (*Fagopyrum esculentum* Moench.). Plant Cell Rep 6:423–426. https://doi.org/10.1007/bf00272773

Ning Y, Hu B, Yu H, Liu X, Jiao B, Lu X (2022) Optimization of protoplast preparation and establishment of genetic transformation

system of an arctic-derived fungus *Eutypella* sp. Front Microbiol 13:769008. https://doi.org/10.3389/fmicb.2022.769008
Noguchi S, Shimura G, Kawai M, Suga Y, Samejima H (1978) Properties of partially purified cellulolytic and plant tissue macerating enzymes of *Irpex lacteus* Fr. in special reference to their application Agric Biol Chem 42(2):339–345. https://doi.org/10.1271/bbb1961.42.339
Papadakis AK, Roubelakis-Angelakis KA (2005) Polyamines inhibit NADPH oxidase-mediated superoxide generation and putrescine prevents programmed cell death induced by polyamine oxidase-generated hydrogen peroxide. Planta 220(6):826–837. https://doi.org/10.1007/s00425-004-1400-9
Rahmani M-S, Pijut PM, Shabanian N (2016) Protoplast isolation and genetically true-to-type plant regeneration from leaf- and callus-derived protoplasts of *Albizia julibrissin*. Plant Cell Tiss Org 127(2):475–488. https://doi.org/10.1007/s11240-016-1072-8
Reed KM, Bargmann BOR (2021) Protoplast regeneration and its use in new plant breeding technologies. Front Genome Ed 3:734951. https://doi.org/10.3389/fgeed.2021.734951
Reustle G, Natter I (1994) Effect of polyvinylpyrrolidone and activated charcoal on formation of microcallus from grapevine protoplasts (*Vitis* sp.). Vitis 33(3):117–121. https://doi.org/10.5073/vitis.1994.33.117-121
Robertson D, Earle ED (1986) Plant regeneration from leaf protoplasts of *Brassica oleracea* var. *italica* CV Green Comet broccoli. Plant Cell Rep. 5(1):61–64.
Rumyanzeva NI, Lozovaya VV (1988) Isolation and culture of buckwheat (*Fagopyrum esculentum* Moench.) callus protoplasts. In: Puite KJ, Dons JJM, Huizing HJ, Kool AJ, Koornneef M, Krens FA (eds) Progress in plant protoplast research: proceedings of the 7th international protoplast symposium, Wageningen, the Netherlands, December 6–11, 1987. Springer Netherlands, Dordrecht, pp 45–46
Rumyantseva NI, Sal'nikov VV, Lebedeva VV (2005) Structural changes of cell surface in callus of *Fagopyrum esculentum* Moench. during induction of morphogenesis. Russ J Plant Physl+ 52(3):381–387. https://doi.org/10.1007/s11183-005-0057-y
Sakamoto S, Matsui K, Oshima Y, Mitsuda N (2020) Efficient transient gene expression system using buckwheat hypocotyl protoplasts for large-scale experiments. Breed Sci 70(1):128–134. https://doi.org/10.1270/jsbbs.19082
Saxena PK, Gill R (1986) Removal of browning and growth enhancement by polyvinylpolypyrrolidone in protoplast cultures of *Cyamopsis tetragonoloba* L. Biol Plant 28(4):313–315. https://doi.org/10.1007/BF02902302
Shaikh N, Guan L, Adachi T (2001) Ultrastuctural analyses on breeding barriers in post-fertilization of interspecific hybrids of buckwheat. In: Proceding of the VIII internatiol an symposium on Buckwheat, pp 319–329
Slawinska J, Obendorf RL (2001) Buckwheat seed set *in planta* and during *in vitro* inflorescence culture: evaluation of temperature and water deficit stress. Seed Sci Res 11(3):223–233
Slawinska J, Kantartzi SK, Obendrof RL (2009) *In vitro* organogenesis of *Fagopyrum esculentum* Moench (Ploygonaceae) as a method to study seed set in buckwheat. J Plant Sci Biotechnol 3(1):73–78
Takahata Y & Jumonji E (1985) Plant regeneration from hypocotyl section and callus in buckwheat (*Fagopyrum esculentum* Moench.). Ann Rep Fac Educ Iwate Univ 45(1)
Taylor DP, Obendorf RL (2001) Quantitative assessment of some factors limiting seed set in buckwheat. Crop Sci 41(6):1792–1799. https://doi.org/10.2135/cropsci2001.1792
Tomasiak A, Zhou M, Betekhtin A (2022) Buckwheat in tissue culture research: current status and future perspectives. Int J Mol Sci 23(4):2298. https://doi.org/10.3390/ijms23042298
Woo S-H, Adachi T, Jong SK, Campbell CG (1999) Isolation of protoplasts from viable sperm cells of common buckwheat (Fagopyrum esculentum Moench.). Can J Plant Sci 80:583–585
Woo SH, Nair A, Adachi T, Campbell CG (2000) Plant regeneration from cotyledon tissues of common buckwheat (*Fagopyrum esculentum* Moench). In Vitro Cell Dev-Pl 36(5):358–361. https://doi.org/10.1007/s11627-000-0063-x
Woo S, Ohmoto T, Campbell C, Adachi T, Jong S (2001) Pre-and post-fertilization to backcrossing in interspecific hybridization between *Fagopyrum esculentum* and *F. homotropicum* with *F. esculentum*. In: Proceedings of the 8th international symposium on Buckwheat, Chunchon, Korea. Citeseer, pp 450–455
Woo S, Takaoka M, Kim H, Park C, Adachi T, Jong S (2004) Plant regeneration via shoot organogenesis from leaf callus culture of common buckwheat (*Fagopyrum esculentum* Moench.). In: Proceedings of the 9th international symposium on Buckwheat, pp 61–65
Woo S-H, Kamal AM, Tatsuro S, Campbell CG, Adachi T, Yun Y-H, Chung K-Y, Choi J-S (2010) Buckwheat (*Fagopyrum esculentum* Moench.): concepts, prospects and potential. Eur J Plant Sci Biotechnol 4(1):1–16
Yamane Y (1974) Induced differentiation of buckwheat plants from subcultured calluses *in vitro*. Jpn J Genetics 49(3):139–146
Yang X, Lü J, da Silva JAT, Ma G (2012) Somatic embryogenesis and shoot organogenesis from leaf explants of *Primulina tabacum*. Plant Cell Tiss Org 109(2):213–221. https://doi.org/10.1007/s11240-011-0087-4
Zhang Q, Chen J, Henny RJ (2005) Direct somatic embryogenesis and plant regeneration from leaf, petiole, and stem explants of Golden Pothos. Plant Cell Rep 23(9):587–595. https://doi.org/10.1007/s00299-004-0882-z
Zhang G, Xu Z, Gao Y, Huang X, Zou Y, Yang T (2015) Effects of germination on the nutritional properties, phenolic profiles, and antioxidant activities of buckwheat. J Food Sci 80(5):H1111–H1119. https://doi.org/10.1111/1750-3841.12830

9.3. Comparison of the cell wall regeneration in the parental protoplasts of *F. tataricum, F. esculentum* and the *F. tataricum* (+) *F. esculentum* hybrid cells

Publication P3:

Sala-Cholewa, K., Milewska-Hendel, A., **Pérez-Pérez, R.**, Grzebelus, E., Betekhtin, A.

Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells.

Plant Cell, Tissue and Organ Culture (PCTOC), **2024,** 157 (26)

https://doi.org/10.1007/s11240-024-02740-6

IF_{2024}: 2.3

Ministry of Science and Higher Education points: 100

A spatio-temporal analysis of cell wall regeneration in protoplasts derived from MC and EC of *F. tataricum* and *F. esculentum*, respectively, and their hybrid cells *Ft* (+) *Fe* (heterokaryon) was carried out. Hybrid cells were obtained via protoplast electrofusion, selected manually using a micromanipulator and cultivated into agarose beads with BM supplemented with 100 nM PSK (Figure 1, Supplementary Table 1). A set of primary antibodies targeting the main cell wall components was used: AGPs (JIM13, JIM16), extensin (JIM20), xyloglucan (LM25) and pectins (LM5, LM6, LM20) (Table 1). In addition, FB28 was used for cellulose staining to assess the timing of cellulose reconstruction, which serves as the structural framework of the cell wall.

The protoplasts were analysed at different time points of the culture: 0 hour, 4 hours, 12 hours, 24 hours, 48 hours and 72 hours (Figure 2). Overall, comparing the regeneration pattern of the different cell wall components in *Ft* (+) *Fe* and parental protoplasts did not show major differences; however, differences were observed among the evaluated time points (Figures 3 – 9).

AGP epitopes, recognised by the JIM13 antibody, were present in all types of analysed protoplasts and were detected at each time interval. This epitope was initially located in intracellular compartments and, at later stages, in the cell membrane and cell wall. In addition, it was observed that the JIM13 signal, from 12 hours to 72 hours, correlated with the arrangement of cellulose microfibrils visualised with FB28 (Figure 3 and Supplementary videos 1 – 6). Similarly, the JIM16 antibody, directed against the AGP, was detected at all stages of

cell wall development in each cell type (Figure 4 and Supplementary videos 7 – 12). In *F. tataricum*, the JIM20, which detects extensin epitopes, was observed in several cells at the early stages of cell wall regeneration (0 hours and 4 h), localised in intracellular compartments. At 12 hours and 24 hours, an increase in the number of cells with extensin epitope was observed, followed by a decrease at 48 hours and 72 hours. In *F. esculentum*, the JIM20 signal was less frequent in the early stages but distinctly increased at 12 hours, followed by a significant decrease at 24 hours. In *Ft* (+) *Fe*, extensin was present in most cells at 12 hours and 24 hours but only in a few cells at 0 hours and 72 hours (Figure 5, Supplementary Table 3 and Supplementary videos 13 – 18). Xyloglucan, recognised by the LM25 antibody, was absent in a minority of protoplasts at 0 hours but was detected in most cells from 12 hours onwards, regardless of their type (Supplementary Table 3). The fluorescence signal was observed in the cell wall matrix as continuous strands that matched the arrangement of cellulose microfibrils (Figure 6 and Supplementary videos 19 – 24). In the case of pectins, the epitope recognised by the LM20 antibody (methyl-esterified homogalacturonan) was predominantly detected in *Ft* (+) *Fe* at 0 hours, with a punctual signal in the cytoplasmic compartments. In *Ft*, the LM20 epitope was abundantly present in the intracellular compartments between 12 hours and 48 hours, while in *F. esculentum* and *Ft* (+) *Fe*, its presence decreased significantly after 24 hours (Figure 7, Supplementary Table 3 and Supplementary Videos 25 – 30). On the other hand, the LM5 epitope (galactan side chain of rhamnogalacturonan I) was detected in the intracellular compartments of *F. tataricum* until 12 hours and in the cell wall matrix after 24 hours, while in *F. esculentum* and *Ft* (+) *Fe*, it was located in the cell wall matrix at 12 hours and 4 hours, respectively (Figure 8 and Supplementary videos 31 – 36). Finally, the LM6 epitope (arabinan side chain of rhamnogalacturonan I) was abundantly detected in the intracellular compartments of *F. tataricum* until 24 hours and in the cell wall matrix at 48 hours and 72 hours. In *F. esculentum* and *Ft* (+) *Fe*, this epitope appeared in the cell wall matrix from 4 hours, coinciding with cellulose deposition (Figure 9 and Supplementary videos 37 – 42).

In summary, this study provides a replicable protocol for obtaining and selecting hybrid protoplasts of *Fagopyrum* using electrofusion and a detailed view of the dynamics of cell wall regeneration, highlighting the differences in the composition and distribution of its components. These findings contribute to a better understanding of the molecular mechanisms involved in cell wall regeneration, the first step for protoplast development. Moreover, due to the lack of significant differences in cell wall regeneration between the hybrid cells and the parental

protoplasts, it can be concluded that cell wall reconstruction in the hybrids proceeds correctly and follows normal physiological processes.

Supplementary material available online

Table S1_ https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM1_ESM.docx

Supplementary video_1 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM1_ESM.mp4

Supplementary video_2 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM2_ESM.mp4

Supplementary video_3 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM3_ESM.mp4

Supplementary video_4 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM4_ESM.mp4

Supplementary video_5 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM5_ESM.mp4

Supplementary video_6 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM6_ESM.mp4

Supplementary video_7 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM7_ESM.mp4

Supplementary video_8 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM8_ESM.mp4

Supplementary video_9 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM9_ESM.mp4

Supplementary video_10 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM10_ESM.mp4

Supplementary video_11 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM11_ESM.mp4

Supplementary video_12 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM12_ESM.mp4

Supplementary video_14 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM14_ESM.mp4

Supplementary video_15 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM15_ESM.mp4

Supplementary video_16 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM16_ESM.mp4

Supplementary video_17 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM17_ESM.mp4

Supplementary video_18 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM18_ESM.mp4

Supplementary video_19 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM19_ESM.mp4

Supplementary video_20 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM20_ESM.mp4

Supplementary video_21 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM21_ESM.mp4

Supplementary video_22 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM22_ESM.mp4

Supplementary video_23 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM23_ESM.mp4

Supplementary video_24 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM24_ESM.mp4

Supplementary video_25 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM25_ESM.mp4

Supplementary video_26 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM26_ESM.mp4

Supplementary video_27 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM27_ESM.mp4

Supplementary video_28 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM28_ESM.mp4

Supplementary video_29 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM29_ESM.mp4

Supplementary video_30 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM30_ESM.mp4

Supplementary video_31 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM31_ESM.mp4

Supplementary video_32 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM32_ESM.mp4

Supplementary video_33 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM33_ESM.mp4

Supplementary video_34 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM34_ESM.mp4

Supplementary video_35 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM35_ESM.mp4

Supplementary video_36 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM36_ESM.mp4

Supplementary video_37 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM37_ESM.mp4

Supplementary video_38 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM38_ESM.mp4

Supplementary video_39 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM39_ESM.mp4

Supplementary video_40 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM40_ESM.mp4

Supplementary video_41 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM41_ESM.mp4

Supplementary video_42 https://static-content.springer.com/esm/art%3A10.1007%2Fs11240-024-02740-6/MediaObjects/11240_2024_2740_MOESM42_ESM.mp4

Plant Cell, Tissue and Organ Culture (PCTOC) (2024) 157:26
https://doi.org/10.1007/s11240-024-02740-6

ORIGINAL ARTICLE

Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells

Katarzyna Sala-Cholewa[1] · Anna Milewska-Hendel[1] · Reneé Pérez-Pérez[1] · Ewa Grzebelus[2] · Alexander Betekhtin[1]

Received: 7 December 2023 / Accepted: 21 March 2024 / Published online: 16 April 2024

Abstract

The cell wall rebuilding is one of the first stage of protoplast development that enables further mitotic divisions and differentiation. Therefore, this work focuses on the comparison of the cell wall regeneration in the parental protoplasts of *Fagopyrum tataricum*, *F. esculentum* and the *F. tataricum* (+) *F. esculentum* hybrids, which are promising materials in terms of future breeding and research programmes. It is worth emphasizing that the preparation of buckwheat hybrids using electrofusion was described for the first time. The results indicate that cell wall rebuilding exhibited a common mechanism for parent protoplasts and the heterokaryon as all analysed cell wall components recognising arabinogalactan proteins (JIM13, JIM16), extensin (JIM20), xyloglucan (LM25) and pectins (LM20, LM5, LM6) were detected during the process of wall regeneration. However, there were certainly differences in the spatio-temporal appearance or disappearance of individual epitopes during the 72 h of the cell culture, which have been discussed in the paper.

Key message

The hybrid protoplasts similarly restore their wall to *F. tataricum* and *F. esculentum* protoplasts despite some qualitative and quantitative differences in epitope distribution.

Keywords Buckwheat · Cell wall · Heterokaryon · Hybrid · Immunocytochemistry · Protoplasts

Abbreviations

AC	alternating current
AGPs	arabinogalactan proteins
BB	blocking buffer
DC	direct current
dH_2O	distilled water
Fb28	fluorescent brightener 28
Fe	*Fagopyrum esculentum*
Ft	*Fagopyrum tataricum*
Ft+Fe	*F. tataricum* and *F. esculentum* hybrid
HG	homogalacturonan
HRGP	hydroxyproline rich proteins
RG-I	rhamnogalacturonan I
Rhd	rhodamine B
RT	room temperature

Katarzyna Sala-Cholewa and Anna Milewska-Hendel contributed equally to this work.

Communicated by Danny Geelen.

✉ Anna Milewska-Hendel
anna.milewska@us.edu.pl

✉ Alexander Betekhtin
alexander.betekhtin@us.edu.pl

[1] Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Jagiellonska 28, 40-032 Katowice, Poland

[2] Department of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, al. Mickiewicza 21, 31-120 Krakow, Poland

Introduction

Buckwheat (*Fagopyrum* spp.) is described as a neglected and underutilised species of cultivated plants; however, it is an attractive crop; it is a good source of, among others, flavonoids, gluten-free proteins, amino acids, dietary fibre, vitamins, minerals (Huda et al. 2021; Li et al. 2019). Of the 23 species of *Fagopyrum*, only two are *F. esculentum* and *F. tataricum* are cultivated (Tomasiak et al. 2022). *F. tataricum* is characterised by over 100-fold higher rutin

content than *F. esculentum* (Fabjan et al. 2003; Zhang et al. 2021). However, low grain content and strong bitter taste limit its use as food. *F. esculentum* is more widely distributed than *F. tataricum;* however the seed yield of this species is often relatively low and unstable (Sytar et al. 2016). One of the most crucial reasons for the low yield is self-incompatibility, as *F. esculentum* plants are dimorphic with two types of flowers: Thrum (pistils shorter than stamens) and Pin (pistils longer than stamens) (Cawoy et al. 2009). *F. tataricum* produces only homostylous flowers capable of self-fertilization.

A somatic hybridization technique has been proposed to overcome breeding barriers in buckwheat (Nešković et al. 1987). Among the different cell fusion methods for developing somatic hybrids in plants, PEG-mediated fusion has been the most popular and used so far. Lachmann et al. (1994) obtained the hybrid calli of *F. esculentum* (+) *F. tataricum* without further plant regeneration. The other method is protoplast electrofusion, which acquired a preferential position due to its rapidity, efficiency and low cytotoxicity (Rems et al. 2013). Since the first description of the electrofusion process by (Senda et al. 1979), it has been successfully applied to different species of agricultural importance (Mackowska et al. 2023; Sedlak et al. 2022).

In *Fagopyrum* spp., plant regeneration from protoplasts has been described for *F. esculentum* from hypocotyl- (Adachi et al. 1989) and callus-derived protoplasts (Zaranek et al. 2023b) while for *F. tataricum* from hypocotyl- (Lachmann and Adachi 1990) and callus-derived protoplasts (Zaranek et al. 2023a). However, to obtain plant regenerants from protoplast cultures, one of the first and crucial stages is cell wall re-synthesis and deposition, which enables the first cell divisions. The cell wall is a dynamic and highly controlled structure mainly composed of cellulose microfibrils, matrix polysaccharides, polyphenolic compounds, and structural proteins in variable amounts (Showalter 1993). Due to its compounds' structural and enzymatic rearrangements, the cell walls play an important role in regulating the morphogenetic processes and maintaining the cellular differentiation status of cells (Knox et al. 1991; Potocka et al. 2018). Cellulose, hemicellulose and pectins, arabinogalactan proteins (AGPs) and extensins (EXTs) all participate (to different degrees) in cell expansion or adhesion and as regulatory molecules they are key determinants of the physical properties of the cell wall (Willats et al. 2001).

Cell wall regeneration from protoplasts has been studied in several species using various techniques (Kuki et al. 2020; Parmentier et al. 1995; Wiśniewska and Majewska-Sawka 2007). However, due to the complexity of the cell wall structure, the most informative method is immunocytochemical detection of specific cell wall antigens (Godel-Jędrychowska et al. 2019; Majewska-Sawka and Münster 2003). To date, there is no data about cell wall reconstruction from protoplasts in any of the *Fagopyrum* species, as well as in their hybrids or the hybrid protoplasts at all, independently of the species. Thus, our goal was to check whether there are differences between those two species and their hybrids (*Ft* + *Fe*) during wall regeneration that could point out the potential recalcitrance of hybrid protoplasts. Since xyloglucan, pectins, extensins, and AGPs are essential components of dicotyledonous walls, the specific antibodies that recognise their epitopes were chosen to follow the process of cell wall rebuilding: LM20, LM5 and LM6 recognising pectic epitopes; JIM13 and JIM16 recognising AGPs epitopes; JIM20 recognised extension epitope and LM25 recognising xyloglucan epitope. It should be emphasized that this is the first study that illustrates and compares the regeneration of the wall of *Fagopyrum* parental protoplasts and their hybrids. Moreover, this is the first record of obtaining hybrids in buckwheat by electrofusion.

Materials and methods

Protoplast isolation

Embryogenic calli of *F. esculentum* and morphogenic calli of *F. tataricum* were used as sources of protoplasts. The callus lines were obtained from immature embryos of both species and maintained in the dark on RX medium (Table S1) at 26 ± 1 °C as Betekhtin et al. (2017) described. The subcultures were carried out every two weeks under the same conditions.

The isolation was performed following the protocol of Zaranek et al. (2023b) with minor modifications. Two grams of 10-day-old callus of *F. tataricum* and 1 g of 12-day-old callus of *F. esculentum* were incubated for 1 h in 10 ml of PSII/F plasmolysis solution (Table S1) in the dark at room temperature (RT). Then, the solution was removed, and 10 ml of E1 and E2 enzyme solutions (Table S1) were added to *F. tataricum* and *F. esculentum*, respectively. The material was incubated for 16 h with gentle shaking (50 rpm, RT). The suspensions were filtered through a 100 µm pore mesh and centrifuged for 5 min (1000 rpm, RT). The pellet was resuspended in 8 ml of Suc/MES solution (Table S1). Next, 2 ml of W5 solution (Table S1) was carefully overlaid to create a gradient and centrifuged for 10 min (1200 rpm, RT). The ring of viable protoplasts between the two phases of the gradient was collected, resuspended in W5 solution up to 10 ml and centrifuged for 5 min (1000 rpm, RT). The pellet was resuspended in 1.5 ml of mannitol solution (Table S1), and the protoplast concentration was adjusted to 8×10^5 cells ml^{-1}. The isolation was performed twice for both species, and one set was kept as control and as nurse culture.

Electrofusion

The electrofusion methodology was adapted from the protocol proposed by Mackowska et al. (2023). One ml of the *F. esculentum* and *F. tataricum* protoplast suspensions were added to the tubes with Rhodamine B (Rhd) and 3,3'-dihexyloxacarbocyanine iodide (DiOC6), respectively and incubated for 10 min in the dark at RT. The preparation of dyes is listed in the Supplementary Information: Table S2. The following washing steps took place on ice. The stained protoplasts were centrifuged for 5 min (1000 rpm, 4 °C), and the pellet was resuspended in 10 ml of ice-cold mannitol solution. These steps were repeated two times more. The pellets were resuspended in 0.9 ml of ice-cold mannitol. The protoplast concentration was adjusted again to 8×10^5 cells ml^{-1}, adding ice-cold mannitol solution if needed. For parental protoplasts analysis, 1 ml of the protoplast solution of each species was stained with 100 µl of calcofluor white (Table S2) and kept in the dark for 10 min.

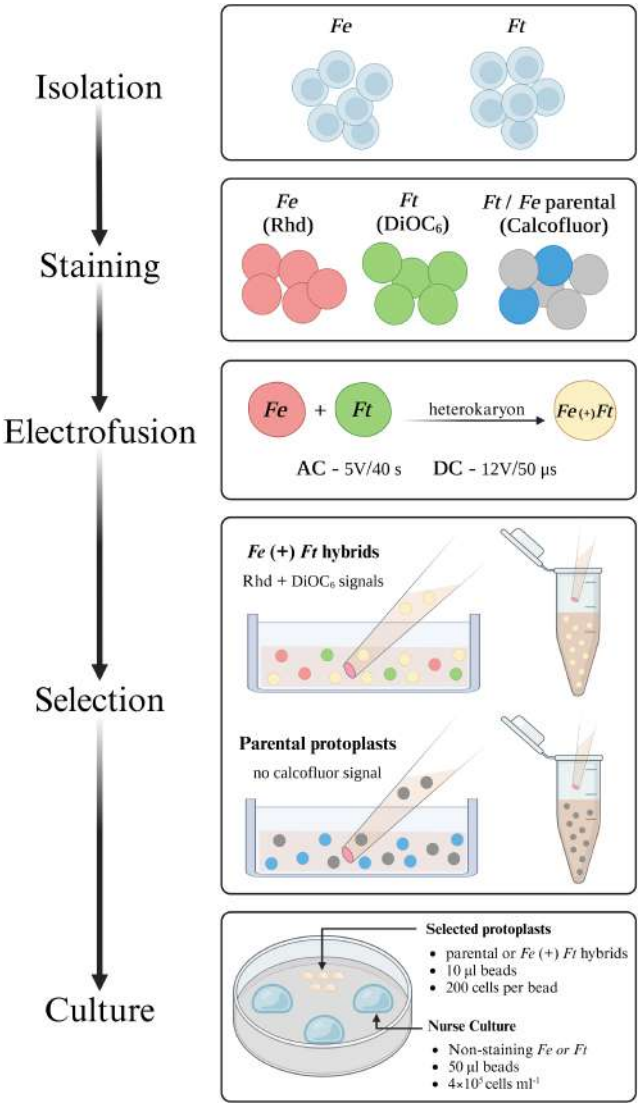


Fig. 1 General scheme of the protoplast electrofusion and selection technique. Once the protoplasts are isolated, they are stained with two contrasting fluorochrome solutions (Rhd– red; DiOC6– green) and fused using periodic electrical pulses. Electrofusion is a random process, so the hybrid protoplasts (yellow) must be selected, one by one, under the microscope. In the case of the parental protoplasts, calcofluor staining allows the subsequent selection of those from which the cell wall was completely removed (no calcofluor signal– grey). The culture of the hybrid and parental protoplasts must be developed after embedding them in agarose beads, and they must be accompanied by a nurse culture that contributes to their development. AC– alternating current; DC– direct current; DiOC6–3,3'-dihexyloxacarbocyanine iodide; Rhd– rhodamine B. This figure was created with BioRender.com. BioRender certificate confirming the publication rights is available upon request from the authors.

Respectively, 0.4 ml of protoplasts stained with Rhd and 0.4 ml stained with $DiOC_6$ were carefully mixed to combine *F. tataricum* (+) *F. esculentum* (*Fe*+*Ft*, heterokaryon), as shown in Fig. 1. The electrofusion was performed in the Super Electro Cell Fusion Generator 21 (NEPAGEN, Japan) using parameters optimised by our team, standardised for *Fagopyrum* protoplasts. The 0.8 ml CUY497P2 electrode was used with 5 V of alternating current (AC) for 40 s and 12 V of direct current (DC) for 50 µs. The fused protoplasts were transferred into a sterile tube covered with foil and kept on ice.

Selection of protoplasts

These steps were performed entirely in the dark. After electrofusion, 0.25 ml of the protoplast suspension was resuspended in 4.5 ml of ice-cold mannitol and carefully mixed. In a sterile Ibidi µ-Dish $^{35\ mm,\ low}$, 0.3 ml of this suspension was placed and allowed to settle for 5 min in the inverted microscope. The protoplasts that simultaneously showed fluorescence for Rhd and $DiOC_6$ were collected using a stripper tip MXL3-75 (ORIGIO Inc, USA) and micromanipulator TransferMan® 4r (Eppendorf, Germany). About 200 hybrid cells were collected and kept on ice until the next steps were performed. The selection process was applied for the parental protoplasts, *F. tataricum* and *F. esculentum*. Approximately 200 protoplasts that did not show fluorescence for calcofluor white were collected.

Culture of selected protoplasts

The parental and hybrid protoplasts selected into Eppendorf tubes were resuspended in 9 µl of LMPA (Table S1), and about 10 µl beads were made on polystyrene Petri dishes (Ø6 cm × 1.2 cm) at the rate of five beads per dish (Fig. 2). Non-stained *Fe* and *Ft* protoplasts were used as nurse culture during parental and *Fe* (+) *Ft* hybrid cells culture. For this, equal volumes of LMPA and the protoplast solution with a concentration of 8×10^5 cells ml^{-1} were mixed and

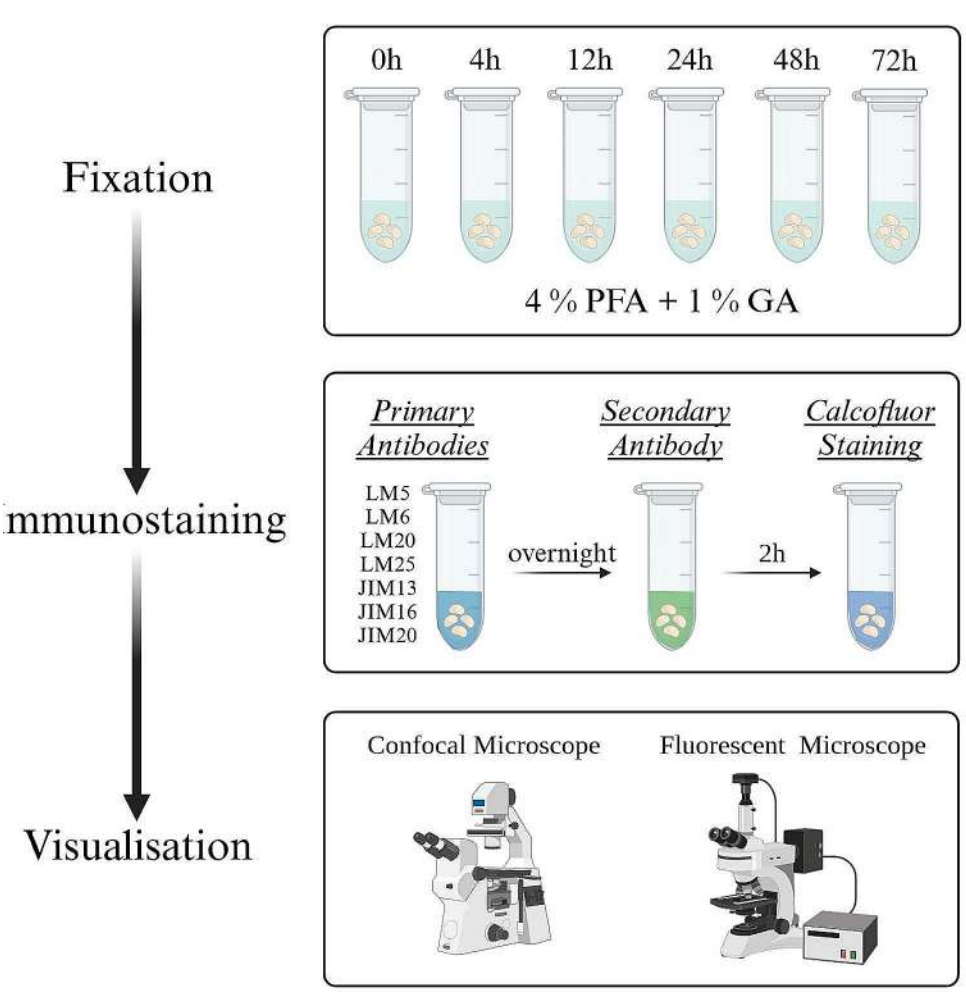


Fig. 2 General scheme of the protoplasts immunostaining process. This figure was created with http://BioRender.com. BioRender certificate confirming the publication rights is available upon request from the authors.

Table 1 Antibodies used for immunostaining of protoplasts and their respective epitopes in the cell wall

Antibody	Epitope	Reference
LM5	linear tetrasaccharide in (1→4)-β-D-galactans (RG I side chain)	(Jones et al. 1997)
LM6	linear pentasaccharide in (1→5)-α-L-arabinans (RG I side chain)	(Willats et al. 1998)
LM20	(1→4)-α-MeGalA (methyl-esterified HG)	(Verhertbruggen et al. 2009)
LM25	XLLG, XXLG and XXXG oligosaccharides of xyloglucan	(Pedersen et al. 2012)
JIM13	Arabinogalactan/Arabinogalactan protein, carbohydrate epitope (β)GlcA1→3(α)GalA1→2Rha	(Yates et al. 1996)
JIM16	Arabinogalactan/ AGP glycan	(Knox et al. 1991)
JIM20	Extensin/Hydroxyproline-rich glycoproteins	(Knox 1995)

RG I: rhamnogalacturonan I; HG: homogalacturonan

placed in three 50 µl beads around the parental and hybrid cells as shown in Fig. 1. Then 4 ml of BM medium supplemented with 4 µl of PSK and 2 µl of Timentin (Table S1) were added to each Petri dish and incubated for 4, 12, 24, 48 and 72 h at 26 ± 1 °C in the dark.

Protoplasts immunostaining

After each time point, the beads with the *F. tataricum* and *F. esculentum* control and hybrid cells were transferred to a 2 ml Eppendorf and fixed with 4% paraformaldehyde (PFA) and 1% glutaraldehyde (GA) in phosphate-buffered saline (PBS, pH = 7.2) overnight at 4 °C (Table S3; Fig. 2). For the 0 h time point; the beads were fixed immediately after they were made. Next, samples were washed with PBS and incubated with blocking buffer (BB) for 30 min (Table S3) and with primary antibodies (Table 1) for 1.5 h.

Negative controls were performed by incubation in BB instead of the primary antibodies. After several rinses with the BB, a secondary antibody conjugated with Alexa Fluor 488 goat anti-rat IgG antibody was applied for 1.5 h in the

dark. Next, the material was washed with PBS and counterstained with 0.01% Fluorescent Brightener 28 (calcofluor white) in PBS solution for 20 min.

Observations

The protoplast beads were individually placed on a glass slide and flattened by the coverslip. The observations and photographic documentation were carried out using an Olympus FV-1000 confocal system (Olympus, Hamburg, Germany) equipped with an Olympus IX81 inverted microscope, a 405-nm diode laser, and a multi-line argon ion laser (excitations 457/488/515 nm; Melles Griot BV, Didam, Netherlands). A series of two-dimensional images of z-stacks (the optical sections through the cells) were taken using two separate photomultipliers. 2D image processing was performed using ImageJ version 1.53s software (Wayne Rasband, National Institutes of Health, USA), and 3D images were performed with Imaris 9.5 software (Bitplane, Zürich, Switzerland). Moreover, 50 cells per antibody were also visualised for statistical analysis with an epifluorescence microscope Nikon Eclipse Ni-U microscope equipped with a Nikon Digital DS-Fi1-U3 camera with the corresponding software (Nikon, Tokyo, Japan). Cells have been scanned in two channels for excitation of Alexa 488 fluorochrome; the 488 nm laser was used, and for FB28, the 405 nm laser. To facilitate the interpretation of the obtained results, the percentage of signal presence from specific epitopes was calculated (number of cells containing signal per total number of cells). Thus, three ranges were specified, where 0–30% means the absence or presence of a signal in a small number of cells; 31–65% occurrence of a signal in the average number of cells; 66–100% occurrence of a signal in most or all cells.

Results

In the presented studies, a spatio-temporal analysis of cell wall regeneration was carried out on protoplasts derived from the callus of two cultivated species of buckwheat (*F. tataricum* and *F. esculentum*) and on their hybrid (heterokaryon: *Ft*+*Fe*). Protoplasts were analysed at various time points: 0 h, 4 h, 12 h, 24 h, 48 h, and 72 h. The wall components that were studied included cellulose (calcofluor staining), pectins (LM5, LM6, LM20), arabinogalactan proteins (JIM13, JIM16), xyloglucan (LM25) and extensin (JIM20). A comparison of protoplasts from two species and hybrids revealed similarities and differences in the cell wall composition at different time points (Table S4).

Arabinogalactan proteins

AGP epitope recognised by the JIM13 antibody occurred in all analysed types of protoplasts (*F. tataricum*, *F. esculentum* and hybrids) and was present at each of the analysed time points, from 0 h to 72 h (Fig. 3A– F, G– L, M– R; Table S4; Supplementary videos S1– S6). This epitope was located within the cell and cell membrane/cell wall at later stages. It was also noticed that the signal from the JIM13 antibody, from 12 h to 72 h, correlated with the arrangement of cellulose microfibrils that were visualised with calcofluor staining (Fig. 3 compare D and D', E and E', F and F', K and K', L and L', P and P, Q and Q'). The presence of JIM13 was also observed outside the cells (Fig. 3, e.g. E, I, J, O). The character of the signal was mainly continuous.

JIM16 antibody, directed against the AGP glycan component, was also observed at every stage of cell wall development for each type of analysed cell (Fig. 4A– F, G– L, M– R; Supplementary videos S7– S12). However, a reduced number of cells (average range) with the presence of JIM16 was observed for *F. tataricum* at 72 h, *F. esculentum* at 24 h and heterokaryons at 48 h (Fig. 4; Table S4). This AGP epitope outside the cell was also observed at each stage of development examined (Fig. 4, e.g., J, K, O, P). Moreover, a difference in the JIM16 distribution was observed. For *F. tataricum*, from 0 h to 24 h, the JIM16 signal was continuous with few areas of higher intensity (Fig. 4A– D). At 48 h and 72 h, the JIM16 epitope did not occur on the entire cell wall but was only present in some regions (Fig. 4E, F). In the case of *F. esculentum*, a continuous signal was observed at 0 h, 4 h, 12 h and 48 h, where areas with increased signal intensity were also observed (Fig. 4G, H, I, K). However, at 24 h and 72 h, the signal was present only in some areas of the cell wall (Fig. 4J, L). For the heterokaryons, the continuous signal was present at 0 h, 12 h, 24 h and 72 h (Fig. 4M, O, P, R); however, for 4 h and 48 h, JIM16 was localised within the walls in a patch-like manner (Fig. 4N, Q).

In summary, the analysed AGP epitopes were present in all cell types, from removing the cell wall (time 0 h) until its reconstitution (time 72 h; Table S4), and were present in the membrane and/or wall. Moreover, the signal outside the cell was also observed. The observed differences concerned the signal's character, especially for JIM16.

Extensin

In *F. tataricum* protoplasts, the JIM20 antibody, which detects extensin epitopes, was present in several cells at the initial stages of wall regeneration (0 h, 4 h; Table S4). In the cells where the signal was detected, it was present in the intracellular compartments (Fig. 5A, B; Supplementary videos S13– S18). An increase in the number of cells

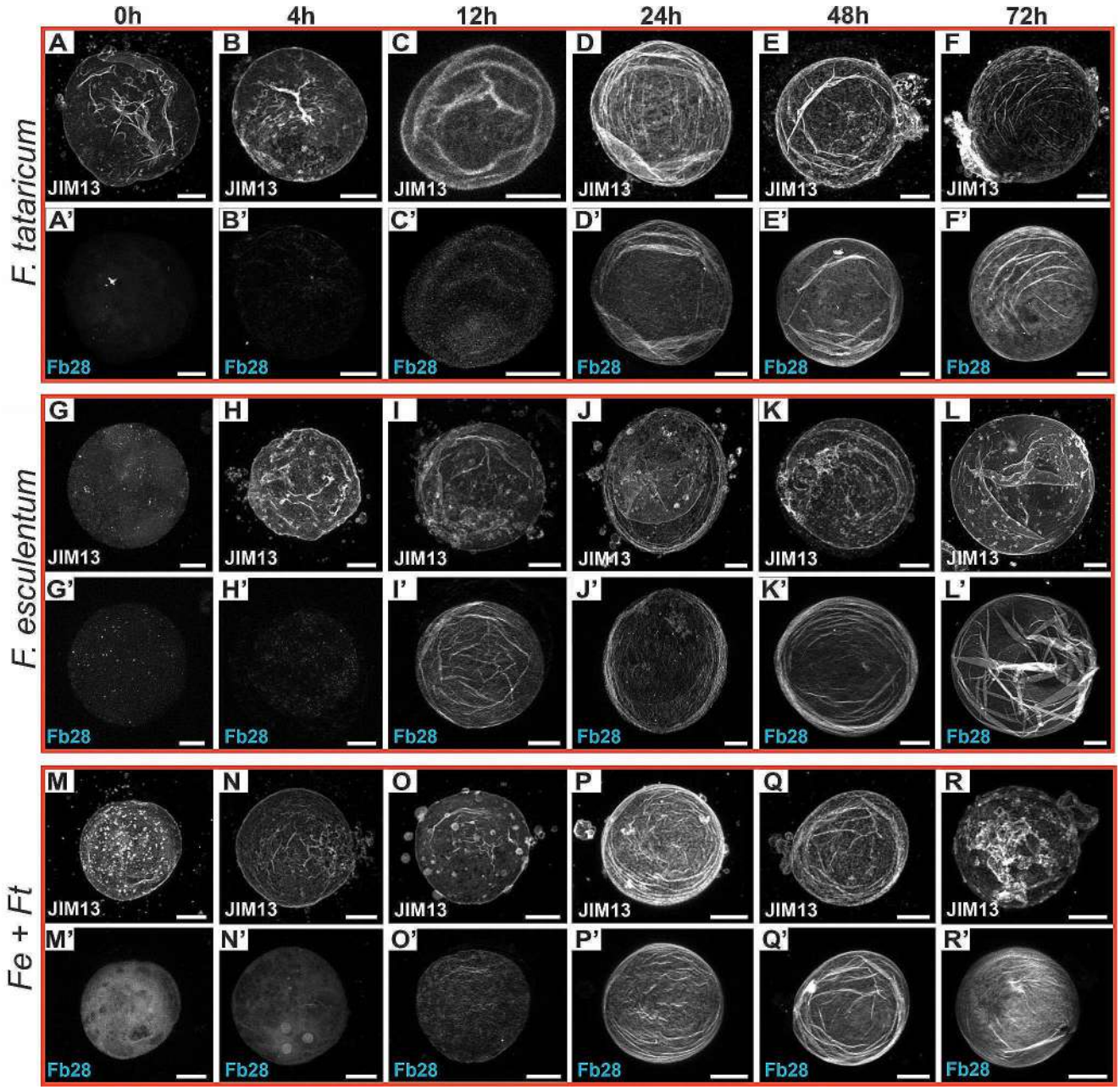


Fig. 3 The presence of JIM13 AGPs epitope during cell wall regeneration in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

with the occurrence of extensins was observed at 12 h and 24 h, followed by a decrease at 48 and 72 h (Fig. 5 compare C– F). If present, the signal from this antibody was continuous except for 24 h, where it had a patch-like character (Fig. 5D). Moreover, it was noticed that at 12 h, 48 h and 72 h in cells where JIM20 was noticed, the signal from the antibody corresponded to the signal from calcofluor staining (Fig. 5 compare, e.g. E and E').

JIM20 in *F. esculentum* protoplasts at 0 h and 4 h was observed in less than half of the analysed cells. However, when the signal was present, it was localised in the intracellular compartments (Fig. 5G, H). Interestingly, at 12 h there was an increase in cells with the JIM20 signal (Fig. 5), followed by a significant decrease at 24 h, and the signal, if present, was dotted (Fig. 5I, J). At 48 h and 72 h, the extensin epitope was observed in some cells, and its character was dotted and patch-like, respectively (Fig. 5K, L).

During wall reconstruction in hybrid protoplasts, the JIM20 epitope was present in most cells at 12 h and 24 h. However, at 0 h and 72 h, the JIM20 signal was observed

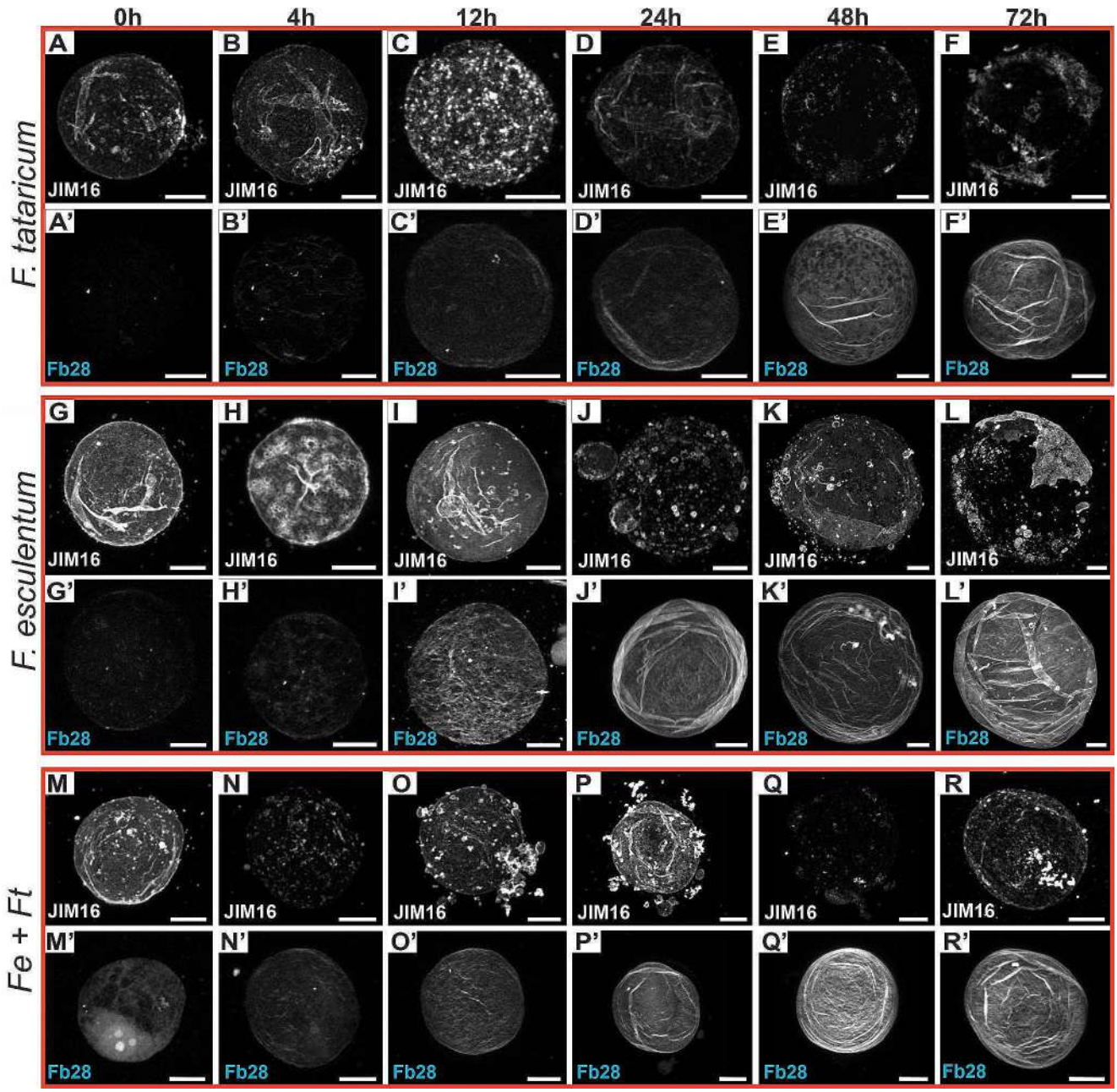


Fig. 4 The distribution of JIM16 AGPs epitope during cell wall reconstruction in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

only in some cells, while at 4 h it was present in a small number of cells, and at 48 h it was absent. At the initial stages of culture, at 0 h and 4 h, the signal, if present, was detected in intracellular compartments (Fig. 5M, N). At 12 h, 24 h, and 72 h, the epitope was present in areas corresponding to those where the calcofluor signal was observed (Fig. 5 compare O and O', P and P', R and R'). In addition, a punctate signal was observed around the cells at all stages (Fig. 5, e.g. O and P).

In summary, the extensin epitope recognised by the JIM20 antibody showed variable occurrence at different time points in all analysed variants (Fig. 5; Table S4). It can be noted that for all cell types at 12 h, the epitope was detected in most cells. At the remaining time points, differences in the number of cells with a positive JIM20 signal were observed between the hybrid and the two *Fagopyrum* species.

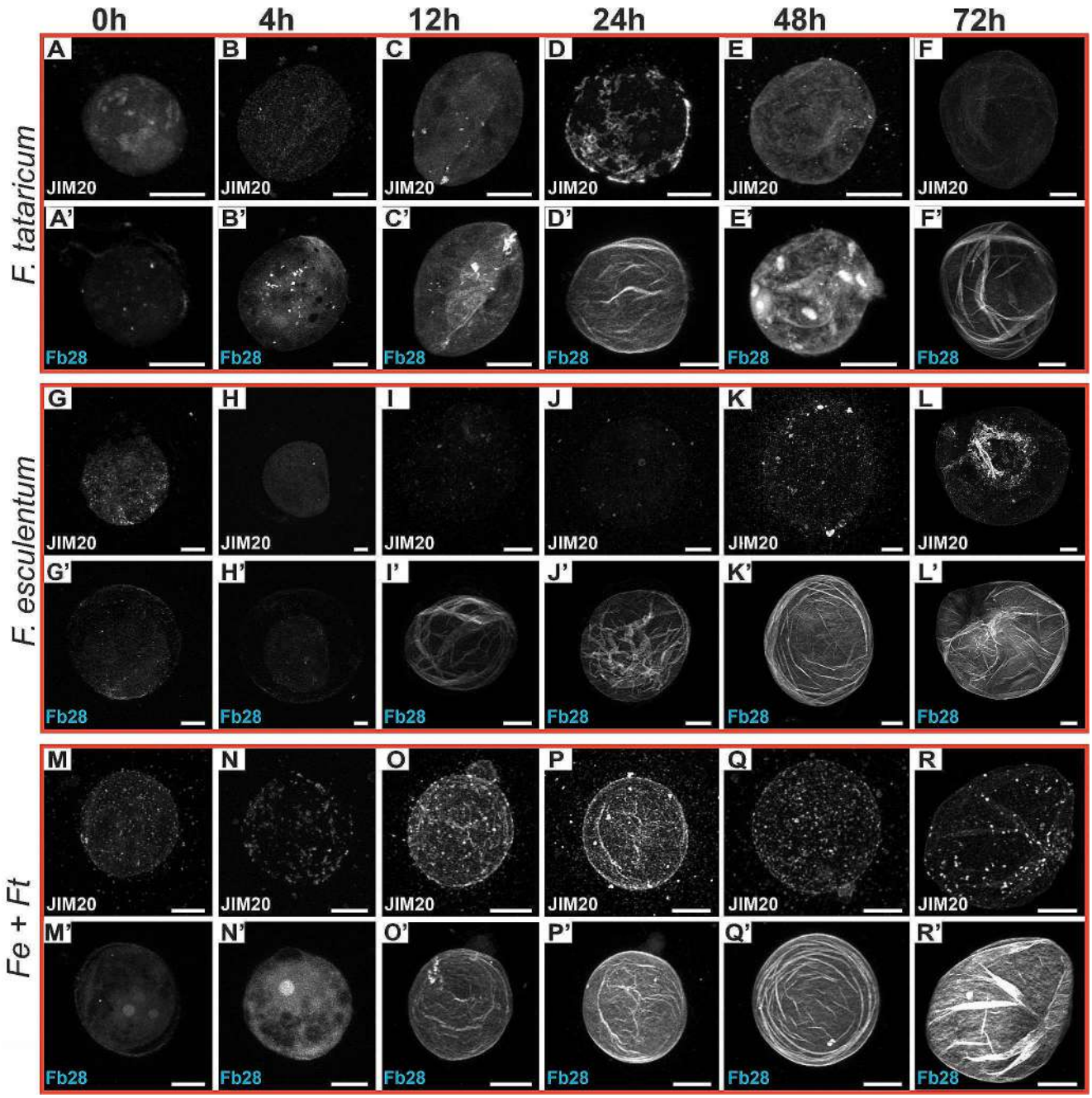


Fig. 5 JIM20 extensin epitope occurrence during wall reconstruction in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with corresponding signal from calcofluor **(A'-R')**

Xyloglucan

At 0 h, the epitope recognised by the LM25 antibody was absent from a minor part of the protoplasts in most of the protoplasts (Fig. 6A– F, G– L, M– R; Supplementary videos S19– S24); however, the fluorescence signal was weak or of moderate intensity (Fig. 6A, G, M), exhibiting a mesh-like pattern. In the next time point, LM25 was detected predominantly in intracellular compartments (Fig. 6B, H, N). From the 12 h time point, the wall was rebuilt by protoplasts (Fig. 6C'– F', I'– L', O'– R') until the 72 h cell wall was restored. The LM25 epitope was present abundantly in all the protoplasts, regardless of genotype, during these time points. The fluorescence signal was observed in the wall matrix as continuous strands that corresponded with the course of cellulose microfibrils (Fig. 6C– F, I– L, O– R).

In summary, at 0 and 4 h, there were differences in the number of protoplasts with detected fluorescence signal

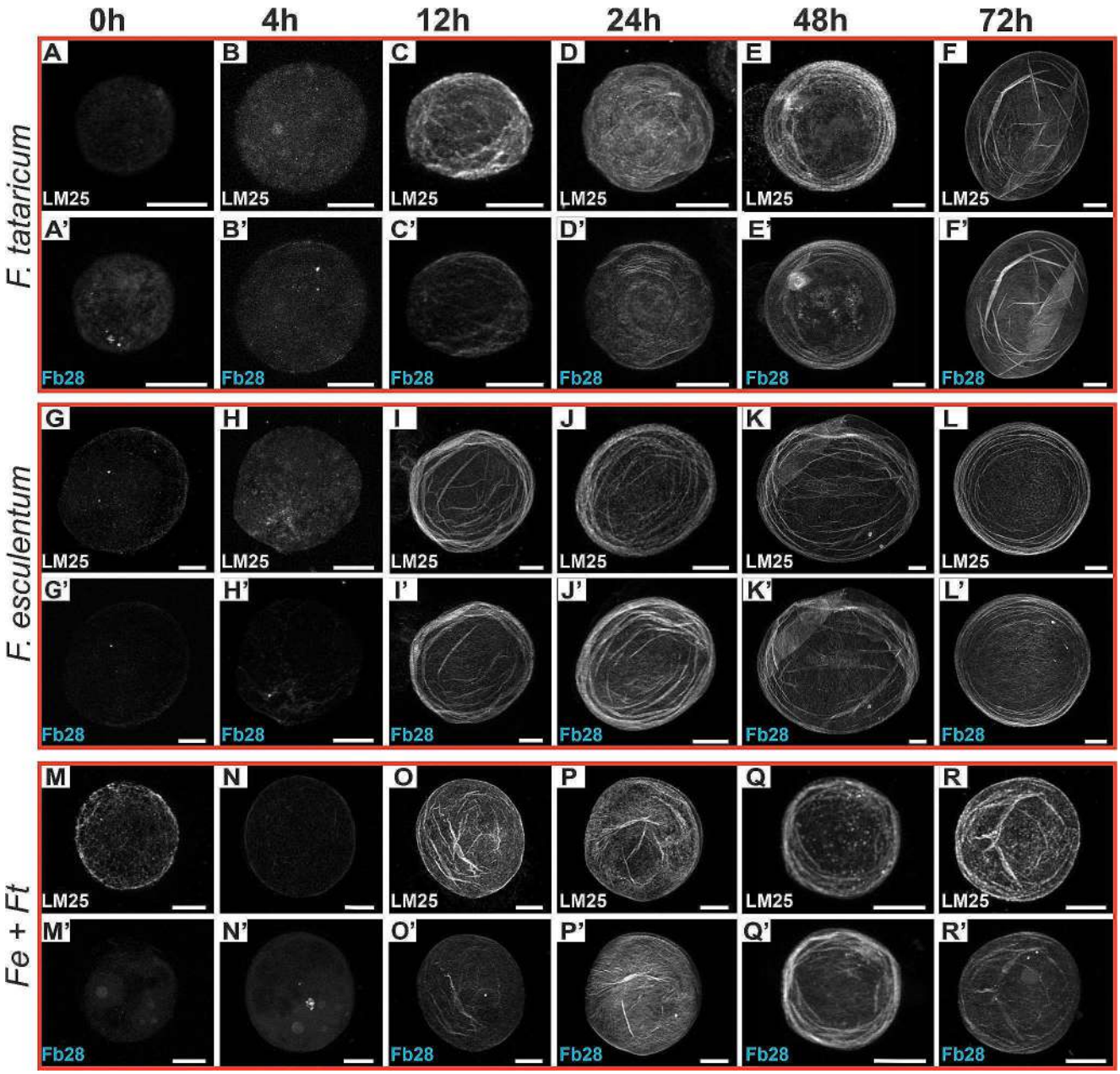


Fig. 6 LM25 xyloglucan epitope distribution during regeneration of cell wall in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

(Fig. 6; Table S4), with *F. esculentum* in average rate at 0 h and *F. esculentum* and hybrid protoplasts in average rate at 4 h. The LM25 epitope was abundantly observed in most cells during the later stages of wall regeneration regardless of the genotype (Fig. 6; Table S4). As mentioned above, no differences in the distribution pattern within the newly arisen wall matrix were observed.

Pectins

Methyl-esterified homogalacturonan (pectins)

The epitope recognised by the LM20 antibody (Fig. 7A–F, G– L, M– R; Supplementary videos S25– S30) at 0 h occurred predominantly in hybrid protoplasts compared to *F. tataricum* and *F. esculentum* protoplasts (Fig. 7A, G, M). At 0 h, the punctate fluorescence signal was detected within cytoplasmic compartments, and in the further time points

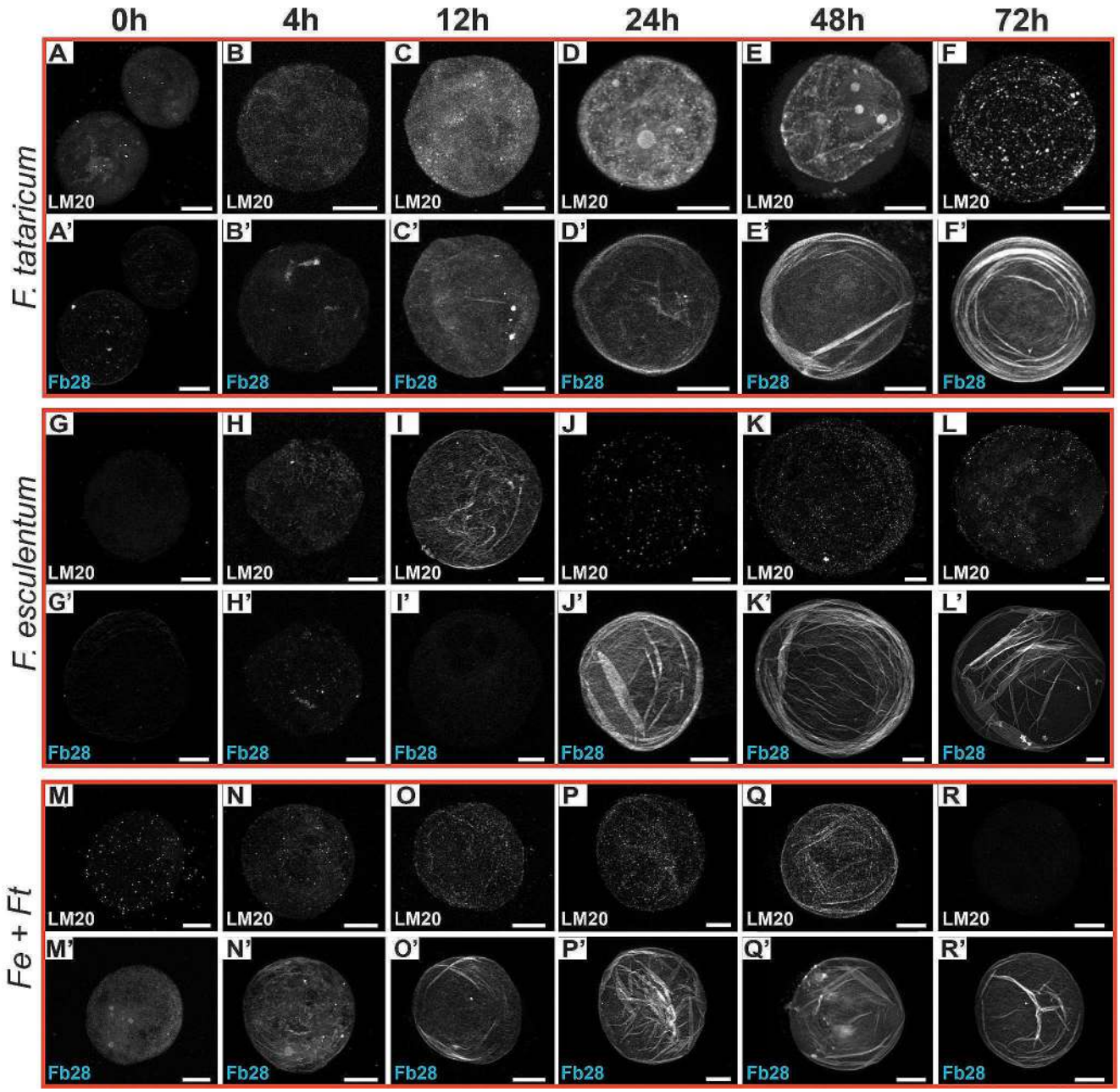


Fig. 7 The presence of LM20 pectic epitope during cell wall rebuilding in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

(4 h, 12 h), this occurrence became more progressed, if present (Fig. 7 compare A– C, G– I, M– O).

In *F. tataricum* protoplasts, the LM20 epitope was observed abundantly in intracellular compartments in the following 12, 24 and 48 h time points (Fig. 7C, D and E). In 72 h, epitope occurred in the wall matrix and was detected in a punctate manner (Fig. 7F; Supplementary video S26). In *F. esculentum* protoplasts, from 24 h and further, a decrease in LM20 epitope occurrence could be seen; namely, epitope was detected but only as a punctate signal within the matrix (Fig. 7J– L).

In hybrid protoplasts at 24 and 48 h, LM20 epitope was detected in the wall matrix; however, at 72 h, only a low number of protoplasts displayed fluorescence signal, with most of the cells that were devoid of LM20 epitope (Fig. 7R; Supplementary video S30). In some of the analysed protoplasts, the fluorescence signal correlated with cellulose microfibril arrangement (Fig. 7 compare F and F', K and K', O and O', P and P', Q and Q'). The LM20 epitope

mainly occurred in a low or average number of cells (Table S4). At 0 and 4 h, hybrid protoplasts exhibited fluorescence signals more frequently than *F. tataricum* or *F. esculentum* protoplasts. From 12 h, the LM20 epitope was detected in *F. tataricum* more often than in *F. esculentum* or hybrid protoplasts. However, unlike other analysed epitopes, the LM20 epitope was not detected abundantly within the wall matrix.

Galactan (RGI, pectins)

The epitope recognised by the LM5 antibody (Fig. 8A– F, G– L, M– R; Supplementary videos S31– S36) was detected in *F. tataricum* protoplasts in intracellular compartments at 0–12 h (Fig. 8A– C), intracellular compartments/wall matrix at 24 h (Fig. 8D) and in wall matrix in the following 48 and 72 h (Fig. 8E, F; Supplementary video S32). A similar pattern of occurrence was observed in *F. esculentum* and hybrid protoplasts; however, the wall-bound epitope

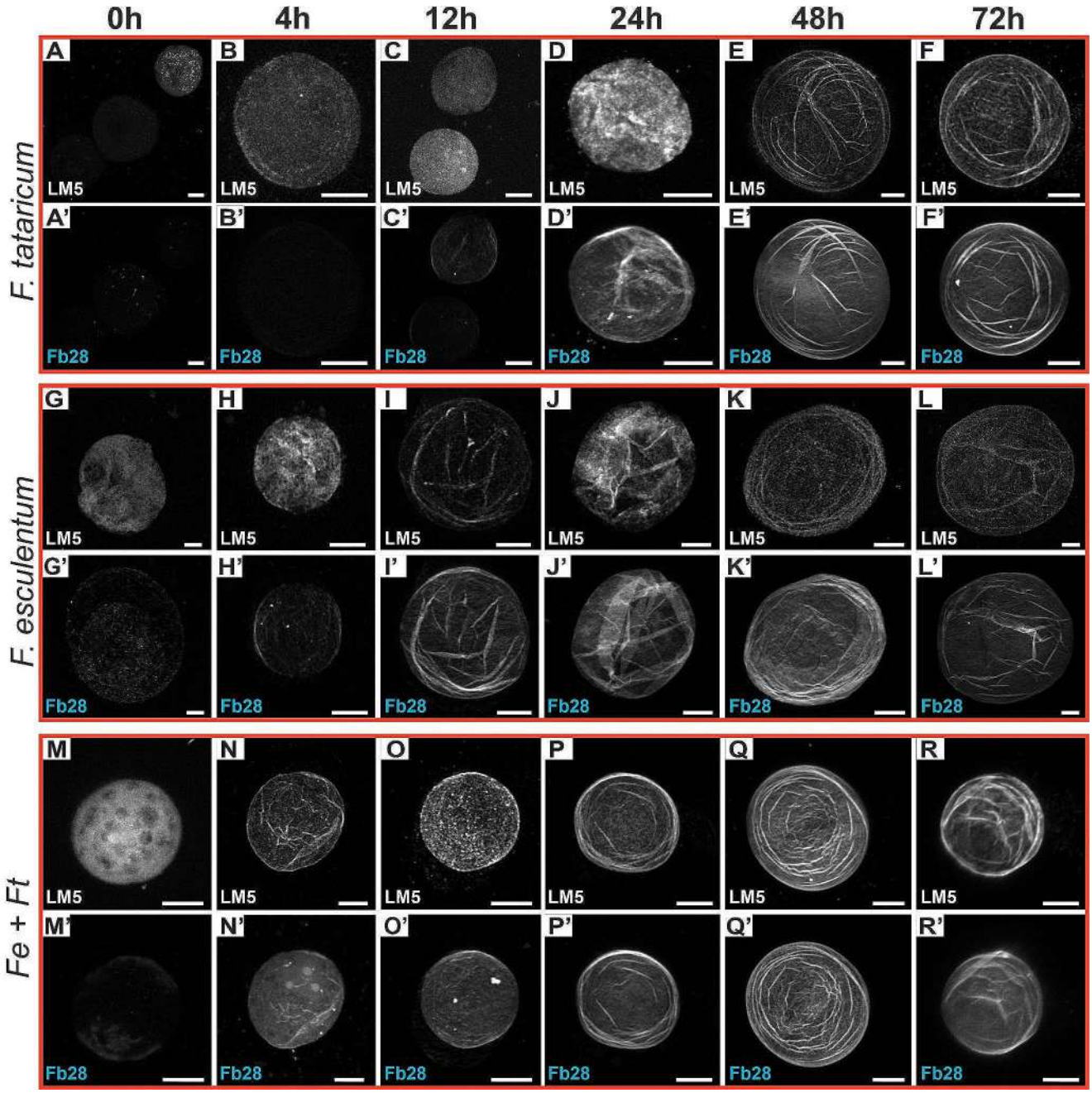


Fig. 8 LM5 pectic epitope distribution during wall reconstruction in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with corresponding signal from calcofluor **(A'-R')**

appeared earlier, from 12 h time point for *F. esculentum* (Fig. 8I– L) and even 4 h for heterokaryons (Fig. 8N– R). The alignment of cellulose microfibrils corresponded with the fluorescence signal, which was depicted in LM5 epitope distribution in different time points regardless of genotype (Fig. 8 compare E and E', F and F', I and I', K and K', L and L', P and P', Q and Q', R and R').

In summary, the fluorescence signal was detected in significant part of the protoplasts (Table S4) with some differences at 0 h (*F. tataricum* and *F. esculentum* belonging to the average range, in hybrid protoplasts– expressed in more prominent number of cells), 24 h (average range for *F. tataricum* in comparison to *F. esculentum* and hybrid) and 48 h (average range for *F. esculentum* in comparison to *F. tataricum* and hybrid).

Arabinan (RGI, pectins)

The epitope recognised by the LM6 antibody (Fig. 9A– F, G– L, M– R; Supplementary videos S37– S42) was detected abundantly in intracellular compartments of *F. tataricum* protoplasts up to 24 h time point (Fig. 9A– D). At 48 and 72 h, the fluorescence signal was localised within the wall matrix (Fig. 9E, E', F, F') and corresponded with the course of some cellulose microfibrils (Fig. 9F, F').

In *F. esculentum* and hybrid protoplasts, the LM6 epitope in the wall matrix was exhibited earlier. Beginning from the 4 h time point, the LM6 epitope appeared at the protoplast surface even before apparent cellulose incorporation (Fig. 9H and H'), and during further stages, the fluorescence signal corresponded with cellulose microfibril deposition (Fig. 9I– L).

In hybrid protoplasts, the cytoplasmic occurrence was noticed only at 0 h, with a significant decrease in the 4 h time point (Fig. 9; Table S4). From the subsequent time points, the occurrence of LM6 was observed in the wall matrix, where the fluorescence signal corresponded with the deposited cellulose component (Fig. 9O– R).

In summary, the occurrence of LM6 epitope was widespread in protoplasts that regenerated cell walls, except 4 h time point where significant differences were noticed. Namely, only an average number of *F. tataricum* contained this compound, most of *F. esculentum* and a few hybrid protoplasts (Table S4). Also, at 24 h, in *F. tataricum*, a fluorescence signal was detected in an average number of protoplasts compared to the predominant distribution in *F. esculentum* and hybrid protoplasts.

Discussion

Isolated protoplasts can regenerate new walls developed enough in structure and composition to permit cell division and differentiation into specific cellular types, tissues, and even whole plants. The protocols for successful protoplast isolation and plant regeneration from protoplast-derived cells have been established for *F. tataricum* and *F. esculentum* (Zaranek et al. 2023a, b).

Structural proteins

The involvement of different AGP epitopes in wall formation during protoplast culture is well documented. In sugar beet, JIM8, JIM13 and LM2 epitopes were detected abundantly in incipient of cell walls and labelling increased as the rebuilding of the walls progressed (Butowt et al. 1999; Majewska-Sawka and Münster 2003). Similar observations with JIM8 and JIM13 epitopes were reported for *Daucus* protoplast cultures (Godel-Jędrychowska et al. 2019; Mock et al. 1990). Another study that involved guard cell- and mesophyll-derived protoplasts from sugar beet and tobacco implied some species-specific differences (Wiśniewska and Majewska-Sawka 2008). Namely, LM2, MAC207, and JIM13 were detected abundantly in sugar beet protoplasts in contrast to JIM4, JIM8, and JIM15 epitopes, while in tobacco, only JIM13 epitope from all epitopes mentioned was present in abundance (Wiśniewska and Majewska-Sawka 2008). Our results are consistent with these findings– both JIM13 and JIM16 epitopes are present during all stages of wall rebuilding in *Fagopyrum* protoplasts, regardless of protoplast source. AGPs have the properties of binding to β-glycans; thus, these plasma-membrane-associated glycoproteins may act as cell surface attachment sites for cell wall matrix polysaccharides (Pennell et al. 1989). Our findings are also in accordance with the reported extracellular localization of AGPs around the regenerating protoplast (Mock et al. 1990), as we also observed fluorescence signals at the surface and around protoplasts. *Fagopyrum* protoplasts are another system where the significant involvement of AGPs in wall formation is postulated (Cassab 1998; Sadava and Chrispeels 1973; Ye and Varner 1991). Unlike AGPs, which are easy to extract from the wall and are considered "mobile", extensins are glycoproteins incredibly resistant to extraction. After being secreted into the wall, they are immediately immobilised by covalent binding with other extensin molecules (Cooper and Varner 1984; Lamport 1986) or wall polymers, presumably pectins (RG-I, Qi et al. 1995). Recent studies, however, indicate that extensins may also be correlated with the increase in cell size (Moore et al. 2014) or the initiation of growth (Cannon et al. 2008).

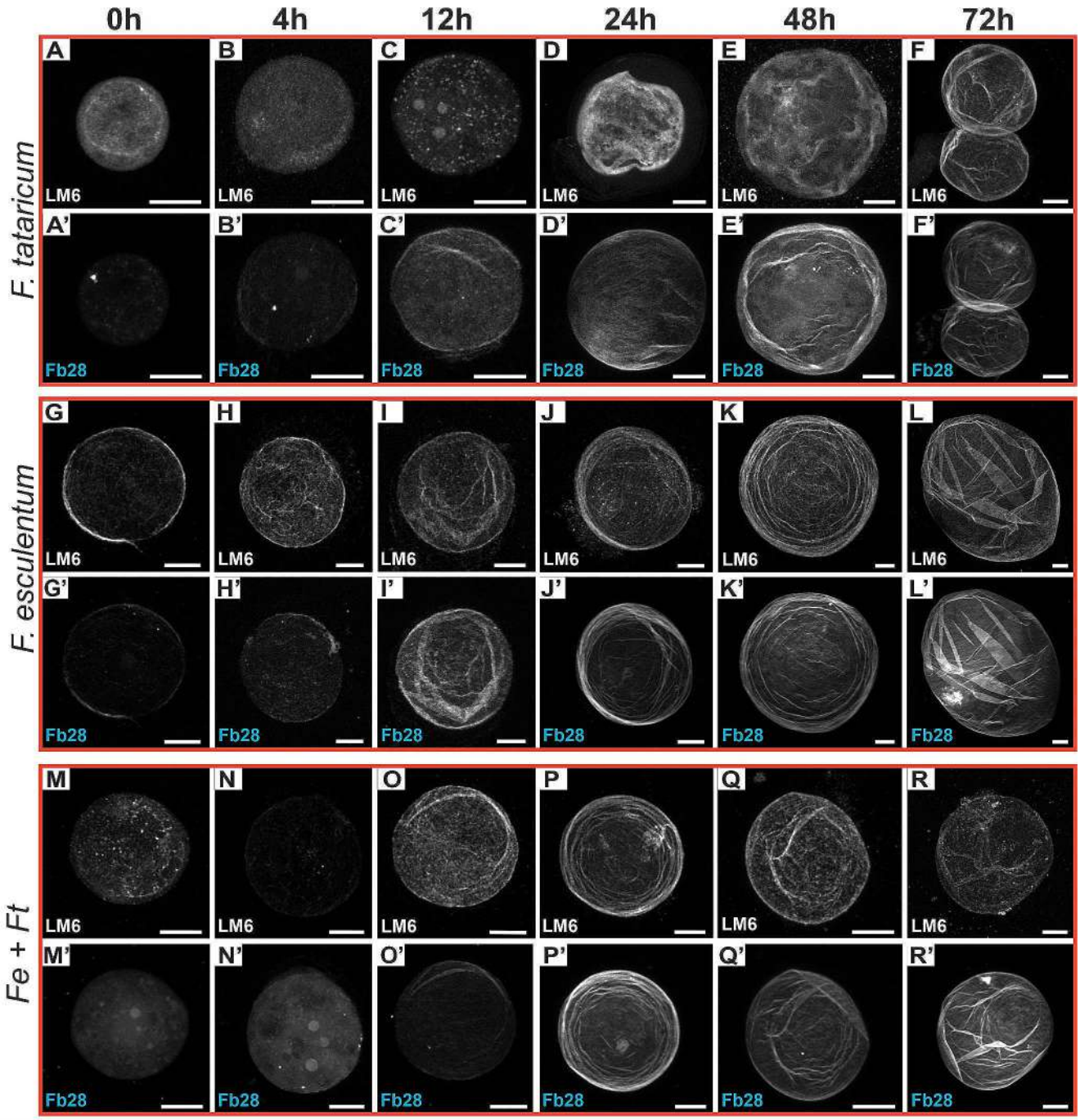


Fig. 9 The occurrence of LM6 pectic epitope during cell wall reconstruction in *F. tataricum* **(A-F)**, *F. esculentum* **(G-L)**, and *Ft* + *Fe* hybrid **(M-R)** with the corresponding signal from calcofluor **(A'-R')**

It was shown that in *Nicotiana sylvestris* protoplasts, the genes encoding the extensins are successively transcribed in a few hours after the isolation procedure (Parmentier et al. 1995). In carrot's protoplast-derived cells, a JIM12 extensin epitope was detected only between 10 and 20 days of culture in the already differentiated cells (Godel-Jędrychowska et al. 2019). Our study shows the occurrence of another extensin epitope, JIM20, in intracellular compartments at the early stages (0 h, 4 h) of wall regeneration and the wall-bound localization during the further time points (12–72 h). Despite differences in the number of protoplasts exhibiting the fluorescence signal, a 12 h time frame is common for all genotypes and points to the most abundant JIM20 occurrence. Thus, it can be stated that JIM20 is involved in the wall regeneration process in *Fagopyrum* species.

Wall polysaccharides

Xyloglucan is the dominant hemicellulose of the primary cell walls of dicotyledonous plants (Brett and Waldron 1996). The network created between cellulose microfibrils and xyloglucan stabilises the spatial structure of the entire cell wall (Park and Cosgrove 2012; Peña et al. 2004; Ryden et al. 2003). However, it was suggested that the formation of a cellulose network during wall rebuilding is independent of xyloglucan as this compound was not essential during the early stages of cellulose assembly and, during the early stages of pea protoplast cultures, it was shown that the binding between cellulose and xyloglucan was not as strong as in the intact pea cell walls (Hayashi et al. 1986). Nevertheless, our results indicate the co-localization of xyloglucan LM25 epitope and cellulose microfibrils. In *Fagopyrum* protoplasts, xyloglucan synthesis and incorporation into the wall matrix accompany the cellulose network formation. Methyl-esterified homogalacturonan (HG) recognized by LM20 was present in intracellular compartments, which indicates the ongoing synthesis. In *F. tataricum* this cytoplasmic distribution was detected even up to 48 h, in contrast to *F. esculentum* and hybrid protoplasts, in which the wall-bound occurrence was observed earlier. However, our results showed a relatively moderate distribution within the wall matrix in the examined time frames. Other results indicate that the amount of esterified HG increases with time during the wall regeneration. For example, in carrot protoplast culture, methyl-esterified pectins were abundantly present after 4 days of culture, and the amount increased along with the culture age (Godel-Jędrychowska et al. 2019). Similarly, in flax hypocotyl-derived protoplasts at 6th day of culture, the amount of methyl-esterified pectins increased compared to analysis from 3rd day (David et al. 1995). Fresh protoplasts of sugar beet showed trace content of methyl-esterified pectin, in contrast to mesophyll protoplast-derived cells (Majewska-Sawka and Münster 2003; Wiśniewska and Majewska-Sawka 2008).

The side chains of RG-I, arabinan and galactan influence the mechanical properties and porosity of walls (Verhertbruggen et al. 2009; Willats et al. 2001) as well as cell adhesion (Iwai et al. 2001; Leboeuf et al. 2004; Orfila et al. 2001; Peña and Carpita 2004). Arabinan and galactan often show different distributions within cells or tissues (Bush et al. 2001; Orfila and Knox 2000) and may have different functions depending on the plant species or cell type (McCartney et al. 2000; Orfila and Knox 2000; Willats et al. 1999). (McCartney et al. 2003; Willats et al. 1999) The occurrence of the LM5 epitope in the walls is correlated with their strengthening (McCartney and Knox 2002; McCartney et al. 2000), in contrast to arabinan (LM6 epitope), the presence of which ensures the elasticity of cell walls (Jones et al. 2003, 2005; Moore et al. 2008, 2013).

Surprisingly, both epitopes were present during wall regeneration in *Fagopyrum* protoplasts. However, it is noteworthy that in hybrid protoplasts, the LM6 occurrence was significantly reduced at a 4 h time point compared to *F. tataricum* and *F. esculentum.* Similarly to the HG, LM5 and LM6 were detected in intracellular compartments and subsequently within the wall, which suggests increased synthesis followed by their incorporation into the wall matrix. So far, LM5 and LM6 have been detected in guard and mesophyll cell protoplasts of tobacco, in contrast to sugar beet protoplasts, in which LM5 was detected in trace amounts, and LM6 occurred only in protoplast-derived callus (Majewska-Sawka and Münster 2003; Wiśniewska and Majewska-Sawka 2008). Thus, the occurrence of galactan and arabinan during wall regeneration seems to be species-specific. Since regenerating walls have to be competent to contain the expansion of the protoplast, withstand the osmotic pressure and, consequently, allow for cell division (Burgess and Linstead 1976), they have to be elastic and strengthened. It could explain the concurrent presence of both LM5 and LM6 epitopes during wall re-establishment in *Fagopyrum* protoplasts.

Final remarks

We want to highlight that the regenerated walls of protoplasts should not be considered equivalent to the walls of cells from which protoplasts were isolated. The sugar composition of cell walls from protoplasts and walls of their corresponding parent tissue differs, and the type of regeneration media (solid or liquid) affects polysaccharide content (David et al. 1995; Takeuchi and Komamine 1978, 1982). Protoplast walls could be compared, in terms of composition, to a very young cell wall formed during cytokinesis (Franz and Blaschek 1985). Indeed, xyloglucan, pectins, AGPs, and extensins participate in cell plate formation (Shibaya and Sugawara 2009; Sinclair et al. 2022). Additionally, during the early stages of wall formation, wall compounds disintegrate from the surface of the cells and are lost into the medium (Franz and Blaschek 1985) which explains the extracellular occurrence of some analysed epitopes in the current study.

Conclusion

In the presented study, we focused on the process of wall rebuilding for *Fagopyrum* spp., a new yet subsequent system introduced in this basic research area. Surprisingly, the early stages of wall formation in hybrid protoplasts were

not impaired compared to those observed in parental protoplasts of *F. tataricum* or *F. esculentum*. These results can contribute to further research on somatic hybrids, as understanding the development patterns may impact the successful modulation of yield productivity and yield stability for future buckwheat breeding programmes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-024-02740-6.

Author contributions Conceptualization: A.B., K.S-C., A.M-H., R.P.P.; Data curation: A.B., K.S-C., A.M-H, R.P.P.; Formal analysis: K.S-C., A.M-H, R.P.P.; Funding acquisition: A.B.; Investigation: K.S-C., A.M-H, R.P.P; Methodology: A.B, E.G., K.S-C., A.M-H, R.P.P; Project administration: A.B.; Supervision: A.B., K.S-C., A.M-H.; Visualization: K.S-C., A.M-H, R.P.P; Roles/Writing - original draft: K.S-C., A.M-H, R.P.P; and Writing - review & editing: A.B., E.G., K.S-C., A.M-H, R.P.P.

Funding This research was funded by the National Science Centre, Poland. Research project Sonata Bis (2020/38/E/NZ9/00033 awarded to AB) and the Research Excellence Initiative of the University of Silesia in Katowice.

Data availability Data supporting this study are included within the article and/or supporting materials.

Declarations

Ethics approval and consent to participate The use of all plant materials in this study complies with relevant institutional, national, and international guidelines and legislation. Seeds of *F. tataricum* (sample k-17) are from the N. I. Vavilov Institute of Plant Genetic Resources collections, Saint Petersburg, Russia. The Plant Cytogenetic and Molecular Biology Group Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Poland multiplied the obtained seeds. *F. tataricum* sample k-17 is a common cultivated landrace of *F. tataricum*, and seeds are available upon request from the publication's authors. *F. esculentum* cultivar Panda seeds are commercially available and purchased from the Malopolska Plant Breeding Company (Poland).

Consent for publication Not applicable.

Conflict of interest All the authors declare that there is no conflict of interest. Ewa Grzebelus is one of the journal's associate editors. As such, she was fully excluded during all the evaluation period of this work, had no access to its handling during peer-refereeing, and her status had no bearing on the editorial consideration of the manuscript.

References

Adachi T, Yamaguchi A, Miike Y, Hoffmann F (1989) Plant regeneration from protoplasts of common buckwheat (*Fagopyrum esculentum*). Plant Cell Rep 8(4):247–250. https://doi.org/10.1007/BF00778544

Brett CT, Waldron KW, Brett CT, Waldron KW (1996) 2nd edn. edn. Chapman & Hall, London

Betekhtin A, Rojek M, Jaskowiak J, Milewska-Hendel A, Kwasniewska J, Kostyukova Y, Kurczynska E, Rumyantseva N, Hasterok R (2017) Nuclear genome stability in long-term cultivated callus lines of *Fagopyrum tataricum* (L.) Gaertn. PLOS ONE 12 (3):e0173537.

Burgess J, Linstead PJ (1976) Scanning electron microscopy of cell wall formation around isolated plant protoplasts. Planta 131(2):173–178. https://doi.org/10.1007/BF00389991

Bush MS, Marry M, Huxham MI, Jarvis MC, McCann MC (2001) Developmental regulation of pectic epitopes during potato tuberisation. Planta 213(6):869–880. https://doi.org/10.1007/s004250100570

Butowt R, Niklas A, Rodriguez-Garcia MI, Majewska-Sawka A (1999) Involvement of JIM13- and JIM8-responsive carbohydrate epitopes in early stages of cell wall formation. J Plant Res 112(1):107–116. https://doi.org/10.1007/PL00013851

Cannon MC, Terneus K, Hall Q, Tan L, Wang Y, Wegenhart BL, Chen L, Lamport DTA, Chen Y, Kieliszewski MJ (2008) Self-assembly of the plant cell wall requires an extensin scaffold. Proc. Natl. Acad. Sci. U.S.A. 105(6):2226–2231 https://doi.org/10.1073/pnas.0711980105

Cassab GI (1998) Plant cell wall proteins. Annu Rev Plant Physiol Plant Mol Biol 49(1):281–309. https://doi.org/10.1146/annurev.arplant.49.1.281

Cawoy V, Ledent J-F, Kinet J-M, Jacquemart A-L (2009) Floral biology of common buckwheat (*Fagopyrum esculentum* Moench). Eur J Plant Sci Biotechnol 3(1):1–9

Cooper JB, Varner JE (1984) Cross-linking of soluble extensin in isolated cell walls. Plant Physiol 76(2):414–417. https://doi.org/10.1104/pp.76.2.414

David H, David PB, Savy A, Demazy C C, Van Cutsem P (1995) Pectins in walls of protoplast-derived cells imbedded in agarose and alginate beads. Protoplasma 186:122–130. https://doi.org/10.1007/BF01281322

Fabjan N, Rode J, Košir IJ, Wang Z, Zhang Z, Kreft I (2003) Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) As a source of dietary rutin and quercitrin. J Agric Food Chem 51(22):6452–6455. https://doi.org/10.1021/jf034543e

Franz G & Blaschek W Wall regeneration in protoplasts of higher plants. In, Berlin, Heidelberg, 1985. The physiological properties of Plant Protoplasts. Springer Berlin Heidelberg, p 171–183

Godel-Jędrychowska K, Maćkowska K, Kurczyńska E, Grzebelus E (2019) Composition of the reconstituted cell wall in protoplast-derived cells of daucus is affected by phytosulfokine (PSK). Int J Mol Sci 20(21). https://doi.org/10.3390/ijms20215490

Hayashi T, Polonenko DR, Camirand A, Maclachlan G (1986) Pea xyloglucan and cellulose: IV. Assembly of Beta-glucans by pea protoplasts. Plant Physiol 82(1):301–306. https://doi.org/10.1104/pp.82.1.301

Huda MN, Lu S, Jahan T, Ding M, Jha R, Zhang K, Zhang W, Georgiev MI, Park SU, Zhou M (2021) Treasure from garden: bioactive compounds of buckwheat. Food Chem 335:127653. https://doi.org/10.1016/j.foodchem.2020.127653

Iwai H, Ishii T, Satoh S (2001) Absence of arabinan in the side chains of the pectic polysaccharides strongly associated with cell walls of *Nicotiana plumbaginifolia* non-organogenic callus with loosely attached constituent cells. Planta 213(6):907–915. https://doi.org/10.1007/s004250100559

Jones L, Seymour GB, Knox JP (1997) Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1->4)-beta-D-galactan. Plant Physiol 113(4):1405–1412. https://doi.org/10.1104/pp.113.4.1405

Jones L, Milne JL, Ashford D, McQueen-Mason SJ (2003) Cell wall arabinan is essential for guard cell function. Proc. Natl. Acad. Sci. U.S.A. 100(20):11783–11788 https://doi.org/10.1073/pnas.1832434100

Jones L, Milne JL, Ashford D, McCann MC, McQueen-Mason SJ (2005) A conserved functional role of pectic polymers in stomatal guard cells from a range of plant species. Planta 221(2):255–264. https://doi.org/10.1007/s00425-004-1432-1

Knox JP (1995) Developmentally regulated proteoglycans and glycoproteins of the plant cell surface. FASEB J 9(11):1004–1012. https://doi.org/10.1096/fasebj.9.11.7544308

Knox JP, Linstead PJ, Cooper JPC, Roberts K (1991) Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. Plant J 1(3):317–326. https://doi.org/10.1046/j.1365-313X.1991.t01-9-00999.x

Kuki H, Yokoyama R, Kuroha T, Nishitani K (2020) Xyloglucan is not essential for the formation and integrity of the cellulose network in the primary cell wall regenerated from *Arabidopsis* protoplasts. Plants (Basel) 9(5):629. https://doi.org/10.3390/plants9050629

Lachmann S, Adachi T (1990) Callus regeneration from hypocotyl protoplasts of tartary buckwheat (*Fagopyrum tataricum* Gaertn). Fagopyrum 10:62–64

Lachmann S, Kishima Y, Adachi T (1994) Protoplast fusion in buckwheat: preliminary results on somatic hybridisation. Fagopyrum 14:7–12

Lamport D (1986) The primary cell wall: a new model. Structure, Modification and Hydrolysis, John Wiley & Sons Inc, New York, Cellulose

Leboeuf E, Thoiron S, Lahaye M (2004) Physico-chemical characteristics of cell walls from *Arabidopsis thaliana* Microcalli showing different adhesion strengths. J Exp Bot 55(405):2087–2097. https://doi.org/10.1093/jxb/erh225

Li J, Zhang K, Meng Y, Li Q, Ding M, Zhou M (2019) FtMYB16 interacts with Ftimportin-α1 to regulate rutin biosynthesis in tartary buckwheat. Plant Biotechnol J 17(8):1479–1481. https://doi.org/10.1111/pbi.13121

Mackowska K, Stelmach-Wityk K, Grzebelus E (2023) Early selection of carrot somatic hybrids: a promising tool for species with high regenerative ability. Plant Methods 19(1):104. https://doi.org/10.1186/s13007-023-01080-4

Majewska-Sawka A, Münster A (2003) Cell-wall antigens in mesophyll cells and mesophyll-derived protoplasts of sugar beet: possible implication in protoplast recalcitrance? Plant Cell Rep 21(10):946–954. https://doi.org/10.1007/s00299-003-0612-y

McCartney L, Knox JP (2002) Regulation of pectic polysaccharide domains in relation to cell development and cell properties in the pea testa. J Exp Bot 53(369):707–713. https://doi.org/10.1093/jexbot/53.369.707

McCartney L, Ormerod AP, Gidley MJ, Knox JP (2000) Temporal and spatial regulation of pectic (1–4)-β-D-galactan in cell walls of developing pea cotyledons: implications for mechanical properties. Plant J 22(2):105–113. https://doi.org/10.1046/j.1365-313x.2000.00719.x

McCartney L, Steele-King CG, Jordan E, Knox JP (2003) Cell wall pectic (1→4)-β- D-galactan marks the acceleration of cell elongation in the *Arabidopsis* seedling root meristem. Plant J 33(3):447–454. https://doi.org/10.1046/j.1365-313X.2003.01640.x

Mock HP, Emmerling M, Seitz HU (1990) Cell wall synthesis in carrot cells: comparison of suspension-cultured cells and regenerating protoplasts. Physiol Plant 79(2):347–353. https://doi.org/10.1111/j.1399-3054.1990.tb06752.x

Moore JP, Farrant JM, Driouich A (2008) A role for pectin-associated arabinans in maintaining the flexibility of the plant cell wall during water deficit stress. Plant Signal Behav 3(2):102–104. https://doi.org/10.4161/psb.3.2.4959

Moore JP, Nguema-Ona EE, Vicré-Gibouin M, Sørensen I, Willats WGT, Driouich A, Farrant JM (2013) Arabinose-rich polymers as an evolutionary strategy to plasticize resurrection plant cell walls against desiccation. Planta 237(3):739–754. https://doi.org/10.1007/s00425-012-1785-9

Moore JP, Fangel JU, Willats WGT, Vivier MA (2014) Pectic-β(1,4)-galactan, extensin and arabinogalactan–protein epitopes differentiate ripening stages in wine and table grape cell walls. Ann Botany 114(6):1279–1294. https://doi.org/10.1093/aob/mcu053

Nešković M, Vujičić R, Budimir S (1987) Somatic embryogenesis and bud formation from immature embryos of buckwheat (*Fagopyrum esculentum* Moench). Plant Cell Rep 6(6):423–426. https://doi.org/10.1007/BF00272773

Orfila C, Knox JP (2000) Spatial regulation of pectic polysaccharides in relation to pit fields in cell walls of tomato fruit pericarp. Plant Physiol 122(3):775–782. https://doi.org/10.1104/pp.122.3.775

Orfila C, Seymour GB, Willats WG, Huxham IM, Jarvis MC, Dover CJ, Thompson AJ, Knox JP (2001) Altered middle lamella homogalacturonan and disrupted deposition of (1–5)-alpha-L-arabinan in the pericarp of Cnr, a ripening mutant of tomato. Plant Physiol 126(1):210–221. https://doi.org/10.1104/pp.126.1.210

Park YB, Cosgrove DJ (2012) Changes in cell wall biomechanical properties in the xyloglucan-deficient *xxt1*/*xxt2* mutant of *Arabidopsis*. Plant Physiol 158(1):465–475. https://doi.org/10.1104/pp.111.189779

Parmentier Y, Fleck J, Jamet E (1995) A novel wound-inducible extensin gene is expressed early in newly isolated protoplasts of *Nieotiana Sylvestris*. Plant Mol Biol 29(2):279–292. https://doi.org/10.1007/BF00043652

Pedersen HL, Fangel JU, McCleary B, Ruzanski C, Rydahl MG, Ralet MC, Farkas V, von Schantz L, Marcus SE, Andersen MC, Field R, Ohlin M, Knox JP, Clausen MH, Willats WG (2012) Versatile high resolution oligosaccharide microarrays for plant glycobiology and cell wall research. J Biol Chem 287(47):39429–39438. https://doi.org/10.1074/jbc.M112.396598

Peña MJ, Carpita NC (2004) Loss of highly branched arabinans and debranching of Rhamnogalacturonan I accompany loss of firm texture and cell separation during prolonged storage of apple. Plant Physiol 135(3):1305–1313. https://doi.org/10.1104/pp.104.043679

Peña MJ, Ryden P, Madson M, Smith AC, Carpita NC (2004) The galactose residues of xyloglucan are essential to maintain mechanical strength of the primary cell walls in *Arabidopsis* during growth. Plant Physiol 134(1):443–451. https://doi.org/10.1104/pp.103.027508

Pennell RI, Knox JP, Scofield GN, Selvendran RR, Roberts K (1989) A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. J Cell Biol 108(5):1967–1977. https://doi.org/10.1083/jcb.108.5.1967

Potocka I, Godel K, Dobrowolska I, Kurczynska EU (2018) Spatio-temporal localization of selected pectic and arabinogalactan protein epitopes and the ultrastructural characteristics of explant cells that accompany the changes in the cell fate during somatic embryogenesis in Arabidopsis thaliana. Plant Physiol Biochem 127:573–589. https://doi.org/10.1016/j.plaphy.2018.04.032

Qi X, Behrens BX, West PR, Mort AJ (1995) Solubilization and partial characterization of extensin fragments from cell walls of cotton

suspension cultures (evidence for a covalent cross-link between extensin and pectin). Plant Physiol 108(4):1691–1701. https://doi.org/10.1104/pp.108.4.1691

Rems L, Usaj M, Kanduser M, Rebersek M, Miklavcic D, Pucihar G (2013) Cell electrofusion using nanosecond electric pulses. Sci Rep 3:3382. https://doi.org/10.1038/srep03382

Ryden P, Sugimoto-Shirasu K, Smith AC, Findlay K, Reiter W-D, McCann MC (2003) Tensile properties of *Arabidopsis* cell walls depend on both a xyloglucan cross-linked microfibrillar network and Rhamnogalacturonan II-borate complexes. Plant Physiol 132(2):1033–1040. https://doi.org/10.1104/pp.103.021873

Sadava D, Chrispeels MJ (1973) Hydroxyproline-rich cell wall protein (extensin): role in the cessation of elongation in excised pea epicotyls. Develop Biol 30(1):49–55. https://doi.org/10.1016/0012-1606(73)90047-X

Sedlak P, Sedlakova V, Vasek J, Zeka D, Cilova D, Melounova M, Orsak M, Domkarova J, Dolezal P, Vejl P (2022) Phenotypic, molecular and biochemical evaluation of somatic hybrids between *Solanum tuberosum* and *S. bulbocastanum*. Sci Rep 12(1):4484 https://doi.org/10.1038/s41598-022-08424-5

Senda M, Takeda J, S A, Nakamura T (1979) Induction of cell fusion of plant protoplasts by electrical stimulation. Plant Cell Physiol 20:1441–1443

Shibaya T, Sugawara Y (2009) Induction of multinucleation by β-glucosyl Yariv reagent in regenerated cells from *Marchantia polymorpha* protoplasts and involvement of arabinogalactan proteins in cell plate formation. Planta 230(3):581–588. https://doi.org/10.1007/s00425-009-0954-y

Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell 5(1):9–23. https://doi.org/10.1105/tpc.5.1.9

Sinclair R, Hsu G, Davis D, Chang M, Rosquete M, Iwasa JH, Drakakaki G (2022) Plant cytokinesis and the construction of new cell wall. FEBS Lett 596(17):2243–2255. https://doi.org/10.1002/1873-3468.14426

Sytar O, Zivcak M, Brestic M (2016) Noninvasive methods to support metabolomic studies targeted at plant phenolics for food and medicinal use. In: Hakeem KR, Tombuloğlu H, Tombuloğlu G (eds) Plant Omics: Trends and Applications. Springer International Publishing, Cham, pp 407–443

Takeuchi Y, Komamine A (1978) Composition of the cell wall formed by protoplasts isolated from cell suspension cultures of *Vinca rosea*. Planta 140(3):227–232. https://doi.org/10.1007/bf00390252

Takeuchi Y, Komamine A (1982) Effects of culture conditions on cell division and composition of regenerated cell walls in *Vinca rosea* protoplasts. Plant Cell Physiol 23(2):249–255. https://doi.org/10.1093/oxfordjournals.pcp.a076344

Tomasiak A, Zhou M, Betekhtin A (2022) Buckwheat in tissue culture research: current status and future perspectives. Int J Mol Sci 23(4). https://doi.org/10.3390/ijms23042298

Verhertbruggen Y, Marcus SE, Haeger A, Ordaz-Ortiz JJ, Knox JP (2009) An extended set of monoclonal antibodies to pectic homogalacturonan. Carbohydr Res 344(14):1858–1862. https://doi.org/10.1016/j.carres.2008.11.010

Willats WG, Marcus SE, Knox JP (1998) Generation of monoclonal antibody specific to (1–>5)-alpha-L-arabinan. Carbohydr Res 308(1–2):149–152. https://doi.org/10.1016/s0008-6215(98)00070-6

Willats WGT, Steele-King CG, Marcus SE, Knox JP (1999) Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. Plant J 20(6):619–628. https://doi.org/10.1046/j.1365-313X.1999.00629.x

Willats WGT, McCartney L, Mackie W, Knox JP (2001) Pectin: cell biology and prospects for functional analysis. In: Carpita NC, Campbell M, Tierney M (eds) Plant cell walls. Springer Netherlands, Dordrecht, pp 9–27

Wiśniewska E, Majewska-Sawka A (2007) Arabinogalactan-proteins stimulate the organogenesis of guard cell protoplasts-derived callus in sugar beet. Plant Cell Rep 26(9):1457–1467. https://doi.org/10.1007/s00299-007-0348-1

Wiśniewska E, Majewska-Sawka A (2008) The differences in cell wall composition in leaves and regenerating protoplasts of *Beta vulgaris* and *Nicotiana tabacum*. Biol Plant 52(4):634–641. https://doi.org/10.1007/s10535-008-0124-2

Yates EA, Valdor JF, Haslam SM, Morris HR, Dell A, Mackie W, Knox JP (1996) Characterization of carbohydrate structural features recognized by anti-arabinogalactan-protein monoclonal antibodies. Glycobiol 6(2):131–139. https://doi.org/10.1093/glycob/6.2.131

Ye ZH, Varner JE (1991) Tissue-specific expression of cell wall proteins in developing soybean tissues. Plant Cell 3(1):23–37. https://doi.org/10.1105/tpc.3.1.23

Zaranek M, Pérez-Pérez R, Milewska-Hendel A, Betekhtin A, Grzebelus E (2023a) Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn. BMC Plant Biol 23(1):385. https://doi.org/10.1186/s12870-023-04402-9

Zaranek M, Pérez-Pérez R, Milewska-Hendel A, Grzebelus E, Betekhtin A (2023b) Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench. Plant Cell Tiss Organ Cult 154(3):673–687. https://doi.org/10.1007/s11240-023-02542-2

Zhang L-L, He Y, Sheng F, Hu Y-F, Song Y, Li W, Chen J, Zhang J, Zou L (2021) Towards a better understanding of *Fagopyrum dibotrys*: a systematic review. Chin Med 16(1):89. https://doi.org/10.1186/s13020-021-00498-z

9.4. Improvement of *F. tataricum* regeneration efficiency using adsorbent and phenolic compounds inhibitors.

Publication P4:

Pérez-Pérez, R.*, Piński, A.*, Zaranek, M., Beckmann, M., Mur, L., Nowak, K., Rojek-Jelonek, M., Kostecka-Gugała, A., Petryszak, P., Grzebelus, E., Betekhtin, A.

Effect of potent inhibitors of phenylalanine ammonia-lyase and PVP on *in vitro* morphogenesis of *Fagopyrum tataricum*.

equal contribution

BMC Plant Biology **2025,** 25, 469

https://doi.org/10.1186/s12870-025-06440-x

IF$_{2023}$: 4.3

Ministry of Science and Higher Education points: 140

In this research, a detailed analysis of the response of protoplast culture (Figure 1) and MC regeneration (Figure 2) of *F. tataricum* to the addition of phenolic compound inhibitors and adsorbents was performed. The protoplasts were isolated from two different lines of MC (Figure 1A, B), embedded in low-melting-point agarose beads and cultivated in BM medium supplemented with 1 mM, 3 mM, 5 mM and 10 mM of AOA, 0.2 µM of AOPP, 10 µM and 50 µM of OBHA, 10 µM and 100 µM of AIP and 1% and 3% of PVP. These additive concentrations were kept in the different used media until plant regeneration. However, during the regeneration of MC, the regeneration medium was supplemented with similar concentrations of AIP and PVP. In both experiments, a medium without additives was taken as the control.

The protoplast culture efficiency ranged from 25% to 44% using most of the additives, except in those cultures supplemented with AOA (all tested concentrations) and 3% PVP, which inhibited cell division (Table 1). During the callus multiplication stage, calli derived from protoplast culture media containing 0.2 mM AOPP (Figure 1C), 10 µM OBHA (Figure 1D), 10 µM AIP (Figure 1F), and 1% PVP (Figure 1H) developed PECCs. In contrast, tissues from treatments with 50 µM OBHA (Figure 1E) and 100 µM AIP (Figure 1G) produced callus-like tissue without PECCs. Once on the regeneration medium, somatic embryos were observed on the 10th day of culture (Figure 1I), and multiple shoots developed after two weeks (Figure 1J). Finally, within two months, these structures regenerated into whole plants (Figure 1K).

Based on relative DNA content determined by flow cytometry, diploid and tetraploid plants were identified. In the 0.2 mM AOPP variant, 25% of the regenerated plants were tetraploid, while 75% were diploid. In the medium supplemented with 10 μM OBHA, equal amounts of tetraploids and diploids were identified (Table 1, Supplementary Figure S3), and media supplemented with 10 μM AIP and 1% PVP regenerated exclusively diploid plants (Table 1).

Regarding plant regeneration from MC, significant differences were observed in the appearance and mass of the calli after seven days on regeneration media supplemented with AIP and PVP (Figure 2). The control calli, grown on medium without additives, were the largest and heaviest, showing robust somatic embryo development, although they also presented extensive browning and reddish discolouration (Figure 2B, H, M). In contrast, the PVP-treated calli showed brown or reddish spots and developed abundant somatic embryos, but their size and mass varied significantly compared to the control (Figure 2C, D, I, J, M). The AIP-treated calli appeared greener and larger than the PVP treatment, although its mass was lower than the control (Figure 2E, F, K, L, M). Flow cytometry analysis confirmed that all plants regenerated on media supplemented with PVP or AIP were diploid (Table 2, Supplementary Figure S4). After 20 days on the rooting medium, treatment with PVP resulted in poor shooting and tissue browning (Figure 2O, P), whereas 100 μM AIP showed improved shoot and root development (Figure 2Q, R). The highest levels of total phenolics were observed in PVP-treated calli, with a 65% increase over the control, while AIP treatment showed a 33% reduction (Figure 3A). The total flavonoid content accounted for 1 – 7% of the total phenolics, showing the lowest concentrations in both AIP treatments with approximately an 80% decrease compared to the control (Figure 3B).

Untargeted metabolomics revealed significant global metabolic changes in morphogenic callus cultures treated with PVP and AIP. Principal component analysis (PCA) showed a clear separation between control without additives and treated groups, with PC1 explaining 37.1% of the variance and PC2 14.4%, highlighting a dose-dependent response stronger for AIP than PVP (Figure 3C). Around 15,328 significantly altered mass-ions, including key metabolites linked to phenylalanine metabolism, were identified (Figure 3D, E, F****). Notably, melatonin was uniquely detected in AIP-treated callus, while glutathione remained stable across the treatments except for a slight decrease under 3% PVP. In response to PVP, oxidised glutathione

******** In Figure 3 of the P4 published version, the x-axis labels of the graphs (panels D, E, and F) incorrectly represent the experimental conditions. Two treatments were labelled as "1% PVP" and two as "10 μM AIP", whereas the correct order in all panels should be: Control, 1% PVP, 3% PVP, 10 μM AIP, 100 μM AIP. However, the results and conclusions remain unaffected.

decreased, whereas AIP caused a more pronounced dose-dependent reduction, resulting in a higher glutathione/oxidised glutathione ratio (Figure 3E, F). This suggests reduced oxidative stress within the cells, particularly in the AIP-treated callus, where the demand for glutathione oxidation was lower. A high glutathione/oxidised glutathione ratio is often associated with rapid cell growth.

On the other hand, HPLC profiling revealed dramatic reductions in polyphenolic synthesis, where *trans*-cinnamic acid dropped from 18.76 µg/g of dry mass (control) to 0.48 µg/g (100 µM AIP). Most of the analysed compounds reached their peak levels in control, particularly rutin (4185.98 ± 151.61 µg/g), (–)-epicatechin (5674.62 ± 260.04 µg/g), and (+)-catechin (2311.51 ± 476.45 µg/g). In contrast, AIP treatments led to the lowest concentrations, particularly at 100 µM, where rutin levels fell to 21.97 ± 1 µg/g, (–)-epicatechin to 80.46 ± 1.45 µg/g, and (+)-catechin to 6.66 ± 0.76 µg/g, demonstrating a potent inhibitory effect on polyphenolic synthesis. PVP treatments showed reductions in rutin and chlorogenic acid but increased the synthesis of small phenolic acids like syringic, ferulic, and sinapic acids. Collectively, AIP strongly inhibited polyphenolic synthesis, while PVP exhibited milder compound-specific effects (Supplementary Material 1; Supplementary Material 2).

LEC1, *LEC2*, *BBM*, *WUS*, *CLV3*, and *FUS3* gene expression revealed differential expressions in response to PVP and AIP treatments compared to the control callus. The *LEC2* and *BBM* expressions increased approximately 2-fold and 3.3-fold higher in both AIP treatments and 3% PVP compared to the control and 1% PVP. *FUS3* expression was highest in 3% PVP, reaching 3.1-fold higher than the control, but was lower in both 1% PVP and 100 µM AIP, with 2.7-fold and 3.7-fold decreases, respectively. *WUS* expression increased in AIP treatments approximately 2.4-fold compared to the control, while *LEC1* and *CLV3* expressions remained stable across treatments (Figure 4A). Finally, in the gene expression analysis of the phenylalanine ammonia-lyase genes (*PAL1* and *PAL2*), a significant increase in *PAL1* expression was observed in response to treatment with 100 µM AIP. At the same time, *PAL2* showed a drastic decrease in both PVP treatments (Figure 4B). In summary, this study shows the significant impact of the phenolic compounds inhibitors and adsorbents on the viability, development and metabolism of *in vitro* cultures derived from *F. tataricum*. AIP treatments showed a more pronounced effect in reducing phenolic levels and modulating gene expression, suggesting an important role of these compounds in promoting protoplast development and regulating somatic embryogenesis and secondary metabolism in this species.

Supplementary material available online

Supplementary Material 1: https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-025-06440-x/MediaObjects/12870_2025_6440_MOESM2_ESM.pdf

Supplementary Material 2: https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-025-06440-x/MediaObjects/12870_2025_6440_MOESM2_ESM.pdf

BMC Plant Biology

RESEARCH Open Access

Effect of potent inhibitors of phenylalanine ammonia-lyase and PVP on in vitro morphogenesis of *Fagopyrum tataricum*

Reneé Pérez-Pérez[1†], Artur Pinski[1*†], Magdalena Zaranek[1], Manfred Beckmann[2], Luis A. J. Mur[2], Katarzyna Nowak[1], Magdalena Rojek-Jelonek[1], Anna Kostecka-Gugała[3], Przemysław Petryszak[3], Ewa Grzebelus[3] and Alexander Betekhtin[1*]

Abstract

Background *Fagopyrum tataricum* (Tartary buckwheat) is known for its high phenolic content, particularly rutin. High concentrations of these compounds secreted in the tissue culture medium can lead to its darkening and the eventual death of explants in in vitro cultures. This study aims to enhance the morphogenesis of *F. tataricum* callus cultures by utilising phenylalanine ammonia-lyase (PAL) inhibitors and polyvinylpyrrolidone (PVP) to mitigate oxidative browning and improve tissue viability.

Results We analysed the response of protoplasts isolated from morphogenic callus to media supplemented with varying concentrations of PAL inhibitors (AIP, AOPP, OBHA) and PVP. The flow cytometry results revealed that 10 µM AIP and 1% PVP yielded exclusively diploid plants, whereas higher concentrations (100 µM AIP and 3% PVP) failed to regenerate plants. Moreover, AOPP and OBHA addition resulted in the regeneration of tetraploid plants. Further analysis of proembryogenic cell complexes (PECCs) isolated from Tartary buckwheat morphogenic calli responses to AIP and PVP indicated that 100 µM AIP was most effective for plant regeneration. Metabolomic analysis showed that AIP treatments reduced phenolic compounds, notably rutin, and increased the GSH/GSSG ratio, indicating reduced oxidative stress. Gene expression analysis highlighted elevated expression of somatic embryogenesis-related genes (*LEC2*, *BBM*) and *WUSCHEL* in AIP-treated callus.

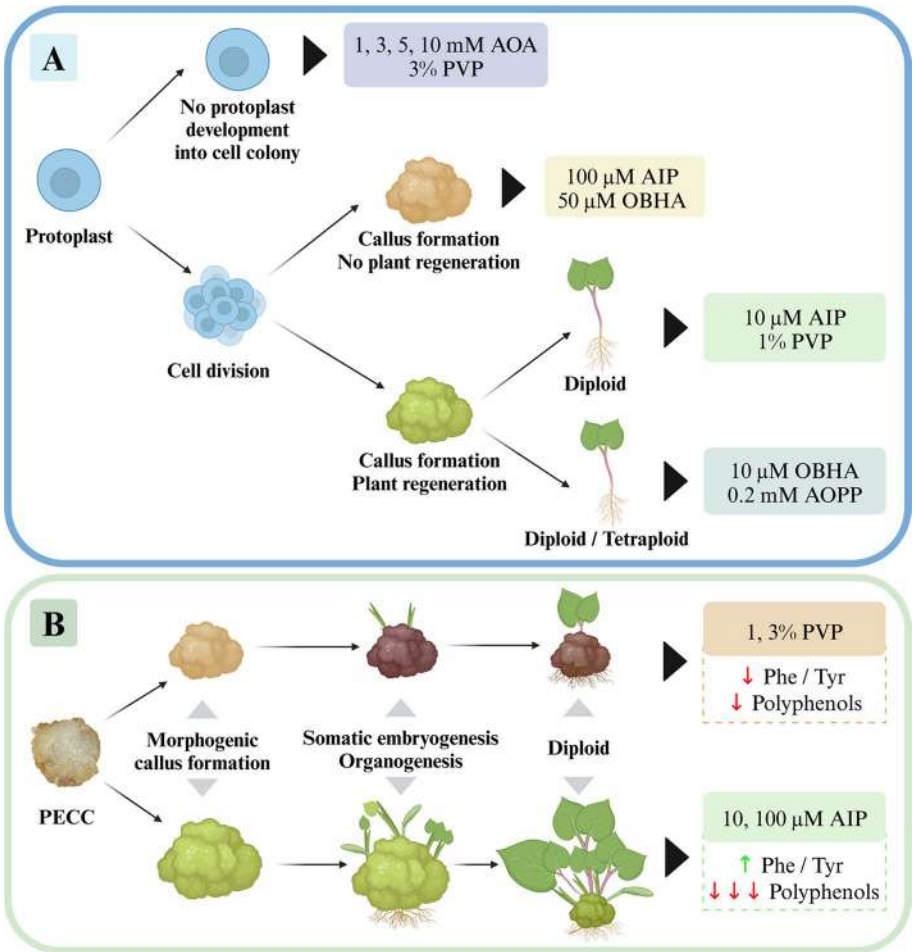
Conclusions This study demonstrates that AIP enhances the regeneration potential of *F. tataricum* callus cultures, offering valuable insights for optimising tissue culture techniques for industrial crops. Additionally, we have detailed the metabolomic changes in calli treated with PVP and AIP, highlighting their impact on metabolism.

Keywords 2-aminoindan- 2-phosphonic acid (AIP), Fagopyrum, In vitro cultures, Polyvinylpyrrolidone, Phenylalanine ammonia-lyase, Lyase

[†]Reneé Pérez-Pérez and Artur Pinski contributed equally to this work.

*Correspondence:
Artur Pinski
artur.pinski@us.edu.pl
Alexander Betekhtin
alexander.betekhtin@us.edu.pl
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

The genus *Fagopyrum*, commonly known as buckwheat, comprises several species, among which *Fagopyrum esculentum* Moench. (common buckwheat) and *Fagopyrum tataricum* (Tartary buckwheat) are the most cultivated. Both species are known for their nutritional value, particularly high content of proteins, fibers, vitamins, and phenolic compounds such as flavonoids [32]. The major flavonoid present in buckwheat is rutin, with a concentration more than 100-fold higher in Tartary, compared to the common buckwheat [38]. Generally, the production of phenolic compounds in Tartary buckwheat is considerably higher [59] and their levels depend on the stage of development, or cultivar [28].

The improvement of Tartary buckwheat callus cultures is important in developing agronomically relevant traits, as they allow for efficient *Agrobacterium*-mediated transformation, protoplast isolation and subsequent plant regeneration [54, 65]. Calli induced from immature embryos of Tartary buckwheat can regenerate entire plants via morphogenesis (morphogenic callus, MC), which occurs either through somatic embryogenesis (formation of somatic embryos) or organogenesis (in the case of Tartary buckwheat only shoots). These calli exhibit genetic stability, crucial for effective propagation, and retain the morphogenic potential over prolonged periods of culture despite possible changes in their morphology [5, 64]. Tartary buckwheat MC consists of proembryogenic cell complexes (PECCs) and a 'soft' callus. According to histological studies, PECCs are embryos arrested at the preglobular stage by supplementing the culture medium with auxin. The PECCs contain phenolic-containing, meristematic and parenchymatous cells. During the cyclical

callus development, the mature PECCs disintegrate, giving rise to young PECCs and 'soft' callus cells. Phenolic-containing cells separate from the PECCs and, as they become senescent, accumulate phenolics until the disruption of the tonoplast leads to their release in the cell and the culture medium. The 'soft' callus is formed during the loosening of the PECCs and consists of elongated cells that are highly vacuolated, metabolically active, but unable to divide; and whose function is to support the growth of the PECCs by providing sugars, proteins, and other conditioning factors. Due to their high phenolic content, Tartary buckwheat callus cultures are prone to browning. This phenomenon leads to medium darkening and can subsequently result in the death of the explants, thus limiting the success of in vitro plant genetic manipulation and multiplication [5].

The biosynthesis of phenolic compounds is controlled by a key enzyme, phenylalanine ammonia-lyase (PAL), that catalyses the deamination of phenylalanine to trans-cinnamic acid [23, 35]. Inhibitors of PAL like α-aminooxyacetic acid (AOA), α-aminooxy-β-phenylpropionic acid (AOPP), 2-aminoindan-2-phosphonic acid (AIP), O-benzylhydroxylamine hydrochloride (OBHA) have been shown to reduce the production of phenylpropanoids thus limiting the cell wall lignification and increase the susceptibility of plants to pathogens [57]. Cinnamic acid and following *p*-coumaric acid are the precursors for various phenolics that are substrates for the polyphenol oxidase (PPO) and peroxidase (POD) [12]. These enzymes catalyse the oxidation of phenolics to quinones, which undergo non-enzymatic polymerisation with amino acids and proteins forming brown or black pigments known as melanins [34], resulting in oxidative browning [6, 52]. The melanins disrupt tissue metabolism, inhibit growth, and can lead to the death of the explants [24, 64]. The *p*-coumaric acid can also be produced by tyrosine ammonia-lyase (TAL) by deamination of tyrosine [63]. However, the activity of TAL is usually much lower in most plants compared to PAL [15]. Therefore, any disruption in PAL activity typically results in noticeable phenotypic changes, including decreased levels of phenolic compounds and associated changes in plant growth, development, and stress responses [8, 15].

The most common strategy to avoid tissue damage by phenolic accumulation is frequent transference of the explants to a fresh medium [40]. Other approaches include adjusting growth conditions, pre-treating explants, or supplementing the culture media with specific additives [31, 39, 46]. Most of those additives can be grouped into three categories: i) antioxidants, which is attributed to the capacity of the phenolics to inactivate free radicals, donating hydrogen atoms or electrons (i.e. ascorbic acid, citric acid, glutathione) [21, 56]; ii) adsorbents, that bind phenolics due to different kinds of interactions like hydrogen bonding and π-π interactions, as well as hydrophobic interactions (i.e. activated charcoal, PVP, PVPP) [33, 41], and iii) inhibitors, which work by targeting and neutralising the enzymes or intermediates involved in the browning process (i.e. tropolone and cysteine – PPO inhibitors, salicylhydroxamic acid – POD inhibitor, phosphonic analogues of phenylglycine – PAL inhibitors) [22, 48, 58].

Polyvinylpyrrolidone (PVP) was the first polymer used against oxidative browning and nowadays remains the first option in the tissue culture [9]. The repeating unit of PVP is a five-membered lactam ring (pyrrolidone) attached to a vinyl group. Phenolic compounds bind to PVP primarily through hydrogen bonding but can also occur by hydrophobic interactions [3]. Hydrogen bonding between the carbonyl group of the pyrrolidone ring and the multiple hydroxyl groups of phenolic compounds is particularly strong [40]. PVP can also interact with amino acids, proteins, and other secondary metabolites, but its affinity for those compounds is generally lower than phenolic compounds [51]. In in vitro cultures, PVP has been shown to reduce phenolic compounds and promote callus regeneration in tree peony. Transcription factors such as MYB, bHLH, and WRKY were shown to play a role in callus browning as indicated by transcriptome analysis [17]. Further analysis indicated that the combined use of activated carbon and PVP promotes rooting development by enhancing the expression of genes related to cytochrome P450 and phenylpropanoid biosynthesis [11]. However, PVP can also negatively impact the growth of tissue cultures, necessitating a more targeted approach, such as the use of inhibitors. AIP is a synthetic phosphonic analogue of phenylglycine acting as a PAL inhibitor [24, 27]. The indan ring in AIP resembles the aromatic ring of phenylalanine, fitting into the active site of PAL. Alternatively, the phosphonic acid group mimics the carboxyl group of the amino acid but is more electronegative and forms strong hydrogen bonds and ionic interactions with the active site residues, creating a more stable and less likely to dissociate bound complex than the natural substrate [58]. The AIP–PAL binding is often considered irreversible [4] and AIP significantly reduces browning and enhances the viability of callus cultures [23, 29, 37]. Besides the in vitro cultures, the application of AIP in rice plants reduced infection by the root-knot nematode *Meloidogyne graminicola*. Transcriptomic and metabolomic analyses indicated that AIP activates the phenylpropanoid pathway, thereby enhancing defence in rice through the activation of jasmonate-mediated defence mechanisms [36]. In *A. thaliana*

plants, reactive oxygen species regulate fumonisin B1 (FB1)-induced cell death with AIP found to inhibit FB1-induced lesion formation and prevent the increase in salicylic acid levels [61].

This study aimed to use protoplast-derived cultures as a model system to evaluate the full range of PAL inhibitors and phenolic adsorbents by assessing their effects on plating efficiency (the number of cell colonies in one-week-old protoplast-derived cultures) and regenerative capabilities. We assessed the structural, genetic, and metabolic impacts of the AIP inhibitor and adsorbent PVP to establish the most valid approach to improved Tartary buckwheat callus culture.

Materials and methods

Plant material

The MC derived from an immature zygotic embryo of *F. tataricum* (L.) Gaertn was used to assess PAL inhibitors and phenolic adsorbent effects on protoplast-derived cultures' plating efficiency and regenerative capabilities. The callus was maintained in the dark at 25 ± 1 °C on RX medium [5, 45] (Supplementary Table S1, sheet 1).

Protoplast-derived cultures and plant regeneration

Protoplasts were isolated from two lines of *F. tataricum* MC, 12-day-old line L1 (Fig. 1A) and 7-day-old line L2 (Fig. 1B) according to the protocol established by

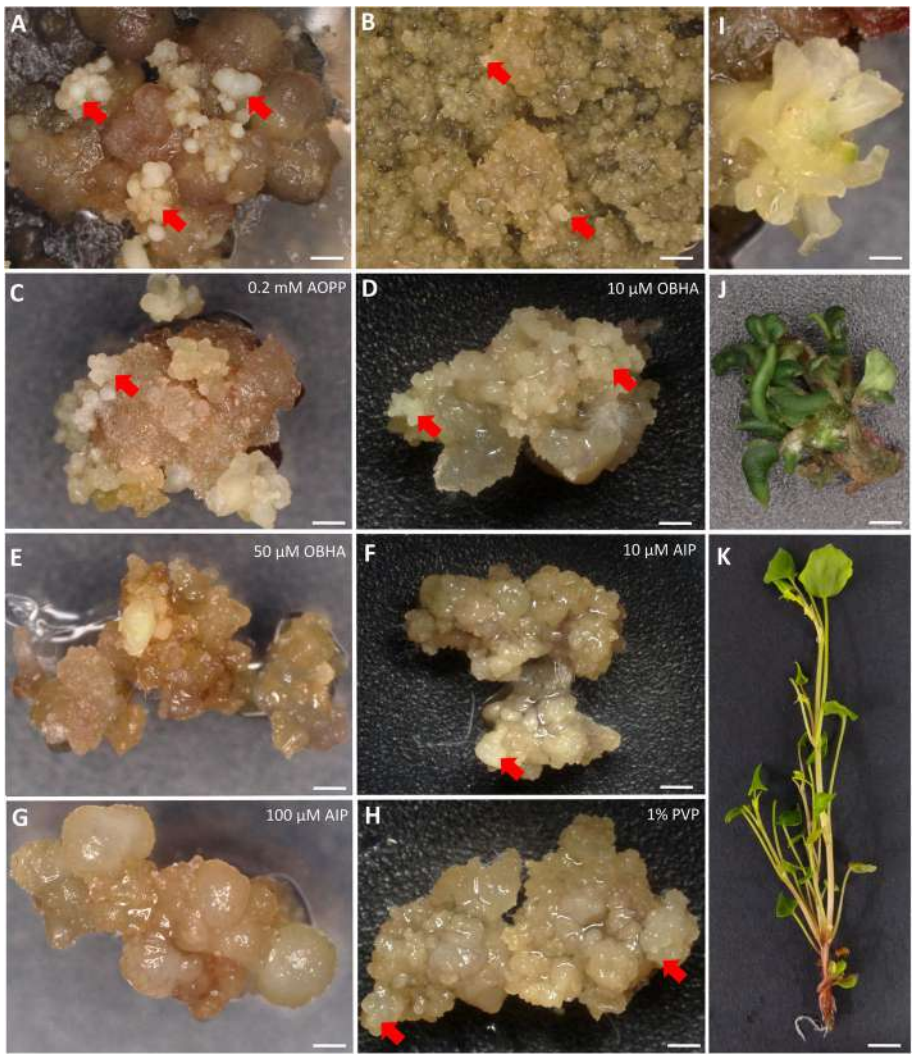


Fig. 1 Lines of morphogenic callus (MC) of *F. tataricum* used for protoplast isolation: L1 (**A**), L2 (**B**). Callus obtained from MC-derived protoplast cultures, protoplast-derived cultures variant: 0.2 mM AOPP (**C**), 10 µM OBHA (**D**), 50 µM OBHA (**E**), 10 µM AIP (**F**), 100 µM AIP (**G**), 1% PVP (**H**). Somatic embryos (**I**), multiple shoots (**J**) and protoplast-derived plants (**K**). Red arrows indicate pro-embryogenic cell complexes. Scale bars: 1 mm (**A**–**H**), 5 mm (**I**–**K**). Abbreviations: AOPP – α -aminooxy-β-phenylpropionic acid; OBHA – O-benzylhydroxylamine hydrochloride; AIP – 2-aminoindan- 2-phosphonic acid; PVP – polyvinyl pyrrolidone

Zaranek et al. [64]. Protoplasts were cultured on basal medium supplemented with 100 nM phytosulfokine-α (PeptaNova GmbH) and different concentrations of phenolics inhibitors: 1 mM, 3 mM, 5 mM, 10 mM of AOA; 0.2 mM of AOPP; 10 µM or 50 µM of OBHA; 10 µM or 100 µM of AIP; and 1% or 3% of the adsorbent PVP. The mechanism of action of AIP and PVP are shown in Supplementary Figures S1 and S2. After 10 days of culture, the medium, with additional supplements, was renewed. Agarose beads overgrown by microcalli were transferred to a callus multiplication medium (CM) [64] enriched with the same additives as in the protoplast-derived cultures. Each variant of the callus culture was maintained at 26 ±1 °C in the dark and subcultured every three weeks. After callus multiplication on the CM medium, the callus was transferred to the RX medium dedicated to *Fagopyrum* cultures. Once PECCs formed on the callus, they were transferred to the regeneration medium (Supplementary Table S1, sheet 1) and subcultured every two weeks. Shoot-like structures were transferred to a rooting medium without plant growth regulators [64].

According to Grzebelus et al. [18], protoplast viability was estimated just after their isolation as the number of protoplasts with apple-green fluorescence per total number of calculated cells (× 100). Plating efficiency was estimated in 10-day-old protoplast-derived cultures and expressed as the number of cell colonies after cell mitotic divisions per total number of observed undivided cells and cell colonies. Microscopic observations were conducted using an Olympus IX81 inverted fluorescence microscope equipped with a filter set suitable for FDA detection (λ_{Ex} = 475 nm, λ_{Em} = 530 nm). Three independent protoplast isolations were performed, with each treatment represented by three Petri dishes, each of which was considered to be a biological replicate. Observations were made on 100–150 cells per Petri dish. Mean values and standard errors were calculated, and the data were analysed using one-way analysis of variance (ANOVA) with Statistica 13 (TIBCO Software Inc.). Tukey's *post-hoc* test was applied to identify significant differences between the means.

Flow cytometry of protoplast-derived plants

Flow cytometry was used to determine the ploidy level of the regenerated *Fagopyrum* plants (Table 1). Fresh and vital leaves from the regenerants and the internal standard *Solanum pseudocapsicum* (2 C DNA = 2,59 pg) [53], were chopped together in a Petri dish in 400 µL of a nuclei extraction buffer (CyStain UV Pr, 05–5002, Sysmex) using a razor blade. The nuclei suspension was filtered through a 30 µm mesh (CellTrics, Sysmex) and stained with DAPI (CyStain UV Precise P, 05–5002, Sysmex) according to the manufacturer's instructions. The samples were incubated for 5 min in the dark and then analysed using a flow cytometer (CyFlow Space, Sysmex) equipped with 365 nm UV LED as the light source. 4000 nuclei were analysed for each sample.

Table 1 Regeneration ability of protoplast-derived callus of *F. tataricum* on culture media supplemented with phenolic inhibitors (AOP, AOPP, OBHA, AIP) and adsorbent (PVP) and ploidy level of regenerated plants

Medium variant	Concentration	Plating efficiency (%) (Mean ± SE)	Regeneration ability	Ploidy level*
Control		33.8 ± 14.6^{abc}	+	diploid
AOA	1 mM	–	–	–
	3 mM	–	–	–
	5 mM	–	–	–
	10 mM	–	–	–
AOPP	0.2 mM	25.2 ± 8.6^{a}	+	tetraploid (25%) diploid (75%)
OBHA	10 µM	44.0 ± 6.8^{c}	+	tetraploid (50%) diploid (50%)
	50 µM	27.9 ± 7.8^{ab}	–	–
AIP	10 µM	40.5 ± 3.5^{bc}	+	diploid
	100 µM	30.0 ± 1.5^{ab}	–	–
PVP	1%	33.3 ± 2.2^{abc}	+	diploid
	3%	–	–	–

^{abc} Means followed by the same letters were not significantly different at $p \leq 0.05$

– not able to regenerate, no obtained plants

* Based on relative DNA content from flow cytometry

Effect of PAL inhibitors and phenolics adsorbents on callus regeneration

A MC obtained from the immature zygotic embryo of *F. tataricum* was used to evaluate the impact of AIP and PVP additives. For plant regeneration, PECCs from the surface of *F. tataricum* callus were transferred onto the regeneration medium (Supplementary Table S1, sheet 1) supplemented with PVP (1 or 3%) or AIP (10 or 100 µM), while medium without any additive was used as control. The PECCs were incubated for seven days in a light chamber at 28 ± 2 °C, 16/8 h (light/dark) photoperiod, and 55 µmol m^{-2} s^{-1} light intensity. Due to the small size of an individual PECC, the mass was determined by grouping ten PECCs, and then the average mass was calculated. On day zero and after seven days on the regeneration medium, the callus derived from the PECCs was individually weighed. The statistical differences were analysed using Statistica ver. 14.0.0.15, using one-way Analysis of Variance (ANOVA) and a *p*-value of < 0.05. Seven-day-old callus of *F. tataricum* cultured on regeneration medium supplemented with PVP (1 or 3%) or AIP (10 or 100 µM) were used for total phenolics analyses, metabolomics, and gene expression analysis.

The calli were kept on the regeneration medium for three weeks, and then the regenerants were transferred onto the rooting medium (Supplementary Table S1, sheet 1), maintaining the same concentration of additives.

Total phenolics and total flavonoids analyses

Total phenolic and total flavonoid contents in the 7-day-old callus of *F. tataricum* were determined spectrophotometrically using the Folin–Ciocâlteu and Aluminium Chloride colourimetric methods respectively [10, 50]. Around 100 mg of tissue was homogenised in a mortar with liquid nitrogen, resuspended in 2 ml cold 95% MeOH, and incubated for 24 h in the dark at room temperature (RT). The samples were centrifuged at 13,000 g for 5 min at RT, twice. For total phenolics analysis, 100 µl of the supernatant was dissolved in 200 µl of Folin–Ciocâlteu reagent and 800 µl of 700 mM of Na_2CO_3. This mixture was incubated for 2 h at RT, centrifuged at 13,000 g for 5 min, and the absorbance was measured at 765 nm in an Eppendorf BioSpectrometer®. Gallic acid was used as the standard, and the results were expressed as millimolar of gallic acid (GA). For total flavonoids analysis, 600 µl of the supernatant was dissolved in 600 µl of $AlCl_3$ and incubated for 1 h at RT. The absorbance was measured at 420 nm. Quercetin was used as the standard and the results were expressed as millimolar of quercetin (Q). The data presented are an average of five measurements per treatment.

Metabolomics

Around 100 mg of 7-day-old callus cultured on different variants of regeneration medium were frozen, and metabolites from grounded material were extracted using a single-phase extraction solution (chloroform/methanol/water, 1:3:1, *v*/*v*/*v*). Frozen samples were homogenised and mixed with 1 mL of the extraction solution for 30 min at 4 °C. Further, the samples were centrifuged for 1 min at 4 °C and 200 µL of sample were lyophilised and resuspended in 50% (*v*/*v*) methanol for further analysis. Metabolite fingerprinting was performed by FIE-HRMS using a Q Exactive Plus Hybrid Quadrupole Orbitrap Mass Analyser with an Acella UHPLC system (Thermo Fisher Scientific). The *m*/*z* (mass-ion) values were generated in both positive and negative ionisation modes, as described by Baptista et al. 2018. The obtained data are provided in Supplementary Table S1 (sheets 2 and 3). Multivariate analysis was performed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml). Data were log_{10}-transformed, and Pareto scaled to the total ion count. The significance of the cross-validated *p*-values, based on the one-way analysis of variance (ANOVA), was set to $p < 0.05$. The multiple comparison and post hoc analysis used Fisher's LSD. Identification was based on the MS peaks to pathway algorithm (tolerance = 5 ppm, reference library; *Gossypium hirsutum*).

Chromatography

Extracts were prepared by vigorously vortexing ~ 10 mg of freeze-dried ground callus (cultivated for seven days on various regeneration media) in 1 ml of 80% methanol for 30 min at room temperature. The homogenates were then centrifuged (15 000 × *g*, 10 min, 4 °C) to remove callus debris. The resulting supernatants were stored in the dark at −20 °C.

Phenolic compounds (except gallic acid) were identified using high-performance liquid chromatography (HPLC). For that, Shimadzu LC–10 AS chromatograph equipped with a C18 RP chromatographic column (LiChrospher® 100 RP- 18, 250–4, 5 µm, Merck) and SPD- 10 AV UV–Vis detector was used. The signal detection was set at the wavelengths of 265 and 325 nm. Chromatographic separation was carried out at 30 ± 1 °C using the following solvents: (A) water with acetic acid (0.1%), (B) methanol with acetic acid (0.1%) and applying the gradient: 90% A, 10% B for 20 min; 75% A, 25% B for 30 min; 65% A, 35% B for 40 min; 55% A, 54% B for 50 min; 50% A, 50% B for 60 min; 30% A, 70% B for 62 min; 100% B to 80 min; 80% A, 10% B up to 85 min. The flow rate was 1 mL/min. The analyses were performed in a minimum of four replicates for each callus and analytical standard sample.

A Shimadzu LC–20 AD chromatograph (HPLC) equipped with a C18 RP chromatographic column (Purospher® STAR, 5 µm, Merck) and the Shimadzu SPDM–20 A–DAD photodiode–array detector was used to identify gallic acid. The signal detection was set at the wavelengths of 265 and 325 nm. Chromatographic separation of calli extracts and gallic acid were performed at 33 °C using a mixture of solvents: (A) 10% methanol in water with acetic acid (0.1%), (B) 50% methanol in water with acetic acid (0.1%), and (C) 100% methanol with acetic acid (0.1%). The solvent gradient conditions were as follows: 100% A for 20 min, then increasing the concentration of solvent B to 100% at 55 min of separation, and solvent C concentration to 100% at 75 min of separation (kept until 81 min). The final step was increasing the concentration of solvent A to 100% at 90 min of separation. The analyses were performed in a minimum of three replicates for each callus and the standard sample.

The identification of phenolics was based on the retention times of analytical standards of syringic acid (ChromaDex), sinapic acid (Fluka), chlorogenic acid, *trans*-cinnamic acid, caffeic acid, ferulic acid (ICN Biomedicals), gallic acid, vanillic acid, (+)-catechin, (–)-epicatechin, rutin, and quercetin (Sigma – Aldrich). The standards were solved in 80% methanol and kept in the dark at –20 °C. Water and methanol were of HPLC gradient grade purity (Sigma–Aldrich, Chromasolv for HPLC) and acetic acid were of analytical grade purity.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The RNA was isolated from 7-day-old callus cultured on different variants of regeneration medium. The callus from media without any additives was used as a control. The RT-qPCR was performed according to the protocol described in Sala-Cholewa et al. [47]. Briefly, the RNA was isolated using FastPure Plant Total TNA Isolation Kit—Polysaccharides and polyphenolics-rich (Vazyme Biotech) and Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and oligo-dT primers were utilised to generate cDNA after removal of DNA from RNA samples. The reactions were performed using a LightCycler® 480 SYBR Green I Master (Roche). The control genes, SAND and ACTIN were used as a reference for the calculation of relative expression level using $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ represents $\Delta C_T^{\text{reference condition}} - \Delta C_T^{\text{compared condition}}$. The two-way ANOVA ($p < 0.05$) followed by Tukey's honestly-significant-difference test (Tukey HSD-test) ($p < 0.05$) was used to identify any significant differences between the experimental combinations. For the primers designee *F. tataricum* "Pinku1" (GWHBJBL00000000) references genome was used (Chinese National Genomics Data Center database, https://bigd.big.ac.cn/) [19].

Results

Protoplast-derived cultures

Protoplasts isolated from two lines of *F. tataricum* showed high viability, ranging between 70–80%, with no significant differences in plating efficiency between the two lines. Plating efficiency across most medium variants ranged from 25–44% (Table 1), except for those supplemented with AOA and 3% of PVP, where no cell division or protoplast culture development was observed. The protoplast medium supplemented with 0.2 mM AOPP displayed a lower (25.2%) plating efficiency than the control (33.8%), while the medium containing 10 µM OBHA resulted in the highest number of cell colonies (44%), although the observed differences were not statically significant.

Upon callus multiplication, PECCs formed in protoplast culture medium variants supplemented with 0.2 mM AOPP (Fig. 1C), 10 µM OBHA (Fig. 1D), 10 µM AIP (Fig. 1F), and 1% PVP (Fig. 1H). However, tissue derived from variants like 50 µM OBHA (Fig. 1E) and 100 µM AIP (Fig. 1G) formed callus-like tissue lacking PECCs. The addition of AOA and 3% PVP resulted in inhibition of protoplast-derived cell division and further development. After ten days on the regeneration medium, somatic embryos (Fig. 1I) were observed. Within two weeks, multiple shoots emerged (Fig. 1J) and after two months, both structures successfully developed into fully matured plants (Fig. 1K). Flow cytometry was used to investigate the effect of phenolic inhibitors on the ploidy of regenerated plants. Obtained data showed a mix of ploidy levels in regenerated plants depending on the treatment. Based on relative DNA content, diploid and tetraploid plants were identified. In the 0.2 mM AOPP variant, 25% of the regenerated plants were tetraploid, while 75% were diploid (Table 1), and for 10 µM OBHA, 50% of the plants were tetraploids (Table 1, Supplementary Figure S3). Media with 10 µM AIP and 1% PVP produced PECCs that regenerated exclusively diploid plants (Table 1).

Macroscopic observations of morphogenesis

After seven days on regeneration media supplemented with phenolic inhibitors/adsorbents, significant differences were observed in the appearance and mass of callus derived from PECCs (Fig. 2A, G, M). The control callus, grown on medium without additives, was the largest and heaviest, demonstrating the development of somatic embryos (Fig. 2B, H, M; somatic embryos marked by red arrows—H). However, it also displayed extensive browning and dark red discolouration. In contrast, callus

supplemented with PVP showed some brownish or dark red spots and developed abundant somatic embryos, but its size and mass varied significantly compared to the control (Fig. 2C, D, J, I, M). AIP-treated callus appeared greener and larger than the PVP-treated one, although its mass was lower than the control (Fig. 2E, F, K, L, M; somatic embryos marked by red arrows—K). Notably, the callus treated with 10 µM AIP had small brownish areas (Fig. 2E, K), while that treated with 100 µM AIP was entirely green and free of discolouration (Fig. 2F, L). After 20 days in the rooting medium, the difference in colouration and shoot and root development remained evident in favour of the treatment with 100 µM of AIP (Fig. 2N-R). In contrast, both treatments with PVP showed poor shoot growth and caused the death of some calli (Fig. 2O, P). In all cases, regeneration primarily occurred via somatic embryogenesis (Fig. 2H–K; somatic embryos marked by red arrows). The flow cytometry analysis showed that all plants regenerated on a rooting medium containing either PVP or AIP were diploids (Table 2, Supplementary Figure S4).

Total phenolics and total flavonoids analyses

Total phenolic and flavonoid concentrations in the calli varied significantly across treatments (Fig. 3A, B). In the case of total phenolics analysis, the control group had 2.2 mM GA per 100 mg of calli. No significant differences were observed between the treatments with the same additive, 1% versus 3% PVP or 10 µM versus 100 µM AIP. However, significant differences were found when comparing different additives. The highest phenolic levels were observed in PVP-treated callus, both showing 3.7 mM GA per 100 mg — a 65% increase over the control. In contrast, AIP-treated calli had 1.5 mM GA, 33% lower than the control (Fig. 3A). The total flavonoid content accounted for 1–7% of the total phenolics in 7-day-old calli. The flavonoids production in the control group represented 6.76% of the total phenolics, while in the treatment with 1% and 3% PVP, and 10 µM and 100 µM AIP, total flavonoids represented 4.2%, 3.67%, 2.13% and 1.25% respectively. No significant differences were observed between the control and both PVP treatments, with flavonoid concentrations ranging from 0.13 to 0.15 mM Q per 100 mg of callus. In contrast, both AIP-treated calli exhibited significantly lower flavonoid concentrations, ranging from 0.018 to 0.031 mM Q per 100 mg of callus, representing an approximately 80% decrease compared to the control (Fig. 3B).

Metabolomics

Untargeted metabolomics revealed global changes in the callus in response to PVP and AIP treatments. Principal component analysis showed clear separations between the control and treated groups, primarily along PC1, accounting for 37.1% of the variation (Fig. 3C). Differences were also observed between the PVP and AIP treatments. Additionally, PC2, which explained 14.4% of the variance, indicated a dose-dependent effect, with higher concentrations of PVP and AIP leading to greater metabolic variation. However, the differences were more pronounced for AIP than for PVP. The sources of variation between treatments were identified using ANOVA ($p < 0.05$) followed by a Fisher's LSD post hoc test, to indicate 15,328 significantly different *m/z* (mass-ions). Key statistically significant metabolites linked to the phenylalanine, the substrate for PAL, were selected (Fig. 3D, E, and F). Specifically, melatonin was detected in the AIP-treated callus but was absent in the other callus samples. Glutathione (GSH) levels remained consistent across treatments, with a slight decrease observed only in the 3% PVP treatment. In contrast, oxidized glutathione (GSSG) showed a slight decrease in response to PVP and a more pronounced, dose-dependent decrease with AIP (Fig. 3E, F).

The untargeted metabolomics analysis was complemented by HPLC profiling of polyphenolics, including rutin, catechin, and epicatechin, along with *trans*-cinnamic acid (Supplementary Figure S5, Supplementary Table S1, sheet 4). *Trans*-cinnamic acid levels decreased significantly across all treatments, with the control showing 18.76 µg/g of dry mass compared to just 0.48 µg/g in the 100 µM AIP treatment. The analysis also revealed significant differences in polyphenolic compound concentrations across treatments. The control consistently exhibited the highest levels of most compounds, particularly rutin (4185.98 ± 151.61 µg/g), (–)-epicatechin (5674.62 ± 260.04 µg/g) and (+)-catechin (2311.51 ± 476.45 µg/g). In contrast, treatments with 1% and 3%

(See figure on next page.)

Fig. 2 Macroscopic observations of *F. tataricum* PECCs and morphogenesis. **A**–**F** General view of the Petri dishes, **G** closer view of PECCs on day zero and **H**–**L** derived calli after seven days of culture on regeneration medium **B**, **H** without supplements (Control) and **C**, **I** supplemented with 1% PVP, **D**, **J** 3% PVP, **E**, **K** 10 µM AIP and **F**, **L** 100 µM AIP. **M** PECCs mass on day zero and callus mass after seven days on the different variants of regeneration medium. **N**–**R** Shoots/shoot-like structures after 20 days on rooting medium **N** without supplements (control) and supplemented with **O** 1% PVP, **P** 3% PVP, **Q** 10 µM AIP and **R** 100 µM AIP. Red arrows indicate somatic embryos. Scale bar: 1 cm (**A**–**F**; **N**—**R**), 1 mm (**G**–**L**). Abbreviations: AIP – 2-aminoindan- 2-phosphonic acid; PECCs – pro-embryogenic cell complexes; PVP – polyvinyl pyrrolidone

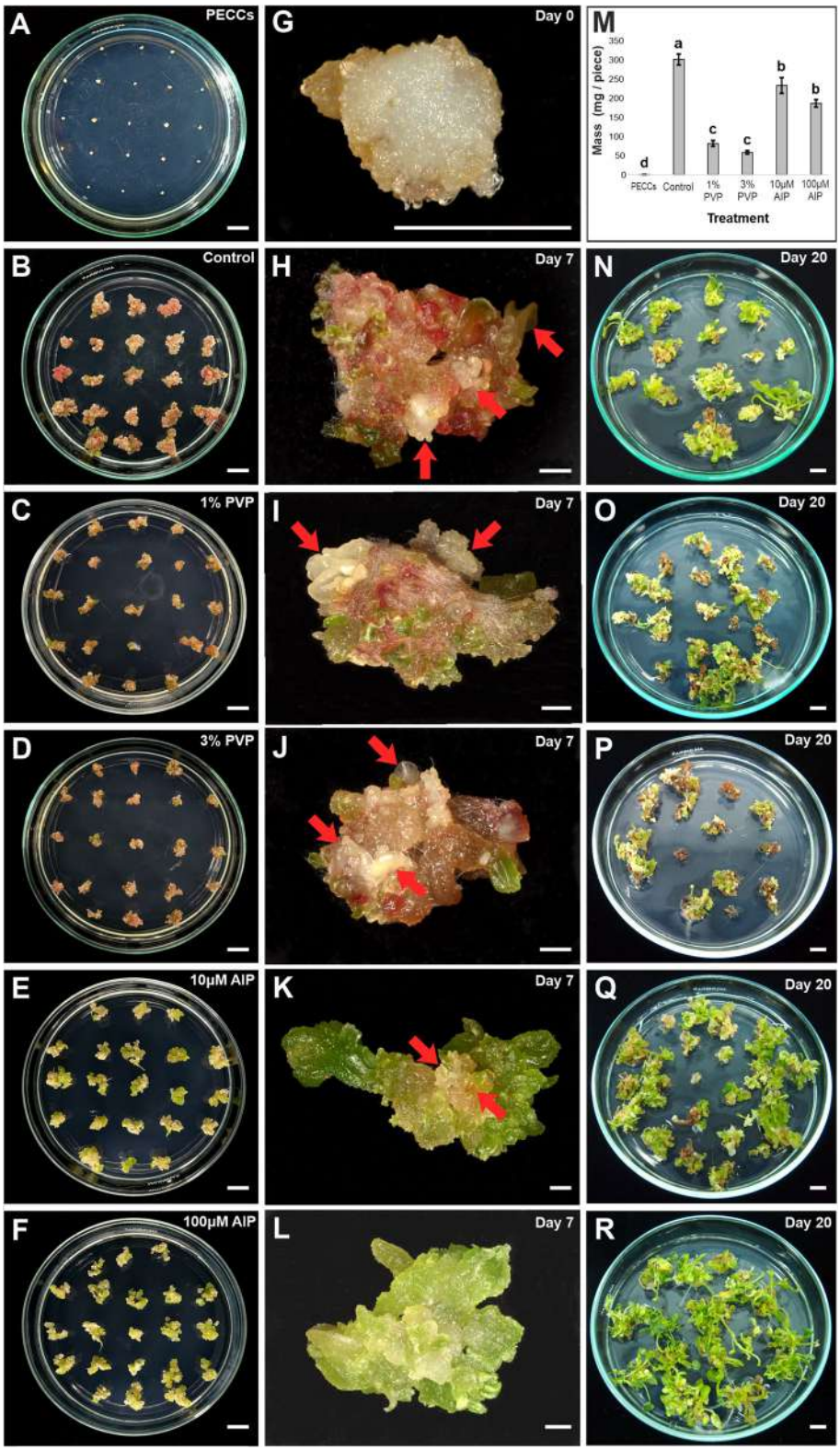


Fig. 2 (See legend on previous page.)

Table 2 The ploidy levels of *F. tataricum* regenerants derived from PECCs, which originated from MC cultivated on regeneration media with or without AIP and PVP

Medium variant	Concentration	Ploidy level[a]
Control		diploid
AIP	10 µM	diploid
	100 µM	diploid
PVP	1%	diploid
	3%	diploid

[a] Based on relative DNA content from flow cytometry

PVP showed reductions in rutin and chlorogenic acid but increased concentrations of syringic, ferulic, and sinapic acids. Notably, AIP treatments resulted in the lowest concentrations for many compounds, especially at 100 µM, where rutin dropped to 21.97 ± 1 µg/g, (−)-epicatechin to 80.46 ± 1.45 µg/g, and (+)-catechin to 6.66 ± 0.76 µg/g, indicating a strong inhibitory effect on polyphenolic synthesis. Overall, PVP enhanced some acids, while AIP significantly decreased overall polyphenolic levels, with 100 µM having a more significant impact.

Gene expression

Among the key factors involved in somatic embryogenesis, the relevant transcription factor-encoding genes include: *LEAFY COTYLEDON 1* (*LEC1*), *LEAFY COTYLEDON 2* (*LEC2*), BABY BOOM (*BBM*), *WUSCHEL* (*WUS*), *CLAVATA* (*CLV3*), and *FUSCA 3* (*FUS3*) [66]. Given that AIP and PVP influence development and regeneration, we aimed to investigate how these treatments affect the expression of these genes. The analysis of *LEC1*, *LEC2*, *BBM*, *WUS*, *CLV3*, and *FUS3* revealed differential expressions in response to PVP and AIP treatments compared to the control callus (Fig. 4A). We observed that *LEC2* expression increased approximately two-fold in both AIP treatments and 3% PVP compared to the control and 1% PVP, while *LEC1* expression remained relatively stable. The highest *BBM* expression was observed in 3% PVP and 10 µM AIP, with levels 3.9 and 3.3-fold higher than control, respectively. *FUS3* expression was highest in 3% PVP, reaching 3.1-fold higher than control, but was lower in both 1% PVP and 100 µM AIP, with 2.7-fold and 3.7-fold decreases, respectively. *WUS* expression was significantly higher in AIP treatments, approximately 2.4 times that of the control, while PVP treatments showed no statistically significant difference compared to the control. *CLV3* expression remained stable across treatments, except for a peak in response to 100 µM AIP, which was 4.8-fold higher than the control.

Given that AIP is an inhibitor of PAL, and PAL is a key rate-limiting enzyme in the phenylpropanoid pathway, we investigated the changes in PAL expression. Although PVP does not directly influence PAL, we decided to explore its potential effects [67]. Five genes encoding PAL have been identified in the *F. tataricum* genome [19]. The analysis of *PAL1 to PAL4* showed that their expression remained largely constant across treatments, except for *PAL1* and *PAL2*. The expression of *PAL1* was significantly higher in response to 100 µM AIP, being 2.7 times higher than the control, while it was three times lower in 1% PVP. *PAL2* showed a sharp decrease in response to PVP, with expression levels being 9.9 and 4.5 times lower in 1% and 3% PVP, respectively. No expression of *PAL5* was detected in the callus.

Discussion

Protoplast-derived cultures and plant regeneration in response to phenolic inhibitors and PVP

The influence of the phenolic inhibitors (AOP, AOPP, OBHA, AIP) and adsorbent (PVP) on protoplast-derived cultures regeneration varied significantly. AIP at 10 µM concentration promoted culture development and enabled diploid plant regeneration, supporting previous findings in *Cannabis sativa* and *Miscanthus*×*giganteus*, where AIP reduced phenolic synthesis and improved tissue quality and regeneration rates [13, 37]. However, regeneration potential was lost at higher AIP concentrations (100 µM), suggesting that excessive phenolic inhibition may negatively affect further cell colony development and regenerating ability. For American elm tissue, a reduction in flavonoid content and tissue browning was observed, along with an improvement in protoplast isolation frequency, faster cell wall reconstruction, and a quicker onset of the first cell division [23]. PVP, at 1%, positively influenced protoplast-derived

(See figure on next page.)

Fig. 3 Changes in the metabolomics in the callus of *F. tataricum* after seven days on regeneration medium supplemented with PVP (1 or 3%) or AIP (10 or 100 µM). **A** Total phenolics concentration expressed in millimolar of gallic acid per 100 mg of tissue (mM GA/100 mg of callus), **B** total flavonoids concentration expressed in millimolar of quercetin per 100 mg of tissue (mM Q/100 mg of callus); **C** Principal component analysis scores plot of metabolome distribution of control, PVP and AIP-treated callus; **D** The violin plots of normalised concentrations of arogenate, phenylalanine, and tyrosine; **E** The heatmap of major differentially accumulated metabolites; **F** The violin plots of normalised concentrations of melatonin, glutathione, and oxidized glutathione. Abbreviations: AIP – 2-aminoindan- 2-phosphonic acid; GA – gallic acid; PVP – polyvinyl pyrrolidone, Q – quercetin

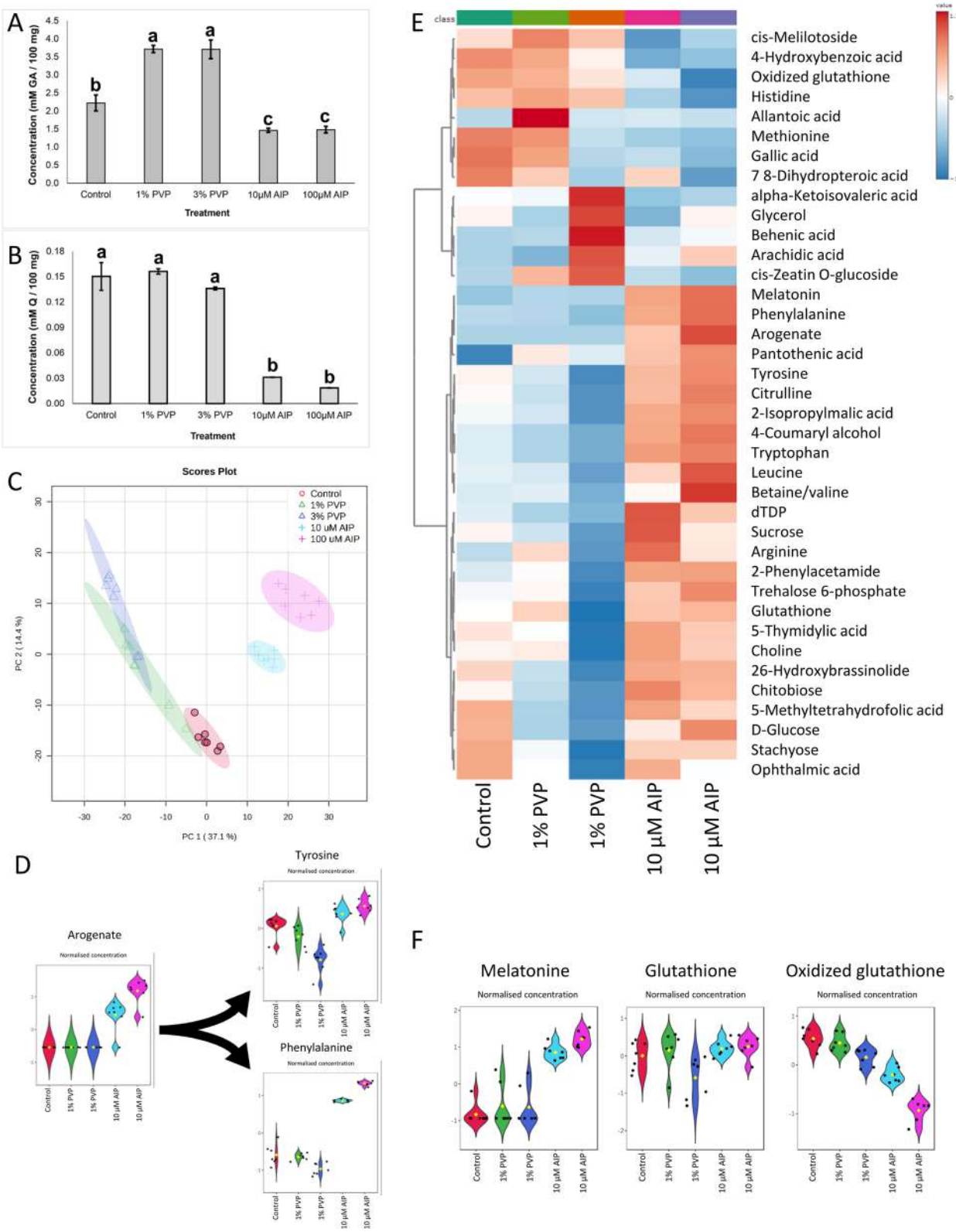


Fig. 3 (See legend on previous page.)

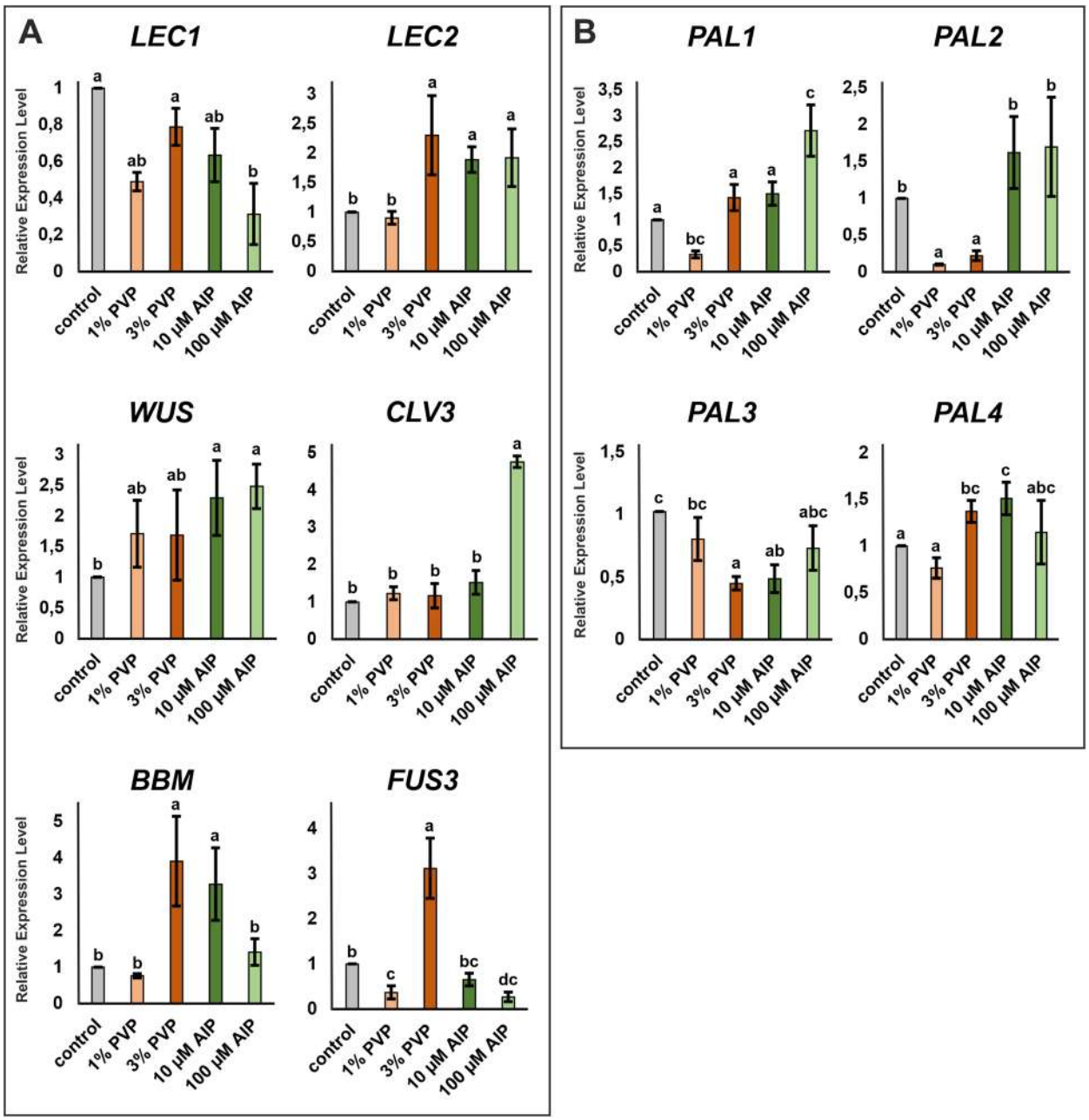


Fig. 4 Expression level of **A** transcription factors *(LEC1, LEC2, FUS3, BBM, WUS* and *CLV3)* and **B** phenylalanine ammonia-lyase *(PAL1, PAL2, PAL3* and *PAL4)* genes in 7-day-old callus cultures on regeneration medium supplemented with PVP (1 or 3%) or AIP (10 or 100 µM). The expression level of genes was calibrated to the expression of reference genes, *ACT* (*actin*) and *SAND* (*SAND DNA-binding protein domain*), and to control *F. tataricum* callus. Different letters indicate a significant difference between treatments according to Tukey's HSD test ($p < 0.05$; $n = 3$; means ± SD are given). Abbreviations: AIP – 2-aminoindane- 2-phosphonic acid; *BBM – BABY BOOM*; *CLV3 – CLAVATA 3*; *FUS3 – FUSCA 3*; *LEC – LEAFY COTYLEDON*, PAL – phenylalanine ammonia-lyase; PVP – polyvinylpyrrolidone; *WUS – WUSCHEL*

cultures regeneration, resulting in regenerated diploid plants, while an increase of PVP to 3% resulted in loss of regeneration potential. Previous studies in grapevine protoplast-derived cultures reported that PVP's adsorptive capacity reduced oxidative stress and supported cell growth [42]. In contrast, the phenolic inhibitor AOA showed no regenerative potential at any tested concentration, likely due to excessive inhibition of phenolic pathways essential for protoplast survival. Interestingly, AOA inhibits PAL activity in hypocotyls of *F. esculentum*

and effectively reduces light-induced anthocyanin formation [20]. Additionally, studies in carnation flowers and tomato plants have shown that AOA inhibits ethylene biosynthesis by blocking the formation of 1-aminocyclopropane- 1-carboxylic acid (ACC), a key ethylene precursor, which may further restrict cell division and regeneration ability [7, 25]. AOPP and OBHA exhibited limited regeneration potential, leading to tetraploid plants forming, which may restrict their application depending on specific tissue culture objectives. Previous studies have shown that AOPP and OBHA effectively inhibit PAL, reducing anthocyanins and lignin content in the cell wall. We could speculate that prolonged exposure to AOPP and OBHA, as seen in protoplast-derived cultures regeneration, may induce polyploidisation, a phenomenon not previously studied in fully grown plants. Notably, AOPP was previously tested for control of the browning of lettuce tissue, aiming to enhance its quality and shelf-life [57]. These findings highlight the importance of optimising compound concentrations to balance phenolic reduction with protoplast viability and successful regeneration of genetically stable plants.

The response of PECCs isolated from Tartary buckwheat morphogenic calli to AIP and PVP

Based on the observed effects of tested inhibitors and adsorbents, we have further investigated the impact of PVP and AIP on plant regeneration from PECCs isolated from Tartary buckwheat morphogenic calli. Both PVP and AIP had distinct effects on *F. tataricum* callus. PVP's role in improving callus regeneration is particularly notable in its capacity to reduce phenolic compound accumulation, which often inhibits tissue growth and viability. In *F. tataricum*, 3% PVP significantly boosted the expression of embryogenesis genes such as *BBM* and *FUS3*, supporting callus morphogenesis and later stages of embryo maturation as *FUS3* is a key regulator of seed storage proteins and other reserved materials [62]. Notably, no protoplast and cell colony development was observed when the medium was supplemented with 3% PVP, suggesting that protoplasts are particularly sensitive to higher concentrations of this adsorbent. A similar sensitivity was observed in the cultures supplemented with 100 µM of AIP. The differing responses to higher concentrations of PVP and AIP could be attributed to the distinct conditions experienced by PECCs and developing protoplasts. While PECCs are cultivated on the surface of the medium, protoplasts remain submerged in the medium containing PVP and AIP, potentially exposing them to higher localised concentrations of these compounds. Previous studies have shown that in sugarcane meristem cultures, 0.03% of PVP (in combination with 0.002% diethyldithiocarbamic acid) resulted in a remarkable increase in regeneration rates and survival, achieving 100% survival and an average of 3.8 shoots per explant [43]. Similarly, in teak, PVP treatment accelerated callus emergence and improved shoot length, showing its positive influence on regeneration [60]. B5 medium combined with PVP supported better callus formation in chestnut compared to MS medium [2]. Conversely, AIP primarily inhibits PAL, reducing phenolic synthesis rather than absorbing existing phenolic compounds. In our study, AIP-treated *F. tataricum* callus exhibited lower phenolic levels and a greener appearance, particularly at a concentration of 100 µM. Our findings on AIP-treated *F. tataricum* callus support this, as they showed upregulation of *WUS*, a key gene in stem cell maintenance, suggesting its potential to promote early-stage callus regeneration by sustaining meristematic activity [26].

AIP and PVP significantly affected callus mass, with a reduction observed in PVP-treated callus and a smaller decrease in AIP-treated callus. It may be linked to the distinct mechanisms of action of these compounds. Overall, PVP reduced the contribution of flavonoids to total phenolics, while AIP led to an even greater decrease. Specifically, metabolomic analyses revealed a pronounced reduction in polyphenols, especially rutin, in AIP treatments, while PVP treatments led to a moderate, dose-dependent reduction. The observed production of polyphenols in AIP-treated callus may be attributed to the activity of TAL, which enables the biosynthesis of *p*-coumaric acid from tyrosine, a compound that was notably increased in the treated callus. This observation is consistent with previous studies showing that AIP presence in *Miscanthus*×*giganteus* reduced phenolic content but was not abolished completely (from 2242.34 µg/g to 1569.71 µg/g of dry weight). Simultaneously, the presence of AIP increased regeneration rates up to 58.3% in combination with specific concentrations of 2,4-D [13]. Concurrently, PVP-treated callus showed decreases in amino acids such as tryptophan, leucine, tyrosine, betaine/valine, and phenylalanine, as well as in saccharides (mono- and disaccharides). The overall reduction in callus mass and amino acid and sugar levels could be attributed to PVP's non-selective binding of nutrients, making them less available to the callus. This could pose a challenge in *F. tataricum* tissue cultures, as PECCs rely on compounds released by the 'soft' callus [5, 44]. The compound often contains hydroxyl groups and thus may be adsorbed by PVP. The reduced nutrient availability seems to decrease *PAL2* expression without affecting other PAL genes. In contrast, AIP-treated callus exhibited a notable accumulation of phenylalanine, a substrate for PAL, with no corresponding increase/decrease in PAL gene expression. As AIP is a potent competitive PAL inhibitor, the buildup of phenylalanine without increased PAL activity

likely explains the observed effects. Simultaneously, we detected arogenate exclusively in AIP-treated callus, with levels increasing in a dose-dependent manner. In plants, phenylalanine is synthesised from prephenate (part of the shikimate-chorismate pathway), which can be converted into phenylalanine via either phenylpyruvate with subsequent transamination or via arogenate through transamination of prephenate followed by dehydration and decarboxylation. Arogenate can also serve as a precursor for tyrosine synthesis [14]. The observed increase in phenylalanine may have led to a downstream build-up of arogenate, potentially redirecting metabolism toward increased tyrosine biosynthesis. Interestingly, in AIP-treated callus, levels of other amino acids like leucine, tryptophan, and betaine/valine were upregulated, while histidine and methionine levels were downregulated. According to HPLC analysis, cinnamic acid, a product of PAL activity, was significantly reduced in both the PVP and AIP treatments. However, AIP proved to be more effective in decreasing cinnamic acid levels.

We also observed changes in the levels of GSH and GSSG in the treated callus, resulting in a higher GSH/GSSG ratio. This suggests reduced oxidative stress within the cells, particularly in the AIP-treated callus, where the demand for glutathione oxidation was lower. A high GSH/GSSG ratio is often associated with rapid cell growth. For example, endogenous GSSG levels decline in maturing somatic embryos of *Eleutherococcus senticosus* [49]. Applying GSH has also been found to promote the proliferation of embryogenic callus cells in Korean pine during somatic embryogenesis [16]. The decrease in GSSG, particularly in AIP-treated callus, may be linked to the presence of melatonin, which was detected exclusively in these samples. Melatonin is a potent, terminal antioxidant that protects cells without depleting cellular GSH levels [30]. Recent research has shown that melatonin dramatically enhances *Agrobacterium*-mediated transformation efficiency in carnation and doubles shoot regeneration rates [1]. Similarly, in rice callus cultures, melatonin supplementation boosted morphogenesis and increased the expression of antioxidant-related genes [55].

Conclusions

This study demonstrates the varied effects of phenolic inhibitors (AOA, AOPP, OBHA, AIP) and the adsorbent PVP on the regeneration potential of *F. tataricum* protoplast-derived cultures. AIP at 10 µM proved effective in promoting protoplast and cell colony development and diploid plant regeneration, while 100 µM inhibited regeneration. Similarly, 1% PVP also supported diploid plant regeneration, but regeneration potential was lost at 3% of PVP. Notably, AOPP and OBHA resulted in the regeneration of polypoid plants, limiting their potential use in in vitro cultures.

Further investigation into AIP and PVP effects on PECCs isolated from Tartary buckwheat MC regeneration highlighted their distinct mechanisms. PVP's ability to adsorb phenolics reduced oxidative stress, leading to nutrient depletion, consequently decreasing callus mass and amino acid availability. In contrast, AIP suppressed phenolic synthesis, increasing the presence of amino acids such as leucine, tryptophan, and betaine/valine. AIP- and PVP-treated callus also showed differences in redox balance with AIP presence, leading to increased GSH/GSSG ratio, which suggests lower oxidative stress. The presence of melatonin in AIP-treated callus likely contributed to this effect, as melatonin acts as an antioxidant, enhancing regeneration and transformation efficiency, as seen in other plant systems. These findings emphasise the importance of carefully balancing phenolic inhibition with nutrient availability and oxidative control for effective *F. tataricum* somatic embryogenesis and plant regeneration.

Abbreviations

ACC	1-Aminocyclopropane- 1-carboxylic acid
AIP	2-Aminoindane- 2-phosphonic acid
AOA	α-Aminooxyacetic acid
AOPP	α-Aminooxy-β-phenylpropionic acid
BBM	*BABY BOOM*
CLV3	*CLAVATA* 3
CM	Callus multiplication medium
FDA	Fluorescein diacetate
FUS3	*FUSCA 3*
GA	Gallic acid
GSH	Glutathione
GSSG	Glutathione oxidised
HPLC	High-performance liquid chromatography
LEC1	*LEAFY COTYLEDON1*
LEC2	*LEAFY COTYLEDON2*
MC	Morphogenic callus
OBHA	O-benzylhydroxylamine hydrochloride
PAL	Phenylalanine ammonia-lyase
PECCs	Proembryogenic cell complexes
POD	Peroxidase
PP0	Polyphenol oxidase
PVP	Polyvinylpyrrolidone
Q	Quercetin
RT	Room temperature
TAL	Tyrosine ammonia-lyase
WUS	*WUSCHEL*

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06440-x.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
Not applicable.

Clinical trial number
Not applicable.

Authors' contributions
Conceptualisation: RPP, AP, MZ, AB; Methodology: RPP, MZ, AP, LM, MB, KN, AKG, EG, PP; Formal analysis: RPP, AP, MZ, MB; Investigation: RPP, MZ, AP; Resources: AB; Writing—original draft: RPP, MZ, AP; Writing—review & editing: all authors; Visualisation: RPP, MZ, AP; Supervision: AP, AB; Project administration: AB; Founding acquisition: AB. All authors have read and approved the final manuscript.

Funding
The authors declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by the National Science Centre, Poland. Research project SONATA BIS 10 (No. Reg. 2020/38/E/NZ9/00033 awarded to AB), and by „jUŚt transition – Potencjał Uniwersytetu Śląskiego podstawą Sprawiedliwej Transformacji regionu" (FESL.10.25-IZ.01.0369/23–003).

Data availability
The datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations

Ethics approval and consent to participate
The plant materials used in this study comply with relevant institutional, national, and international guidelines and legislation. Seeds of *F. tataricum* (sample k- 17) are from the N. I. Vavilov Institute of Plant Genetic Resources collections, Saint Petersburg, Russia. The Plant Cytogenetic and Molecular Biology Group Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Poland, multiplied the obtained seeds. *F. tataricum* sample k- 17 is a common cultivated landrace of *F. tataricum*, and seeds are available upon request from the publication's authors.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Katowice, Poland. [2]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Penglais Campus, Aberystwyth, Wales SY23 2DA, UK. [3]Department of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Ave. Mickiewicza 21, Krakow 31 - 120, Poland.

Received: 3 February 2025 Accepted: 21 March 2025
Published online: 15 April 2025

References

1. Aalami O, Azadi P, Hadizadeh H, Wilde HD, Karimian Z, Nemati H, Samiei L. Melatonin strongly enhances the *Agrobacterium*- mediated transformation of carnation in nitrogen-depleted media. BMC Plant Biol. 2023;23:316.
2. Abenavoli MR, Pennisi AM. The effect of PVP on chestnut callus formation. In: Symposium on plant biotechnology as a tool for the exploitation of mountain lands, vol. 457. Torino: ISHS Acta Horticulturae; 1997. p. 17–20.
3. Amente G, Chimdessa E. Control of browning in plant tissue culture: a review. J Sci Agric. 2021;5:67–71.
4. Bata Z, Qian R, Roller A, Horak J, Bencze LC, Paizs C, Hammerschmidt F, Vértessy BG, Poppe L. A methylidene group in the phosphonic acid analogue of phenylalanine reverses the enantiopreference of binding to phenylalanine ammonia-lyases. Adv Synth Catal. 2017;359:2109–20.
5. Betekhtin A, Rojek M, Jaskowiak J, Milewska-Hendel A, Kwasniewska J, Kostyukova Y, Kurczynska E, Rumyantseva N, Hasterok R. Nuclear genome stability in long-term cultivated callus lines of *Fagopyrum tataricum* (L.) Gaertn. PLoS One. 2017;12:1–17.
6. Bolton JL, Dunlap T. Formation and biological targets of quinones: cytotoxic versus cytoprotective effects. Chem Res Toxicol. 2017;30:13–37.
7. Broun R, Mayak S. Aminooxyacetic acid as an inhibitor of ethylene synthesis and senescence in carnation flowers. Sci Hortic. 1981;15:277–82.
8. Cass CL, Peraldi A, Dowd PF, Mottiar Y, Santoro N, Karlen SD, Bukhman YV, Foster CE, Thrower N, Bruno LC, Moskvin OV, Johnson ET, Willhoit ME, Phutane M, Ralph J, Mansfield SD, Nicholson P, Sedbrook JC. Effects of phenylalanine ammonia lyase (PAL) knockdown on cell wall composition, biomass digestibility, and biotic and abiotic stress responses in *Brachypodium*. J Exp Bot. 2015;66:4317–35.
9. Chai J, Gao Y, Dong Y, Kong L, Zhang Y. Browning treatment in tissue culture of 'hongyang' kiwifruit. IOP Conf Ser Mater Sci Eng. 2018;452:022075.
10. Chandra S, Khan S, Avula B, Lata H, Yang MH, Elsohly MA, Khan IA. Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: a comparative study. Evid Based Complement Altern Med. 2014;2014:253875.
11. Chen X, Yu C, Nie J, Yang H, Ji W, Xu G, Zhu H, Jin S, Zhu X. The effect of anti-browning agent activated carbon and polyvinyl pyrrolidone on the rooting of embryo seedlings of "FengDan" and its transcriptome analysis. Front Plant Sci. 2022;13:832619.
12. Deutch CE. Browning in apples: exploring the biochemical basis of an easily-observable phenotype. Biochem Mol Biol Educ. 2018;46:76–82.
13. Downey CD, Zoń J, Jones AMP. Improving callus regeneration of *Miscanthus giganteus* J.M.Greef, Deuter ex Hodk., Renvoize 'M161' callus by inhibition of the phenylpropanoid biosynthetic pathway. In Vitro Cell Dev Biol Plant. 2019;55:109–20.
14. El-Azaz J, Cánovas FM, Ávila C, de la Torre F. The arogenate dehydratase adt2 is essential for seed development in *Arabidopsis*. Plant Cell Physiol. 2018;59:2409–20.
15. Feduraev P, Skrypnik L, Riabova A, Pungin A, Tokupova E, Maslennikov P, Chupakhina G. Phenylalanine and tyrosine as exogenous precursors of wheat (*Triticum aestivum* L.) secondary metabolism through pal-associated pathways. Plants. 2020;9:476.
16. Gao F, Wang R, Shi Y, Shen H, Yang L. Reactive oxygen metabolism in the proliferation of Korean pine embryogenic callus cells promoted by exogenous GSH. Sci Rep. 2023;13:2218.
17. Gao J, Xue J, Xue Y, Liu R, Ren X, Wang S, Zhang X. Transcriptome sequencing and identification of key callus browning-related genes from petiole callus of tree peony *(Paeonia suffruticosa* cv. Kao) cultured on media with three browning inhibitors. Plant Physiol Biochem. 2020;149:36–49.
18. Grzebelus E, Szklarczyk M, Baranski R. An improved protocol for plant regeneration from leaf- and hypocotyl-derived protoplasts of carrot. Plant Cell Tiss Organ Cult 2012;109:101–109. https://doi.org/10.1007/s11240-011-0078-5.
19. He Q, Ma D, Li W, Xing L, Zhang H, Wang Y, Du C, Li X, Jia Z, Li X, Liu J, Liu Z, Miao Y, Feng R, Lv Y, Wang M, Lu H, Li X, Xiao Y, Wang R, Liang H, Zhou Q, Zhang L, Liang C, Du H. High-quality *Fagopyrum esculentum* genome provides insights into the flavonoid accumulation among different tissues and self-incompatibility. J Integr Plant Biol. 2023;65:1423–41.
20. Horbowicz M, Kosson R, Koczkodaj D, Lahuta L. Effects of alpha-aminooxyacetic acid on the level of polyamines, anthocyanins and photosynthetic pigments in seedlings of common buckwheat (*Fagopyrum esculentum* Moench). Acta Soc Bot Pol. 2011;80:99–104.
21. Igor Otavio M, Cristine Vanz B, Maria Izabela F, Hector Alonzo Gomez G, Chung-Yen Oliver C, Giuseppina Pace Pereira L. Phenolic compounds: functional properties, impact of processing and bioavailability. In: Marcos SH, Mariana PT, Maria del Rosario GM, editors. Phenolic Compounds. Rijeka: IntechOpen; 2017. p. Ch. 1.
22. Jiang S, Penner MH. The Effect of *p*-Coumaric acid on browning inhibition in potato polyphenol oxidase-catalyzed reaction mixtures. Foods. 2022;11:577.
23. Jones AMP, Chattopadhyay A, Shukla M, Zoń J, Saxena PK. Inhibition of phenylpropanoid biosynthesis increases cell wall digestibility, protoplast isolation, and facilitates sustained cell division in American elm (*Ulmus americana*). BMC Plant Biol. 2012;12:75.

24. Jones AMP, Saxena PK. Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L.: a novel approach to reduce oxidative browning in plant tissue culture. PLoS One. 2013;8:e76802.
25. Jones RA, El-Abd SO. Prevention of salt-induced epinasty by α-aminooxyacetic acid and cobalt. Plant Growth Regul. 1989;8:315–23.
26. Kadri A, Grenier De March G, Guerineau F, Cosson V, Ratet P. *WUSCHEL* overexpression promotes callogenesis and somatic embryogenesis in *Medicago truncatula* Gaertn. Plants. 2021;10:715.
27. Kan J, Liu Y, Hui Y, Wan B, Liu J, Qian C, Jin C. 2-aminoindan-2-phosphonic acid alleviates oxidative browning in fresh-cut lily bulbs. J Food Process Preserv. 2022;46:e16449.
28. Kim SJ, Maeda T, Zaidul TS, Matsuura-Endo C, Yamauchi H, Mukasa Y, Saito K, Hashimoto N, Noda T, Saito T, Suzuki T. Identification of anthocyanins in the sprouts of buckwheat. J Agric Food Chem. 2007;55:6314–8.
29. Klejdus B, Kováčik J, Babula P. PAL inhibitor evokes different responses in two *Hypericum* species. Plant Physiol Biochem. 2013;63:82–8.
30. Korkmaz A, Reiter RJ, Topal T, Manchester LC, Oter S, Tan D-X. Melatonin: an established antioxidant worthy of use in clinical trials. Mol Med. 2009;15:43–50.
31. Krishna H, Sairam RK, Singh SK, Patel VB, Sharma RR, Grover M, Nain L, Sachdev A. Mango explant browning: effect of ontogenic age, mycorrhization and pre-treatments. Sci Hortic. 2008;118:132–8.
32. Krkošková B, Mrázová Z. Prophylactic components of buckwheat. Food Res Int. 2005;38:561–8.
33. Kuśmierek K, Świątkowski A. Adsorption of phenols on carbonaceous materials of various origins but of similar specific surface areas. Separations. 2023;10:422.
34. Lee JHJ, Kasote DM, Jayaprakasha GK, Avila CA, Crosby KM, Patil BS. Effect of production system and inhibitory potential of aroma volatiles on polyphenol oxidase and peroxidase activity in tomatoes. J Sci Food Agric. 2021;101:307–14.
35. Liu C, Zhang Z, Dang Z, Xu J, Ren X. New insights on phenolic compound metabolism in pomegranate fruit during storage. Sci Hortic. 2021;285:110138.
36. Liu J, Lefevere H, Coussement L, Delaere I, De Meyer T, Demeestere K, Höfte M, Gershenzon J, Ullah C, Gheysen G. The phenylalanine ammonia-lyase inhibitor AIP induces rice defence against the root-knot nematode *Meloidogyne graminicola*. Mol Plant Pathol. 2024;25:e13424.
37. Monthony AS, Jones AMP. Enhancing protoplast isolation and early cell division from C*annabis sativa* callus cultures via phenylpropanoid inhibition. Plants. 2024;13:130.
38. Nalinkumar A, Singh P. An overview of buckwheat *Fagopyrum* spp an underutilized crop in India-nutritional value and health benefits. Int J Med Res Health Sci. 2020;9:39–44.
39. Ngomuo M, Mneney E, Ndakidemi P. Control of lethal browning by using ascorbic acid on shoot tip cultures of a local *Musa* spp.(Banana) cv. Mzuzu in Tanzania. Afr J Biotechnol. 2014;13:1721-5.
40. Permadi N, Akbari SI, Prismantoro D, Indriyani NN, Nurzaman M, Alhasnawi AN, Doni F, Julaeha E. Traditional and next-generation methods for browning control in plant tissue culture: Current insights and future directions. Curr Plant Biol. 2024;38:100339.
41. Permadi N, Nurzaman M, Alhasnawi AN, Doni F, Julaeha E. Managing lethal browning and microbial contamination in *Musa* spp. tissue culture: synthesis and perspectives. Horticulturae. 2023;9:453.
42. Reustle G, Natter I. Effect of polyvinyl pyrrolidone and activated charcoal on formation of microcallus from grapevine protoplasts (*Vitis* sp.). Vitis. 1994;33:117–21.
43. Roostika I, Wati RPDL, Sukmadjaja D. Pengaruh PVP dan DIECA terhadap regenerasi meristem tebu. Buletin Tanaman Tembakau, Serat dan Minyak Industri. 2015;7:9–14.
44. Rumiantseva NI, Akulov AN, Mukhitov AR. Extracellular polymers in callus cultures of *Fagopyrum tataricum* (L.) Gaertn. with different morphogenic activities: time courses during the culture cycle. Prikl Biokhim Mikrobiol. 2004;40:571–8.
45. Rumyantseva NI, Sergeeva NV, Khakimova L, Sal'nikov VV, Gumerova EA, Lozovaya VV. Organogenesis and somatic embryogenesis in the culture of two buckwheat species. Fiziol Rast. 1989;36:187–94.
46. Safwar G, Abdul F, El Sharbasy S. The effect of some antioxidants on blackening and growth of in vitro culture of banana (*Musa* spp. cv. Grand naine). Egypt J Genet Cytol. 2015;44:47–59.
47. Sala-Cholewa K, Tomasiak A, Nowak K, Piński A, Betekhtin A. DNA methylation analysis of floral parts revealed dynamic changes during the development of homostylous *Fagopyrum tataricum* and heterostylous *F. esculentum* flowers. BMC Plant Biol. 2024;24:448.
48. Samuilov VD, Kiselevsky DB. Salicylhydroxamic acid enhances the NADH-oxidase activity of peroxidase in pea mitochondrial and chloroplast suspensions. Mosc Univ Biol Sci Bull. 2016;71:19–23.
49. Shohael AM, Ali MB, Hahn EJ, Paek KY. Glutathione metabolism and antioxidant responses during *Eleutherococcus senticosus* somatic embryo development in a bioreactor. Plant Cell Tissue Organ Cult. 2007;89:121–9.
50. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965;16:144.
51. Sriyanti I, Marlina L, Fudholi A, Marsela S, Jauhari J. Physicochemical properties and in vitro evaluation studies of polyvinylpyrrolidone/cellulose acetate composite nanofibres loaded with *Chromolaena odorata* (L) King extract. J Market Res. 2021;12:333–42.
52. Tang T, Xie X, Ren X, Wang W, Tang X, Zhang J, Wang Z. A difference of enzymatic browning unrelated to PPO from physiology, targeted metabolomics and gene expression analysis in Fuji apples. Postharvest Biol Technol. 2020;170:111323.
53. Temsch E, Greilhuber J, Krisai R. Genome size in liverworts. Preslia. 2010;82:63–80.
54. Tomasiak A, Zhou M, Betekhtin A. Buckwheat in tissue culture research: current status and future perspectives. Int J Mol Sci. 2022;23:2298
55. Ubaidillah M, Al Ayyubi NNA, Nur Khofifa RA, Dewanti P. Response of rice somatic embryogenesis to exogenous melatonin about its role in scavenging reactive oxygen species. Agrivita. 2024;46:48.
56. Uchendu EE, Brown DCW, Saxena PK. Cryopreservation of shoot tips and cotyledons of the North American ginseng (*Panax quinquefolius* L.). CryoLetters. 2011;32:463–72.
57. Urban K, Hura T. Zastosowanie inhibitorów amoniakoliazy L-fenyloalaniny w badaniach ekofizjologii roślin. Postępy Biochemii. 2023;69:11–7.
58. Wanat W, Talma M, Hurek J, Pawełczak M, Kafarski P. Substituted phosphonic analogues of phenylglycine as inhibitors of phenylalanine ammonia lyase from potatoes. Biochimie. 2018;151:119–27.
59. Wiczkowski W, Szawara-Nowak D, Dębski H, Mitrus J, Horbowicz M. Comparison of flavonoids profile in sprouts of common buckwheat cultivars and wild tartary buckwheat. Int J Food Sci Technol. 2014;49:1977–84.
60. Widhiastuty NS, Anwar S, Rosyida. The effect of pvp (polivinil pirolidon) and bap (6-benzylamino purine) on shoots induction of teak plus perhutani (*Tectona grandis*). IOP Conf Ser Earth Environ Sci. 2023;1246:012011.
61. Xing F, Li Z, Sun A, Xing D. Reactive oxygen species promote chloroplast dysfunction and salicylic acid accumulation in fumonisin B1-induced cell death. FEBS Lett. 2013;587:2164–72.
62. Yang S, Chen Q, Liu S. *FUSCA3*, a multi-role regulator in the process of plant growth and development. Plant Cell Tissue Organ Cult. 2022;150:1–13.
63. Yang YH, Yang MR, Zhu JY, Dong KW, Yi YJ, Li RF, Zeng L, Zhang CF. Functional characterization of tyrosine decarboxylase genes that contribute to acteoside biosynthesis in *Rehmannia glutinosa*. Planta. 2022;255:64.
64. Zaranek M, Pérez-Pérez R, Milewska-Hendel A, Betekhtin A, Grzebelus E. Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn. BMC Plant Biol. 2023;23:385.
65. Zaranek M, Pérez-Pérez R, Milewska-Hendel A, Grzebelus E, Betekhtin A. Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench. Plant Cell Tissue Organ Cult. 2023;154:673–87.
66. Zaranek M, Pinski A, Skupien-Rabian B, Jankowska U, Godel-Jedrychowska K, Sala-Cholewa K, Nowak K, Kurczyńska E, Grzebelus E, Betekhtin A. The cell colony development is connected with the accumulation of embryogenesis-related proteins and dynamic distribution of cell wall components in in vitro cultures of *Fagopyrum tataricum* and *Fagopyrum esculentum*. BMC Plant Biol. 2025;25:102.
67. Zhang F, Wang J, Li X, Zhang J, Liu Y, Chen Y, Yu Q, Li N. Genome-wide identification and expression analyses of phenylalanine ammonia-lyase gene family members from tomato (*Solanum lycopersicum*) reveal their role in root-knot nematode infection. Front Plant Sci. 2023;14:1204990.

Publisher's Note

10. Conclusions

In this doctoral thesis, *Fagopyrum in vitro* regeneration systems have been optimised using protoplasts and calli as starting material. Moreover, the first protocol to generate *F. esculentum* (+) *F. tataricum* hybrid cells via protoplast electrofusion was developed for comparative studies of cell wall regeneration. These findings help to understand the molecular, cellular and physiological mechanisms controlling early regeneration stages while identifying critical efficiency-determining factors. These results provide transformative insights into protoplast technology and tissue culture, demonstrating how culture conditions directly modulate cellular behaviour and regeneration outcomes.

The main conclusions of the thesis are presented below:

P1:

- Hypocotyls and morphogenic calli derived from immature embryos of *F. tataricum* have been demonstrated as highly viable sources for protoplast isolation:
  - **a.** The immobilisation of *F. tataricum* protoplasts in a low-melting-point agarose matrix and using PSK in the culture medium enhance cell division, increase the cell colony formation and favour the development of microcalli.
  - **b.** The hormonal combinations BAP–KIN in regeneration medium stimulate morphogenesis in *F. tataricum*.

P2:

- Embryogenic calli from immature embryos of *F. esculentum* proved a superior source to hypocotyls for high-yield protoplast isolation:
  - **a.** Supplementation of *F. esculentum* protoplast culture medium with PSK is essential to induce cell division and cell colony formation, unlike phenolic inhibitors and adsorbents, which do not influence protoplast development.
  - **b.** The use of TDZ during *F. esculentum* regeneration stimulates somatic embryogenesis and considerably decreases the whole plant regeneration timing to two months.
  - **c.** The protoplast-to-plant system successfully regenerates *F. esculentum* whole plants using embryogenic calli derived from immature embryos as the source of protoplasts within 3–5 months, compared with the long-lasting and poor plant regeneration from hypocotyl-derived protoplasts.

P3:

- Protoplast electrofusion was successfully used to obtain hybrid cells between *F. esculentum* and *F. tataricum*:
  - **a.** *De novo* cell wall reconstruction period in *F. esculentum*, *F. tataricum* and *Fe (+) Ft* hybrid cells is approximately 48 hours.
  - **b.** Immunolabelling of major cell wall components (AGPs, cellulose, pectins, extensin, xyloglucan) showed similar patterns in hybrid and parental cells during early cell wall regeneration. LM20 revealed delayed methylesterified homogalacturonan synthesis in *F. esculentum*, pointing to species-specific pectin dynamics.

P4:

- Regulation of phenolic and flavonoid content using PAL inhibitors (AIP, AOPP, OBHA) and PVP directly influence the response of protoplasts and morphogenic calli during the growth and plant regeneration processes of *F. tataricum*:
  - **a.** Optimal diploid regeneration in calli derived from protoplast was achieved with 10 μM AIP and 1% PVP, whereas higher concentrations (100 μM AIP and 3% PVP) failed to regenerate plants. Treatments with 0.2 mM AOPP and 10 μM OBHA resulted in the regeneration of both diploid and tetraploid plants, suggesting that prolonged exposure may lead to polyploidisation. All AIP and PVP-tested concentrations during PECCs regeneration developed into diploid plants, and the plant regeneration seemed to be more effective under 100 μM AIP treatment.
  - **b.** AIP and PVP effects in Tartary buckwheat PECCs isolated from MC demonstrated distinct mechanisms. While PVP adsorbed phenolics and depleted nutrients, which resulted in suppressing callus growth, AIP inhibited phenolic synthesis, enhancing amino acid levels (e.g., leucine, tryptophan, and betaine/valine).
  - **c.** AIP- and PVP-treated calli showed contrasting redox states. AIP elevated the glutathione/oxidised glutathione ratio, suggesting lower oxidative stress, potentially due to melatonin accumulation in AIP-treated calli.
  - **d.** RT-qPCR analysis showed marked upregulation of somatic embryogenesis-related transcription factors (*LEC2*, *BBM*) and *WUSCHEL* in AIP-treated callus tissues.

Beyond basic science contributions to plant biotechnology, this work delivers practical tools for crop improvement, establishing robust protocols that merge fundamental research with agricultural applications for *Fagopyrum* and related species.

11. Supplement

Part of the methodology used in the doctoral thesis was published in three chapters of the Springer monograph "Buckwheat: Methods in Molecular Biology", edited by Betekhtin, A. and Pinski, A.

1. Zaranek, M., **Pérez-Pérez, R.**, Malec, J., Grzebelus, E. Protoplast isolation, culture, and regeneration in Common and Tartary buckwheat. In: Betekhtin, A., Pinski, A. (eds) buckwheat. Methods in Molecular Biology, vol 2791. Humana, New York, NY. Springer, 2024, Chapter 5, 45 – 56. https://doi.org/10.1007/978-1-0716-3794-4_5

Ministry of Science and Higher Education points: 20

Protoplast technology has diverse applications, including genome editing, genetic manipulation, somatic hybridisation, and fundamental research, such as *de novo* cell wall reconstruction or cell division. This chapter provides a detailed protocol for the isolation, cultivation, and subsequent regeneration of protoplasts from *F. esculentum* and *F. tataricum* into whole plants. The technique uses two main sources for protoplast isolation: 1) hypocotyls derived from seeds germinated under sterile conditions and 2) embryogenic and morphogenic calli derived from immature zygotic embryos of *F. esculentum* and *F. tataricum*, respectively.

For hypocotyl germination, sterilised seeds of *F. esculentum* and *F. tataricum* were transferred to Petri dishes containing FE-germination medium (MS medium with vitamins, 200 mg L^{-1} cefotaxime disodium 30 g L^{-1} sucrose and 7 g L^{-1} plant agar) and FT-germination medium (MS medium with vitamins, 30 g L^{-1} sucrose and 7 g L^{-1} plant agar), respectively. The seeds are incubated in the dark for 10 days at 26 ºC.

Protoplast isolation begins by incubating 1 g of 10-day-old hypocotyls or 2 g of calli in a pre-plasmolysis solution (PSII for hypocotyls: 0.5 M mannitol; PSII/F for calli: 0.6 M mannitol and 5 mM $CaCl_2$) for one hour to facilitate membrane detachment from the cell walls. Then, different enzyme solutions are used to digest the cell wall; the combination and concentrations depend on the tissue type and species. For *F. esculentum* and *F. tataricum* hypocotyls, a mixture of 1% cellulase R10, 0.6% macerozyme R10, and 0.1% disease is used. For *F. esculentum* embryogenic calli, a combination of 1.5% cellulase R10, 0.15% driselase, and 0.1% pectolyase Y-23 is recommended, while *F. tataricum* morphogenic calli require 1% cellulase R10 and 0.1% pectolyase Y-23. Cell wall digestion takes place overnight in the dark at 50 rpm. The remaining steps of the protocol are common for all variants.

After a series of centrifuge steps, the pellet is resuspended in a 0.6M sucrose solution, and a washing solution (150 mM NaCl, 125 mM $CaCl_2$, 5 mM KCL and 5 mM glucose) is carefully layered on top to create a gradient. Following centrifugation, a ring of viable protoplasts forms at the interface between both solutions. The protoplast concentration is determined using a Fuchs-Rosenthal haemocytometer and adjusted to 5×10^5 cells mL^{-1} for hypocotyl-derived protoplasts and 8×10^5 cells mL^{-1} for calli-derived protoplasts.

The protoplasts are immobilised in agarose by mixing a 1.2% low-melting-point agarose solution and then distributing the mixture into four 50 µl beads in a Petri dish. The beads are covered with 4 ml of BM medium supplemented with 100 nM PSK to enhance cell division. The protoplast cultures are incubated at 25 ºC in the dark for approximately two months, with the medium replaced with fresh BM medium on the 10th day of culture. Once microcalli outgrow the agarose beads, they are transferred and multiplied in CM medium supplemented with 100 nM PSK. For regeneration, *F. esculentum*-derived calli are cultured on MS3 medium (MS medium, 3 mg L^{-1} BAP, 1 mg L^{-1} TDZ, 30 g L^{-1} sucrose, and 3 g L^{-1} Phytagel), while *F. tataricum*-derived calli are cultured on MS4 medium (MS medium, 2 mg L^{-1} BAP, 1 mg L^{-1} kinetin, 30 g L^{-1} sucrose, and 3 g L^{-1} Phytagel). Cultures are maintained in a growth room at 28 °C under a 16/8 hours photoperiod, with the regeneration medium refreshed every two to three weeks. Developed shoots are transferred to a rooting medium (MS medium with vitamins, 30 g L^{-1} sucrose and 3 g L^{-1} Phytagel) to stimulate the root system development and reinforcement and kept under the same growth conditions. After the whole plants develop, they are transferred into a moss-coconut fibre substrate and maintained in a greenhouse at 25 ºC with a 16/8 hours photoperiod.

2. Milewska-Hendel, A., Sala-Cholewa, K., **Pérez-Pérez, R.** Immunodetection of cell wall components in studies on cell wall rebuilding in *Fagopyrum esculentum* and *Fagopyrum tataricum.* In: Betekhtin, A., Pinski, A. (eds) buckwheat. Methods in Molecular Biology, vol 2791. Humana, New York, NY. Springer, 2024, Chapter 7, 71 – 80. https://doi.org/10.1007/978-1-0716-3794-4_7

Ministry of Science and Higher Education points: 20

The immunocytochemical analysis provides a powerful approach for visualising cell wall components through epitope-targeted primary antibodies, enabling the detection of pectins, arabinogalactan proteins, hemicelluloses, extensions, and other structural elements. When combined with FB28 staining, this methodology enables the comprehensive characterisation of cell wall regeneration dynamics in protoplast cultures. This chapter contains a detailed protocol for immunostaining-based qualitative analysis of cell wall component deposition during *de novo* wall reconstruction in isolated protoplasts of *F. esculentum* and *F. tataricum*.

The protocol starts with the protoplast isolation, where 2 g of 12-day-old calli from *F. esculentum* and *F. tataricum* are incubated for one hour in pre-plasmolysis solution (PSII for hypocotyls: 0.5 M mannitol; PSII/F for calli: 0.6 M mannitol and 5 mM $CaCl_2$). For cell wall digestion in *F. esculentum* embryogenic calli, a combination of 1.5% cellulase R10, 0.15% driselase, and 0.1% pectolyase Y-23 is used, and for *F. tataricum* morphogenic calli, 1% cellulase R10 and 0.1% pectolyase Y-23. The digestion occurs in the dark at 50 rpm for 14 – 15 hours. The digested solution is centrifuged, and the pellet is resuspended in 0.6 M sucrose solution. Carefully, a gradient is created by adding washing solution (150 mM NaCl, 125 mM $CaCl_2$, 5 mM KCL and 5 mM glucose) on top and centrifuge. The ring of viable protoplasts in the interface between of both solutions is collected, resuspended in washing solution and centrifuged. The pellet is resuspended in a washing solution, and protoplast concentration is adjusted to 8×10^5 cells mL^{-1}.

Following isolation, it is possible to find non-viable protoplasts or protoplasts with remaining cell walls; thus, selecting only viable protoplasts with completely removed cell walls is important to proceed with *de novo* reconstruction analyses. The viability and cell wall removal is verified through dual staining with 0.01% propidium iodide (0.01 g in 100 ml of washing solution; $\lambda_{ex}/_{em}$ = 536/617 nm) and 0.01% Fluorescent Brightener 28 (0.01 g in 100 ml of washing solution; $\lambda_{ex}/_{em}$ = 380/475 nm). After 15 min dark incubation, protoplasts negative for both fluorophores are manually selected using an inverted microscope with a

micromanipulator. Approximately 200 selected protoplasts are transferred to sterile 1.5 mL tubes containing 5 µL BM medium and then mixed with 6 µL of a warm 1.2% low-melting-point agarose solution. The mixture (11 µL total) is pipetted as beads in Petri dishes alongside three 50 µL nurse culture beads (8×10^5 protoplasts mL^{-1} in agarose). Cultures are maintained in 4 mL BM medium supplemented with 100 nM PSK and 2 µL Timentin at 25 °C in darkness, with sampling at 4, 12, 24, 48, and 72 hours.

At each time point, the BM medium is removed, and the agarose beads with the selected protoplasts are transferred to a new 2 ml Eppendorf (three beads per tube) containing a 3% paraformaldehyde and 1% glutaraldehyde fixative and incubated overnight at 4 ºC. The number of tubes depends on the number of antibodies to be analysed. The fixative is washed with phosphate-buffered saline (1×PBS, pH 7.2), and then the beads are incubated with blocking buffer (2% bovine serum albumin and 2% fetal calf serum) for 30 min at room temperature. Later, 250 µl of the primary antibodies (1 µl antibody per 19 µl blocking buffer) are added in each tube, and as the secondary antibody, 250 µl anti-rat IgG H + L conjugated with the fluorochrome AlexaFluor488 (1 µl antibody per 99 µl blocking buffer) is used. The incubation period for primary and secondary antibodies is around 1.5 – 2 hours. The antibodies are washed with PBS, and finally, the beads are stained with FB28 (0.01 g in 100 ml of ultrapure water) for 15 min and washed thoroughly in PBS and then ultrapure water. The beads are placed individually in a glass slide and covered gently with a coverslip, while microscopic observations are performed on a confocal microscope using two channels: Alexa488 fluorochrome (for excitation use 488 nm laser) and FB28 (for excitation use 405 nm laser).

3. **Pérez-Pérez, R.**, Kwasniewska, J. Visualisation of *Fagopyrum esculentum* and *Fagopyrum tataricum* chromosomes and micronuclei. In: Betekhtin, A., Pinski, A. (eds) buckwheat. Methods in Molecular Biology, vol 2791. Humana, New York, NY. Springer, 2024, Chapter 8, 81 – 87. https://doi.org/10.1007/978-1-0716-3794-4_8

Ministry of Science and Higher Education points: 20

This chapter details a squash preparation methodology for chromosome analysis during metaphase in *F. esculentum* and *F. tataricum*. The optimised protocol yields high-quality chromosome spreads with minimal cytoplasmic contamination. Furthermore, adapting this technique facilitates the examination of micronuclei formation in interphase nuclei of both buckwheat species.

The sources of cells for both observations are young root tips 2 cm in length, which can be obtained from plants already established on culture media or from germinated seeds. Seeds from *F. esculentum* are kept in tap water overnight to soften and remove the seed coat. Then, the seeds from both species are individually placed into a Petri dish with filter paper moistened with tap water and incubated at 22 ºC in the dark till the root reaches a length of 2 cm. The roots are detached from the seed with a scalpel.

The technique starts by transferring 1-2 cm root tips to a 5 ml Eppendorf tube with cold tap water and incubating for 24 hours on ice for a low-temperature pretreatment to accumulate the condensed chromosomes. Then, the material is fixed in a fresh acetic acid-ethanol solution (3:1 ethanol and acetic acid) for 2 hours, and the roots are shortened to a length of approximately 0.5 cm. Into a glass maceration container, the tips are washed with 10 mM citrate buffer (0.84 g citric acid monohydrate, 1.76 g of trisodium citrate dihydrate, 100 ml distilled water) and then macerated with a prewarmed to 37 ºC enzyme cocktail (2% cellulase RS, 20% pectinase, 10 mM citrate buffer) during 1.5 hours for chromosome slides and 1.1 hours for nuclei and micronuclei slides. The enzyme solution is washed with 10 mM citrate buffer for 30 min, and the tips are transferred to a new container with 45 % acetic acid solution.

With the help of a dissecting microscope and microneedles, the root cap is removed, and the meristem is carefully dissected. Meristems are placed on the centre of a clean, dry glass slide with a micropipette and squeezed tightly with the help of a 22 × 22 mm coverslip. The preparations are kept on dry ice to fix the material to the slide, and after 30 min, the coverslip is carefully flicked off with a scalpel. The slides are air-dried for 30 min, then the samples are

stained with 10 µl 4′,6-diamidino-2-phenylindole fluorophore (DAPI; $\lambda_{ex}/_{em}$ = 358/460 nm) and covered with a 24 × 24 mm coverslip. The observation of well-spread chromosomes and well-preserved nuclei with micronuclei is performed under a fluorescent microscope equipped with a DAPI filter. The preparations can be stored at 4 °C for up to three months.

The results obtained in the doctoral thesis were presented at three scientific conferences:

Conference: 15th International Buckwheat Symposium: "Buckwheat for health".
Organiser: Institute of Soil Science and Plant Cultivation.
Place: Puławy, Poland.
Date: July, 2-8, 2023.
Title of the presentation: "Development of somatic hybrids in buckwheat using electrofusion of protoplasts". Oral presentation.

Conference: 9th Central European Congress of Life Sciences EUROBIOTECH 2024.
Organiser: Małopolska Centre of Biotechnology of the Jagiellonian University.
Place: Kraków, Poland.
Date: June, 24-25, 2024.
Title of the presentation: "Cell wall reconstruction in hybrid protoplasts derived from *Fagopyrum* calli". Oral presentation.

Conference: 6th Symposium on Physiology and Breeding of Cereals.
Organiser: Institute of Natural Resources and Agrobiology of Salamanca (IRNASA).
Place: Salamanca, Spain.
Date: October, 16-18, 2024.
Title of the presentation: "Electrofusion-driven somatic hybridisation: a dual approach to improve buckwheat crop". Poster.

12. References

1. **Adachi, T., Yamaguchi, A., Miike, Y. and Hoffmann, F.** (1989) Plant regeneration from protoplasts of common buckwheat (*Fagopyrum esculentum*). *Plant Cell Reports*, **8**, 247-250. https://doi.org/https://doi.org/10.1007/BF00778544

2. **Al-Nema, Q. and Al-Mallah, M.** (2020) Electrofusion of mesophyll protoplasts from two varieties of sugar beet, (*Beta vulgaris* L.). *Journal of Life and Bio Sciences Research*, **1**, 22-25. https://doi.org/https://doi.org/10.38094/jlbsr117

3. **Amente, G. and Chimdessa, E.** (2021) Control of browning in plant tissue culture: A review. *Journal of Scientific Agriculture*, **5**, 67-71. https://doi.org/https://doi.org/10.25081/jsa.2021.v5.7266

4. **Betekhtin, A., Pinski, A., Milewska-Hendel, A., Kurczynska, E. and Hasterok, R.** (2019) Stability and instability processes in the calli of *Fagopyrum tataricum* that have different morphogenic potentials. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **137**, 343-357. https://doi.org/https://doi.org/10.1007/s11240-019-01575-w

5. **Betekhtin, A., Rojek, M., Jaskowiak, J., Milewska-Hendel, A., Kwasniewska, J., Kostyukova, Y., Kurczynska, E., Rumyantseva, N.I. and Hasterok, R.** (2017) Nuclear genome stability in long-term cultivated callus lines of *Fagopyrum tataricum* (L.) Gaertn. *PLOS One*, **12**, 1-17. https://doi.org/https://doi.org/10.1371/journal.pone.0173537

6. **Bonafaccia, G., Marocchini, M. and Kreft, I.** (2003) Composition and technological properties of the flour and bran from common and Tartary buckwheat. *Food Chemistry*, **80**, 9-15. https://doi.org/https://doi.org/10.1016/S0308-8146(02)00228-5

7. **Cai, Z., Knorr, D. and Smetanska, I.** (2012) Enhanced anthocyanins and resveratrol production in *Vitis vinifera* cell suspension culture by indanoyl-isoleucine, N-linolenoyl-l-glutamine and insect saliva. *Enzyme and Microbial Technology*, **50**, 29-34. https://doi.org/https://doi.org/10.1016/j.enzmictec.2011.09.001

8. **Cardoso, J.C., Viana, M.L., Matias, R., Furtado, M.T., Caetano, A., Consolaro, H. and Brito, V.** (2018) Towards a unified terminology for angiosperm reproductive systems. *Acta Botanica Brasilica*, **32**, 329-348. https://doi.org/https://doi.org/10.1590/0102-33062018abb0124

9. **Castilleux, R., Plancot, B., Vicré, M., Nguema-Ona, E. and Driouich, A.** (2021) Extensin, an underestimated key component of cell wall defence? *Annals of Botany*, **127**, 709-713. https://doi.org/http://doi.org/10.1093/aob/mcab001

10. **Cawoy, V., Ledent, J.F., Kinet, J.M. and Jacquemart, A.** (2009) Floral biology of common buckwheat (*Fagopyrum esculentum* Moench). *The European Journal of Plant Science and Biotechnology*, **3**, 1-9.

11. **Chen, X., Yu, C., Nie, J., Yang, H., Ji, W., Xu, G., Zhu, H., Jin, S. and Zhu, X.** (2022) The effect of anti-browning agent activated carbon and polyvinyl pyrrolidone on the rooting of embryo seedlings of "FengDan" and its transcriptome analysis. *Frontiers in Plant Science*, **13**, 832619. https://doi.org/https://doi.org/10.3389/fpls.2022.832619

12. **David, H., Bade, P., David, A., Savy, C., Demazy, C. and Van Cutsem, P.** (1995) Pectins in walls of protoplast-derived cells imbedded in agarose and alginate beads. *Protoplasma*, **186**, 122-130. https://doi.org/https://doi.org/10.1007/BF01281322

13. **Eckardt, N.A.** (2008) Role of xyloglucan in primary cell walls. *Plant Cell*, **20**, 1421-1422. https://doi.org/http://doi.org/10.1105/tpc.108.061382

14. **Fawcett, J., Takeshima, R., Kikuchi, S., Yazaki, E.i., Katsube-Tanaka, T., Dong, Y., Li, M., Hunt, H.V., Jones, M.K., Lister, D.L., Ohsako, T., Ogiso-Tanaka, E., Fujii, K., Hara, T., Matsui, K., Mizuno, N., Nishimura, K., Nakazaki, T., Saito, H., Takeuchi, N., Ueno, M., Matsumoto, D., Norizuki, M., Shirasawa, K., Li, C., Hirakawa, H., Ota, T. and Yasui, Y.** (2023) Genome sequencing reveals the genetic architecture of heterostyly and domestication history of common buckwheat. *Nature Plants*, **9**, 1236-1251. https://doi.org/https://doi.org/10.1038/s41477-023-01474-1

15. **Fesenko, I.N., Bondarev, N.I., Rezunova, O.V., Evsyuticheva, D.E. and Fesenko, A.N.** (2022) Hybridisation with *Fagopyrum cymosum* Meisn. as a way to make cultivated Tartary buckwheat (*F. tataricum* Gaertn.) with grain characteristics typical for common buckwheat (*F. esculentum* Moench.). *Breeding Science*, **72**, 232-237. https://doi.org/https://doi.org/10.1270/jsbbs.21086

16. **Gieniec, M., Siwek, J., Oleszkiewicz, T., Maćkowska, K., Klimek-Chodacka, M., Grzebelus, E. and Baranski, R.** (2020) Real-time detection of somatic hybrid cells during electrofusion of carrot protoplasts with stably labelled mitochondria. *Scientific Reports*, **10**, 18811. https://doi.org/https://doi.org/10.1038/s41598-020-75983-w

17. **Godel-Jędrychowska, K., Maćkowska, K., Kurczyńska, E. and Grzebelus, E.** (2019) Composition of the reconstituted cell wall in protoplast-derived cells of *Daucus* affected by phytosulfokine (PSK). *International Journal of Molecular Sciences*, **20**. https://doi.org/https://doi.org/10.3390/ijms20215490

18. **Grison, M., Maucort, G., Dumazel, A., Champelovier, D., Boutté, Y., Fernández-Monreal, M. and Bayer, E.M.** (2024) Plant root expansion microscopy (ROOT-ExM): A streamlined super resolution method for plants. *bioRxiv*, 581184. https://doi.org/https://doi.org/10.1101/2024.02.21.581184

19. **Gumerova, E.A., Akulov, A.N. and Rumyantseva, N.I.** (2015) Effect of methyl jasmonate on growth characteristics and accumulation of phenolic compounds in suspension culture of Tartary buckwheat. *Russian Journal of Plant Physiology*, **62**, 195-203. https://doi.org/https://doi.org/10.1134/S1021443715020077

20. **Gumerova, E.A., Galeeva, E.I., Chuyenkova, S.A. and Rumyantseva, N.I.** (2003) Somatic embryogenesis and bud formation on cultured *Fagopyrum esculentum* hypocotyls. *Russian Journal of Plant Physiology*, **50**, 640-645. https://doi.org/https://doi.org/10.1023/A:1025640107932

21. **Han, M., Kamal, A.H.M., Huh, Y.S., Jeon, A.Y., Bae, J., Chung, K.Y., Lee, M.S., Park, S., Jeong, H.S. and Woo, S.H.** (2011) Regeneration of plantlet via somatic embryogenesis from hypocotyls of Tartary buckwheat (*Fagopyrum tataricum*). *Australian Journal of Crop Science*, **5**, 865-869. https://doi.org/https://doi.org/10.7740/kjcs.2013.58.4.331

22. **He, X.Y., Xu, L.J., Xu, X., Yi, D.D., Hou, S.L., Yuan, D.Y. and Xiao, S.X.** (2024) Embryogenic callus induction, proliferation, protoplast isolation, and PEG induced fusion in *Camellia oleifera*. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **157**, 75. https://doi.org/https://doi.org/10.1007/s11240-024-02800-x

23. **Holländer-Czytko, H. and Amrhein, N.** (1983) Subcellular compartment of shikimic acid and phenylalanine in buckwheat cell suspension cultures grown in the presence of

shikimate pathway inhibitors. *Plant Science Letters*, **29**, 89-96. https://doi.org/https://doi.org/10.1016/0304-4211(83)90027-5

24. **Hou, S., Sun, Z., Linghu, B., Wang, Y., Huang, K., Xu, D. and Han, Y.** (2015) Regeneration of buckwheat plantlets from hypocotyl and the influence of exogenous hormones on rutin content and rutin biosynthetic gene expression *in vitro*. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **120**, 1159-1167. https://doi.org/https://doi.org/10.1007/s11240-014-0671-5

25. **Huda, M., Lu, S., Jahan, T., Ding, M., Jha, R., Zhang, K., Zhang, W., Georgiev, M., Park, S. and Zhou, M.** (2021) Treasure from garden: Bioactive compounds of buckwheat. *Food Chemistry*, **335**, 127653. https://doi.org/https://doi.org/10.1016/j.foodchem.2020.127653

26. **Huh, H., Jayachandran, D., Sun, J., Irfan, M., Lam, E., Chundawat, S. and Lee, S.H.** (2025) Time-resolved tracking of cellulose biosynthesis and assembly during cell wall regeneration in live *Arabidopsis* protoplasts. *Science Advances*, **11**, eads6312. https://doi.org/https://doi.org/10.1126/sciadv.ads6312

27. **Jha, R., Zhang, K., He, Y., Mendler-Drienyovszki, N., Magyar-Tábori, K., Quinet, M., Germ, M., Kreft, I., Meglič, V., Ikeda, K., Chapman, M.A., Janovská, D., Podolska, G., Woo, S.H., Bruno, S., Georgiev, M.I., Chrungoo, N., Betekhtin, A. and Zhou, M.** (2024) Global nutritional challenges and opportunities: Buckwheat, a potential bridge between nutrient deficiency and food security. *Trends in Food Science & Technology*, **145**, 104365. https://doi.org/https://doi.org/10.1016/j.tifs.2024.104365

28. **Jiang, S. and Penner, M.** (2022) The effect of *p*-coumaric acid on browning inhibition in potato polyphenol oxidase-catalyzed reaction mixtures. *Foods*, **11**. https://doi.org/https://doi.org/10.3390/foods11040577

29. **Kao, K.N. and Saleem, M.** (1986) Improved fusion of mesophyll and cotyledon protoplasts with PEG and high pH-Ca^{2+} solutions. *Journal of Plant Physiology*, **122**, 217-225. https://doi.org/https://doi.org/10.1016/S0176-1617(86)80120-1

30. **Kästner, U., Klocke, E. and Abel, S.** (2017) Regeneration of protoplasts after somatic hybridisation of *Hydrangea*. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **129**, 359-373. https://doi.org/https://doi.org/10.1007/s11240-017-1183-x

31. **Kativat, C. and Tantasawat, P.A.** (2017) PEG-mediated symmetric and asymmetric protoplast fusion in sunflower (*Helianthus annuus* L.). *Pakistan Journal of Botany*, **49**, 1903-1910.

32. **Kinnersley, A.M., Racusen, R.H. and Galston, A.W.** (1978) A comparison of regenerated cell walls in tobacco and cereal protoplasts. *Planta*, **139**, 155-158. https://doi.org/https://doi.org/10.1007/bf00387141

33. **Krkošková, B. and Mrázová, Z.** (2005) Prophylactic components of buckwheat. *Food Research International*, **38**, 561-568. https://doi.org/https://doi.org/10.1016/j.foodres.2004.11.009

34. **Kuki, H., Yokoyama, R., Kuroha, T. and Nishitani, K.** (2020) Xyloglucan is not essential for the formation and Integrity of the cellulose network in the primary cell wall regenerated from *Arabidopsis* protoplasts. *Plants*, **9**, 629. https://doi.org/https://doi.org/10.3390/plants9050629

35. **Lachmann, S. and Adachi, T.** (1990) Studies on the influence of photoperiod and temperature on floral traits in buckwheat (*Fagopyrum esculentum* Moench) under controlled stress conditions. *Plant Breeding*, **105**, 248-253. https://doi.org/https://doi.org/10.1111/j.1439-0523.1990.tb01202.x

36. **Lachmann, S., Kishima, Y. and Adachi, T.** (1994) Protoplast fusion in buckwheat: preliminary results on somatic hybridisation. *Fagopyrum*, **14**, 7-12.

37. **Landi, L., B., M., L., P. and Taruschio Lim, K.Y.** (2001) PEG-mediated fusion of *Rubus idaeus* (raspberry) and *R. fruticosus* (blackberry) protoplasts, selection and characterisation of callus lines. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology*, **135**, 63-69. https://doi.org/https://doi.org/10.1080/11263500112331350660

38. **Lerouxel, O., Cavalier, D.M., Liepman, A.H. and Keegstra, K.** (2006) Biosynthesis of plant cell wall polysaccharides - a complex process. *Current Opinion in Plant Biology*, **9**, 621-630. https://doi.org/http://doi.org/10.1016/j.pbi.2006.09.009

39. **Lewis, D. and Jones, D.A.** (1992) The genetics of heterostyly. In *Evolution and function of heterostyly* (Barrett, S.C.H. ed. Berlin, Heidelberg: Springer, pp. 129-150. https://doi.org/https://doi.org/10.1007/978-3-642-86656-2_5

40. **Long, Y., Yang, Y., Pan, G. and Shen, Y.** (2022) New insights into tissue culture plant-regeneration mechanisms. *Frontiers in Plant Science*, **13**. https://doi.org/http://dpi.org/10.3389/fpls.2022.926752

41. **Ma, Y. and Johnson, K.** (2023) Arabinogalactan proteins - Multifunctional glycoproteins of the plant cell wall. *Cell Surf*, **9**, 100102. https://doi.org/https://doi.org/10.1016/j.tcsw.2023.100102

42. **Mackowska, K., Stelmach-Wityk, K. and Grzebelus, E.** (2023) Early selection of carrot somatic hybrids: A promising tool for species with high regenerative ability. *Plant Methods*, **19**, 104. https://doi.org/https://doi.org/10.1186/s13007-023-01080-4

43. **Matsui, K. and Yasui, Y.** (2020) Genetic and genomic research for the development of an efficient breeding system in heterostylous self-incompatible common buckwheat (*Fagopyrum esculentum*). *Theoretical and Applied Genetics*, **133**, 1641-1653. https://doi.org/https://doi.org/10.1007/s00122-020-03572-6

44. **Mutwil, M., Debolt, S. and Persson, S.** (2008) Cellulose synthesis: A complex complex. *Current Opinion in Plant Biology*, **11**, 252-257. https://doi.org/http://doi.org/10.1016/j.pbi.2008.03.007

45. **Nagata, T. and Takebe, I.** (1970) Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. *Planta*, **92**, 301-308. https://doi.org/https://doi.org/10.1007/BF00385097

46. **Nalinkumar, A. and Singh, P.** (2020) An overview of buckwheat *Fagopyrum* spp. an underutilised crop in India-nutritional value and health benefits. *International Journal of Medical Research and Health Sciences*, **9**, 39-44.

47. **Ndakidemi, C.F., Mneney, E. and Ndakidemi, P.A.** (2014) Effects of ascorbic acid in controlling lethal browning in *in vitro* culture of *Brahylaena huillensis* using nodal segments. *American Journal of Plant Sciences*, **5**, 187-191. https://doi.org/http://dx.doi.org/10.4236/ajps.2014.51024

48. **Nešković, M., Ćulafić, L. and Vujičić, R.** (1995) Somatic embryogenesis in buckwheat (*Fagopyrum* Mill.) and sorrel (*Rumex* L.), Polygonaceae. In *Somatic Embryogenesis and Synthetic Seed II* (Bajaj, Y.P.S. ed. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 412-427. https://doi.org/https://doi.org/10.1007/978-3-642-78643-3_33

49. **Ngomuo, M., Mneney, E. and Ndakidemi, P.** (2014) Control of lethal browning by using ascorbic acid on shoot tip cultures of a local *Musa* spp. (banana) cv. Mzuzu in Tanzania. *African Journal of Biotechnology*, **13**, 1721-1725. https://doi.org/https://doi.org/10.5897/AJB2013.13251

50. **Pedersen, G.B., Blaschek, L., Frandsen, K.E.H., Noack, L.C. and Persson, S.** (2023) Cellulose synthesis in land plants. *Molecular Plant*, **16**, 206-231. https://doi.org/https://doi.org/10.1016/j.molp.2022.12.015

51. **Permadi, N., Akbari, S., Prismantoro, D., Indriyani, N., Nurzaman, M., Alhasnawi, A., Doni, F. and Julaeha, E.** (2024) Traditional and next-generation methods for browning control in plant tissue culture: Current insights and future directions. *Current Plant Biology*, **38**, 100339. https://doi.org/https://doi.org/10.1016/j.cpb.2024.100339

52. **Pilet, P.E., Blaschek, W., Senn, A. and Franz, G.** (1984) Comparison between maize root cells and their respective regenerating protoplasts: Wall polysaccharides. *Planta*, **161**, 465-469. https://doi.org/https://doi.org/10.1007/BF00394579

53. **Ranaware, A.S., Kunchge, N.S., Lele, S.S. and Ochatt, S.J.** (2023) Protoplast technology and somatic hybridisation in the family *Apiaceae*. *Plants*, **12**. https://doi.org/https://doi.org/10.3390/plants12051060

54. **Rao, S.R. and Ravishankar, G.A.** (2002) Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances*, **20**, 101-153. https://doi.org/https://doi.org/10.1016/S0734-9750(02)00007-1

55. **Rumyantseva, N.I., Sal'nikov, V.V. and Lebedeva, V.V.** (2005) Structural changes of cell surface in callus of *Fagopyrum esculentum* Moench. during induction of morphogenesis. *Russian Journal of Plant Physiology*, **52**, 381-387. https://doi.org/https://doi.org/10.1007/s11183-005-0057-y

56. **Rumyantseva, N.I., Salnikov, V.V. and Lebedeva, V.V.** (2004) The study of *Fagopyrum esculentum* callus surface during induction of morphogenesis. In *9th International Symposium on Buckwheat* (Faberová, I., Dvořáček, V., Čepková, P., Hon, I., Holubec, V. and Stehno, Z. ed. Research Institute of Crop Production, Prague, pp. 136.

57. **Safwar, G., Abdul, F. and El Sharbasy, S.** (2015) The effect of some antioxidants on blackening and growth of *in vitro* culture of banana (*Musa* spp. cv. Grand naine). *Egyptian Journal of Genetics and Cytology*, **44**.

58. **Sakamoto, S., Matsui, K., Oshima, Y. and Mitsuda, N.** (2020) Efficient transient gene expression system using buckwheat hypocotyl protoplasts for large-scale experiments. *Breeding Science*, **70**, 128-134. https://doi.org/https://doi.org/10.1270/jsbbs.19082

59. **Shaikh, N.Y., Guan, L. and Adachi, T.** (2002) Ultrastructural aspects on degeneration of embryo, endosperm and suspensor sells following interspecific crosses in the genus *Fagopyrum*. *Breeding Science*, **52**, 171-176. https://doi.org/https://doi.org/10.1270/jsbbs.52.171

60. **Shea, E.M., Gibeaut, D.M. and Carpita, N.C.** (1989) Structural analysis of the cell walls regenerated by carrot protoplasts. *Planta*, **179**, 293-308. https://doi.org/https://doi.org/10.1007/BF00391074

61. **Shin, Y., Chane, A., Jung, M. and Lee, Y.** (2021) Recent advances in understanding the roles of pectin as an active participant in plant signaling networks. *Plants (Basel)*, **10**. https://doi.org/http://doi.org/10.3390/plants10081712

62. **Showalter, A.M.** (1993) Structure and function of plant cell wall proteins. *The Plant Cell*, **5**, 9-23. https://doi.org/https://doi.org/10.1105/tpc.5.1.9

63. **Smetanska, I.** (2018) Sustainable production of polyphenols and antioxidants by plant *in vitro* cultures. In *Bioprocessing of plant in vitro systems. Reference series in phytochemistry*. (Pavlov, A., Bley, T. ed: Springer, pp. 225-269. https://doi.org/https://doi.org/10.1007/978-3-319-54600-1_2

64. **Tagawa, S., Yamagishi, Y., Watanabe, U., Funada, R. and Kondo, T.** (2019) Dynamics of structural polysaccharides deposition on the plasma-membrane surface of plant protoplasts during cell wall regeneration. *Journal of Wood Science*, **65**, 47. https://doi.org/https://doi.org/10.1186/s10086-019-1826-0

65. **Takahata, Y. and Jumonji, E.** (1985) Plant regeneration from hypocotyl section and callus in buckwheat (*Fagopyrum esculentum* Moench.). *Annual report of the Faculty of Education of the University of Iwate*, **45**, 137-142. https://doi.org/https://doi.org/10.15113/00011812

66. **Tan, F., Zhang, K., Mujahid, H., Verma, D.P.S. and Peng, Z.** (2011) Differential histone modification and protein expression associated with cell wall removal and regeneration in rice (*Oryza sativa*). *Journal of Proteome Research*, **10**, 551-563. https://doi.org/https://doi.org/10.1021/pr100748e

67. **Taylor, D.P. and Obendorf, R.L.** (2001) Quantitative assessment of some factors limiting seed set in buckwheat. *Crop Science*, **41**, 1792-1799. https://doi.org/https://doi.org/10.2135/cropsci2001.1792

68. **Thomas, T.D.** (2008) The role of activated charcoal in plant tissue culture. *Biotechnology Advances*, **26**, 618-631. https://doi.org/https://doi.org/10.1016/j.biotechadv.2008.08.003

69. **Tomasiak, A., Zhou, M. and Betekhtin, A.** (2022) Buckwheat in tissue culture research: Current status and future perspectives. *International Journal of Molecular Sciences*, **23**. https://doi.org/https://doi.org/10.3390/ijms23042298

70. **Tomaszewska-Sowa, M.** (2011) Immunocytochemical localization of pectin and arabinogalactan protein epitopes in regenerating tissues of sugar beet (*Beta vulgaris* L.). *Biuletyn Instytutu Hodowli i Aklimatyzacji Roślin*, 157-169. https://doi.org/https://doi.org/10.37317/biul-2011-0065

71. **Tudses, N., Premjet, S. and Premjet, D.** (2015) Establishment of method for protoplast fusion with PEG-mediated between *Jatropha curcas* L. and *Ricinus communis* L. *International Journal of Life Sciences Biotechnology and Pharma Research*, **4**, 50-56.

72. **Urban, K. and Hura, T.** (2023) The use of L-phenylalanine ammonia lyase inhibitors in plant ecophysiological studies. *Postepy Biochem*, **69**, 11-17. https://doi.org/https://doi.org/10.18388/pb.2021_471

73. **Varotto, S., Nenz, E., Lucchin, M. and Parrini, P.** (2001) Production of asymmetric somatic hybrid plants between *Cichorium intybus* L. and *Helianthus annuus* L. *Theoretical and Applied Genetics*, **102**, 950-956. https://doi.org/https://doi.org/10.1007/s001220000485

74. **Vogrinčič, V., Kastelec, D. and Murovec, J.** (2024) Phytosulfokine alpha enhances regeneration of transformed and untransformed protoplasts of *Brassica oleracea*. *Frontiers in Plant Science*, **15**. https://doi.org/https://doi.org/10.3389/fpls.2024.1379618

75. **Wanat, W., Talma, M., Hurek, J., Pawełczak, M. and Kafarski, P.** (2018) Substituted phosphonic analogues of phenylglycine as inhibitors of phenylalanine ammonia lyase from potatoes. *Biochimie*, **151**, 119-127. https://doi.org/https://doi.org/10.1016/j.biochi.2018.06.005

76. **Wang, C., Yu, H., Zhang, Z., Yu, L., Xu, X., Hong, Z. and Luo, L.** (2015) Phytosulfokine is involved in positive regulation of *Lotus japonicus* nodulation. *Molecular Plant-Microbe Interactions*, **28**, 847-855. https://doi.org/https://doi.org/10.1094/MPMI-02-15-0032-R

77. **Wen, F., Su, W., Zheng, H., Yu, B., Ma, Z., Zhang, P. and Guo, W.** (2020) Plant regeneration via protoplast electrofusion in cassava. *Journal of Integrative Agriculture*, **19**, 632-642. https://doi.org/https://doi.org/10.1016/S2095-3119(19)62711-5

78. **Wijerathna-Yapa, A. and Hiti-Bandaralage, J.** (2023) Tissue culture-A sustainable approach to explore plant stresses. *Life (Basel)*, **13**. https://doi.org/https://doi.org/10.3390/life13030780

79. **Wiśniewska, E. and Majewska-Sawka, A.** (2007) Arabinogalactan-proteins stimulate the organogenesis of guard cell protoplasts-derived callus in sugar beet. *Plant Cell Reports*, **26**, 1457-1467. https://doi.org/https://doi.org/10.1007/s00299-007-0348-1

80. **Wiśniewska, E. and Majewska-Sawka, A.** (2008) The differences in cell wall composition in leaves and regenerating protoplasts of *Beta vulgaris* and *Nicotiana tabacum*. *Biologia Plantarum*, **52**, 634-641. https://doi.org/https://doi.org/10.1007/s10535-008-0124-2

81. **Woo, S.H., Kamal, A.H.M., Tatsuro, S., Campbell, C.G., Adachi, T., Yun, Y.H., Chung, K.Y. and Choi, J.S.** (2010) Buckwheat (*Fagopyrum esculentum* Moench.): Concepts, prospects and potential. *The European Journal of Plant Science and Biotechnology*, **4**, 1-16.

82. **Woo, S.H., Ohmoto, T., Campbell, C., Adachi, T. and Jong, S.K.** (2001) Pre-and post-fertilisation to backcrossing in interspecific hybridisation between *Fagopyrum esculentum* and *F. homotropicum* with *F. esculentum*. In *8th International Symposium on Buckwheat*. Chunchon, Korea: Citeseer, pp. 450-455.

83. **Yamane, Y.** (1974) Induced differentiation of buckwheat plants from subcultured calluses *in vitro*. *Genetics Society of Japan*, **49**, 139-146. https://doi.org/https://doi.org/10.1266/jjg.49.139

84. **Yang, X., Tu, L., Zhu, L., Fu, L., Min, L. and Zhang, X.** (2008) Expression profile analysis of genes involved in cell wall regeneration during protoplast culture in cotton by suppression subtractive hybridization and macroarray. *Journal of Experimental Botany*, **59**, 3661-3674. https://doi.org/https://doi.org/10.1093/jxb/ern214

85. **Yasui, Y., Mori, M., Aii, J., Abe, T., Matsumoto, D., Sato, S., Hayashi, Y., Ohnishi, O. and Ota, T.** (2012) *S-LOCUS EARLY FLOWERING 3* is exclusively present in the genomes of short-styled buckwheat plants that exhibit heteromorphic self-incompatibility. *PLOS One*, **7**, 1-9. https://doi.org/https://doi.org/10.1371/journal.pone.0031264

86. **Yokoyama, R., Kuki, H., Kuroha, T. and Nishitani, K.** (2016) *Arabidopsis* regenerating protoplast: A powerful model system for combining the proteomics of cell wall proteins and the visualization of cell wall dynamics. In *Proteomes*, pp. 34. https://doi.org/https://doi.org/10.3390/proteomes4040034

87. **Yui, M., Hayashi, T., Yamamori, M. and Kato, M.** (2004) Inter-specific hybridisation between japanese common buckwheat 'yabukawa-zairai'and *Fagopyrum cymosum* Meissn. In *9th International Symposium on Buckwheat* (Faberová, I., Dvořáček, V., Čepková, P., Hon, I., Holubec, V. and Stehno, Z. ed. Research Institute of Crop Production, Prague, pp. 190.

88. **Zaman, M.S. and Parihar, A.** (2023) Development of novel interspecific hybrid between cultivated and wild species of okra [*Abelmoschus esculentus* (L.)] Moench through embryo rescue. *Indian Journal of Genetics and Plant Breeding*, **83**, 422-432. https://doi.org/https://doi.org/10.31742/ISGPB.83.3.15

89. **Zaranek, M., Pinski, A., Skupien-Rabian, B., Jankowska, U., Godel-Jedrychowska, K., Sala-Cholewa, K., Nowak, K., Kurczyńska, E., Grzebelus, E. and Betekhtin, A.** (2025) The cell colony development is connected with the accumulation of embryogenesis-related proteins and dynamic distribution of cell wall components in *in vitro* cultures of *Fagopyrum tataricum* and *Fagopyrum esculentum*. *BMC Plant Biology*, **25**, 102. https://doi.org/https://doi.org/10.1186/s12870-025-06119-3

13. Author declarations of the publications included in the doctoral thesis

Załącznik nr 9
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Katowice, 23.04.2025
miejscowość, data

mgr Reneé Pérez Pérez
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

A STATEMENT OF THE APPLICANT'S AUTHOR OF THEIR CONTRIBUTION TO THE WORK

I declare that for the following work:

Zaranek, M., Pérez-Pérez, R., Milewska-Hendel, A., Betekhtin, A., Grzebelus, E. (2023). Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn. BMC Plant Biology, 385 (23), 743–757.

My participation consisted of optimising the regeneration medium for *Fagopyrum tataricum* morphogenic calli by trying different combinations of hormones and nutrients. I also participated in reviewing the manuscript.

Reneé Pérez Pérez
.....
Signature of the author of the publication

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

mgr Magdalena Zaranek
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Zaranek, M., Pérez-Pérez, R., Milewska-Hendel, A., Betekhtin, A., Grzebelus, E. (2023), Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn. BMC Plant Biology, 385 (23), 743–757.

My participation consisted of optimising and performing studies on the isolation, culture and regeneration of protoplasts derived from morphogenic callus and hypocotyls; preparing material for histochemical studies; microscopic observations; statistical analysis; analysis and interpretation of the obtained results; and preparing the first and final versions of the manuscript.

Magdalena Zaranek
.....
Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr Anna Milewska-Hendel
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Zaranek, M., Pérez-Pérez, R., Milewska-Hendel, A., Betekhtin, A., Grzebelus, E. (2023). Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn. BMC Plant Biology, 385 (23), 743–757

My participation consisted of performing histological analysis, microscopy, processing results, and preparing the final version of the manuscript.

Anna Milewska-Hendel
.....
Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr hab. inż. Ewa Grzebelus, prof. URK
First and last name of co-author of the publication

Uniwersytet Rolniczy w Krakowie
Affiliation

STATEMENT

I declare that for the following work:

Zaranek, M., Pérez-Pérez, R., Milewska-Hendel, A., Betekhtin, A., Grzebelus, E. (2023). Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn. BMC Plant Biology, 385 (23), 743–757

My participation consisted of developing the concept and basic methodology of isolation, culture and regeneration of protoplasts, as well as providing substantive supervision, analysis and interpretation of the obtained results; proofreading subsequent versions of the manuscript and preparing its final version.

.....

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 14.04.2025

dr hab. Alexander Betekhtin, prof. UŚ
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Zaranek, M., Pérez-Pérez, R., Milewska-Hendel, A., Betekhtin, A., Grzebelus, E. (2023). Promotive effect of phytosulfokine - peptide growth factor - on protoplast cultures development in *Fagopyrum tataricum* (L.) Gaertn. BMC Plant Biology, 385 (23), 743–757

My contribution included obtaining project funding, planning and supervising the research, substantively supervising the research, interpreting the results, and editing all versions of the manuscript. I am the corresponding author.

Alexander Betekhtin

.....

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 9
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Katowice, 23.04.2025
miejscowość, data

mgr Reneé Pérez Pérez
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

A STATEMENT OF THE APPLICANT'S AUTHOR OF THEIR CONTRIBUTION TO THE WORK

I declare that for the following work:

Zaranek, M.*, Pérez-Pérez, R.*, Milewska-Hendel, A., Grzebelus, E., Betekhtin, A. (*equal contribution). (2023). Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench. Plant Cell, Tissue and Organ Culture (PCTOC), 154, 673–687.

* Equal contribution

I shared an equal contribution with Magdalena Zaranek in this study. My involvement included optimising the isolation and culture of protoplasts derived from *Fagopyrum esculentum* embryogenic calli, line NL2. This involved testing two different enzyme solutions (E1 and E2) to improve the cell wall digestion in embryogenic calli, which included different concentrations of Cellulase R10 and the presence of Driselase. I also optimised the regeneration media (MS3 and MS4) by evaluating various hormone types like 6-benzylaminopurine, thidiazuron and kinetin and their concentrations. During the regeneration stage, I monitored the development of somatic embryos, shoots and root formation till whole-plant regeneration. Additionally, I prepared microcalli samples derived from hypocotyl and calli protoplast cultures for histological analysis, which involved fixation with glutaraldehyde and paraformaldehyde, dehydration with ethanol solutions and LR-White resin embedding. Subsequently, I trimmed the resin blocks and performed ultramicrotome sectioning with a diamond knife, stained the sections with Toluidine blue, and conducted the following microscopy imaging of them. Additionally, I contributed to data collection and analysis, with a focus on protoplast viability and developmental from the NL2 line (including cell division, colony formation, and microcalli formation). I also collected the image-based track of somatic embryogenesis until whole plants were regenerated. Furthermore, I processed and interpreted the histological sectioning and its microscopy analysis results. My involvement extended to manuscript preparation, where I authored sections of the Materials and Methods, Results, and Discussion related to NL2 embryogenic calli line, its regeneration in optimised media, histological techniques, and microscopy analyses. I also prepared Figures 1 and 4 and Tables 1 and 2 and actively participated in manuscript revisions and corrections.

Reneé Pérez Pérez
.....
Signature of the author of the publication

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

mgr inż. Magdalena Zaranek
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Zaranek, M.*, Pérez-Pérez, R.*, Milewska-Hendel, A., Grzebelus, E., Betekhtin, A. (*equal contribution). (2023). Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench. Plant Cell, Tissue and Organ Culture (PCTOC), 154, 673–687.

* Equal contribution

I shared an equal contribution with Reneé Pérez-Pérez because my participation consisted of optimising and performing studies on the isolation, culture, and regeneration of protoplasts derived from embryogenic callus (L1) and hypocotyls of *F. esculentum*. Test of eight different enzyme solutions for cell wall digestion during protoplast isolation from hypocotyls (enzyme solutions: E1, E3-E9) and from L1 line (enzyme solution: E1). The differences between enzyme solutions are based on the presence and concentrations of Cellulase R10, Cellulase RS, Macerozyme R10, Pectolyase Y23 and Driselase. I also tested different protoplast culture media variants with several growth regulators like α-phytosulfokine, putrescine, chloropyridin phenylurea and polyvinylpyrrolidone. I optimized the callus multiplication medium (CM) by adding 2,4-dichlorophenoxyacetic acid, kinetin and α-phytosulfokine. I also contributed to preparing microcalli material derived from hypocotyl and calli protoplast cultures for histological studies. I performed microscopic observations of the protoplast development, from the first cell division to plant regeneration. I collect data and perform statistical analysis of protoplast yield and viability, plating efficiency, and interpret the obtained results. I also prepared the first and last versions of the manuscript. The first version of the manuscript includes a description of materials and methods, results and a discussion section concerning hypocotyl and calli (line L1) protoplast cultures. I prepared Figures 1-3 and Figure 5 as well as Tables 3, 4 and 5. After that, I actively participated in making manuscript corrections.

Magdalena Zaranek

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr Anna Milewska-Hendel
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Zaranek, M.*, Pérez-Pérez, R.*, Milewska-Hendel, A., Grzebelus, E., Betekhtin, A. (*equal contribution). (2023). Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench. Plant Cell, Tissue and Organ Culture (PCTOC), 154, 673–687.

* Equal contribution

My participation consisted of supervising the histological and microscopy analysis, processing of the results, and manuscript writing.

Anna Milewska-Hendel

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr hab. inż. Ewa Grzebelus, prof. URK
First and last name of co-author of the publication

Uniwersytet Rolniczy w Krakowie
Affiliation

STATEMENT

I declare that for the following work:
Zaranek, M.*, Pérez-Pérez, R.*, Milewska-Hendel, A., Grzebelus, E., Betekhtin, A. (*equal contribution). (2023). Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench. Plant Cell, Tissue and Organ Culture (PCTOC), 154, 673–687.
* Equal contribution

My participation consisted of developing the concept and basic methodology of isolation, culture and regeneration of protoplasts, as well as providing substantive supervision, analysis and interpretation of the obtained results; proofreading subsequent versions of the manuscript and preparing its final version.

.....
Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 14.04.2025

dr hab. Alexander Betekhtin, prof. UŚ
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Zaranek, M.*, Pérez-Pérez, R.*, Milewska-Hendel, A., Grzebelus, E., Betekhtin, A. (*equal contribution). (2023). Efficient and rapid system of plant regeneration via protoplast cultures of *Fagopyrum esculentum* Moench. Plant Cell, Tissue and Organ Culture (PCTOC), 154, 673–687.

* Equal contribution

My contribution included obtaining project funding, planning and supervising the research, substantively supervising the research, interpreting the results, and editing all versions of the manuscript. I am the corresponding author.

Alexander Betekhtin

.....

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 9
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Katowice, 23.04.2025
miejscowość, data

mgr Reneé Pérez Pérez
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

A STATEMENT OF THE APPLICANT'S AUTHOR OF THEIR CONTRIBUTION TO THE WORK

I declare that for the following work:

Sala-Cholewa, K.*, Milewska-Hendel, A*., Pérez-Pérez, R., Grzebelus, E., Betekhtin, A. (2024). Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells. Plant Cell, Tissue and Organ Culture (PCTOC), 157 (26).

* Equal contribution

My participation consisted of isolating the protoplast from *F. esculentum* and *F. tataricum* calli, optimisation and performance of the electrofusion technique, staining and selection of hybrids and parental protoplasts without cell wall using a single cell micromanipulator; protoplasts cultivation, fixation at different time points of the culture and immunostaining with primary and secondary antibodies, as well as the imaging of the protoplasts in the epifluorescence microscope for the statistical analysis. I also performed three-dimensional image analysis and visualisation using Imaris 9.5 software. In addition, I wrote Materials and Methods section, created the schemes used in the paper using BioRender.com, and participated in the manuscript reviewing and edition.

Reneé Pérez Pérez
.....
Signature of the author of the publication

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr Anna Milewska-Hendel
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Sala-Cholewa, K., Milewska-Hendel, A., Pérez-Pérez, R., Grzebelus, E., Betekhtin, A. (2024). Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells. Plant Cell, Tissue and Organ Culture (PCTOC), 157 (26).

* Equal contribution

I was responsible for executing immunohistochemical labeling of arabinogalactan proteins (AGPs) and extensins in protoplasts culture. Fluorescent signal detection was conducted using confocal and epifluorescence microscopy, focusing on signal intensity profiling and spatial distribution metrics. The resulting data were statistically analysed to evaluate differences across experimental groups. I also prepared illustrative figures and contributed to the interpretation of the results, contextualizing them within the framework of cell wall remodeling processes. I contributed substantively to the drafting and critical revision of the manuscript.

Anna Milewska-Hendel

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr Katarzyna Sala-Cholewa
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Sala-Cholewa, K., Milewska-Hendel, A., Pérez-Pérez, R., Grzebelus, E., Betekhtin, A. (2024). Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells. Plant Cell, Tissue and Organ Culture (PCTOC), 157 (26).

* Equal contribution

I conducted immunolabeling of pectins and hemicelluloses in fixed protoplasts cultures, utilizing monoclonal antibodies specific to distinct polysaccharide epitopes. Imaging was performed using epifluorescence and confocal microscopy, I acquired z-stacks and carried out two-dimensional reconstruction of cell wall architecture. I was also involved in the preparation of figure panels and in the critical interpretation of the results in the context of cell wall organization as well as in writing and revising the manuscript.

Katarzyna Sala-Cholewa
.....
Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr hab. inż. Ewa Grzebelus, prof. URK
First and last name of co-author of the publication

Uniwersytet Rolniczy w Krakowie
Affiliation

STATEMENT

I declare that for the following work:

Sala-Cholewa, K., Milewska-Hendel, A., Pérez-Pérez, R., Grzebelus, E., Betekhtin, A. (2024). Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells. Plant Cell, Tissue and Organ Culture (PCTOC), 157 (26).

* Equal contribution

My participation consisted of developing the concept and basic methodology for isolation, culture and electrofusion of protoplasts. Additionally, I helped with the manuscript edition.

.....

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 14.04.2025

dr hab. Alexander Betekhtin, prof. UŚ
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Sala-Cholewa, K., Milewska-Hendel, A., Pérez-Pérez, R., Grzebelus, E., Betekhtin, A. (2024). Reconstruction pattern of the cell wall in *Fagopyrum* protoplast-derived hybrid cells. Plant Cell, Tissue and Organ Culture (PCTOC), 157 (26).

* Equal contribution

My contribution included obtaining project funding, planning and supervising the research, substantively supervising the research, interpreting the results, and editing all versions of the manuscript. I am the corresponding author.

Alexander Betekhtin

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 9
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Katowice, 23.04.2025
miejscowość, data

mgr Reneé Pérez Pérez
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

A STATEMENT OF THE APPLICANT'S AUTHOR OF THEIR CONTRIBUTION TO THE WORK

I declare that for the following work:

Pérez-Pérez, R.*, Piński, A.*, Zaranek, M., Beckmann, M., Mur, L., Nowak, K., Rojek-Jelonek, M., Kostecka-Gugała, A., Petryszak, P., Grzebelus, E., Betekhtin, A. (2025). Effect of potent inhibitors of phenylalanine ammonia-lyase and PVP on *in vitro* morphogenesis of *Fagopyrum tataricum*. BMC Plant Biology, 25 (469).

*Equal contribution

I shared equal contribution with Artur Piński. My participation involved preparing the stocks and working solutions of the different inhibitors and adsorbents used as media additives (AOA, OBHA, AOPP, AIP, PVP) and optimising their concentrations in the medium. Additionally, I performed the regeneration of *F. tataricum* morphogenic calli on media supplemented with PVP and AIP, measured the total phenolic and flavonoid contents and the regenerated calli mass, and prepared the samples used in all performed analyses. In addition, I tracked the development of the regeneration process, taking pictures of the different treatments at different time points. I also created the schemes used in the paper using BioRender.com and participated in the results interpretation and manuscript writing.

Reneé Pérez Pérez
.....
Signature of the author of the publication

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr Artur Piński
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Pérez-Pérez, R.*, Piński, A.*, Zaranek, M., Beckmann, M., Mur, L., Nowak, K., Rojek-Jelonek, M., Kostecka-Gugała, A., Petryszak, P., Grzebelus, E., Betekhtin, A. (2025). Effect of potent inhibitors of phenylalanine ammonia-lyase and PVP on *in vitro* morphogenesis of *Fagopyrum tataricum*. BMC Plant Biology, 25 (469).

*Equal contribution

I share equal contribution with Reneé Pérez-Pérez because I conceptualised the experiments and supervised all steps of the investigation. I also performed the metabolomics analysis and formal analysis of all experiment results. I participated in the manuscript writing, review and edition. I am the corresponding author.

.....

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

mgr Magdalena Zaranek
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Pérez-Pérez, R.*, Piński, A.*, Zaranek, M., Beckmann, M., Mur, L., Nowak, K., Rojek-Jelonek, M., Kostecka-Gugała, A., Petryszak, P., Grzebelus, E., Betekhtin, A. (2025). Effect of potent inhibitors of phenylalanine ammonia-lyase and PVP on *in vitro* morphogenesis of *Fagopyrum tataricum*. BMC Plant Biology, 25 (469).

*Equal contribution

My participation consisted of conducting studies concerning the isolation and culture of protoplast derived from *Fagopyrum tataricum* morphogenic callus using different concentrations of phenolic biosynthesis inhibitors (AOA, OBHA, AOPP, AIP) and adsorbents (PVP). I tracked the development of protoplast culture by taking photos, making statistical analyses of the protoplast growth parameters, interpreting the obtained results and preparing the final versions of the manuscript.

Magdalena Zaranek
.....
Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 23.04.2025

dr Magdalena Rojek-Jelonek
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Pérez-Pérez, R.*, Piński, A.*, Zaranek, M., Beckmann, M., Mur, L., Nowak, K., Rojek-Jelonek, M., Kostecka-Gugała, A., Petryszak, P., Grzebelus, E., Betekhtin, A. (2025). Effect of potent inhibitors of phenylalanine ammonia-lyase and PVP on *in vitro* morphogenesis of *Fagopyrum tataricum*. BMC Plant Biology, 25 (469).

*Equal contribution

I performed flow cytometry analysis on protoplast- and calli-derived plants to determine the ploidy level of the regenerants.

Magdalena Rojek-Jelonek

.....

Signature of the co-author of the publication

* applies to co-authors

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dania 19 lutego 2024 r.

A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK

Katowice, 15.04.2025

dr hab. Alexander Betekhtin, prof. UŚ
First and last name of co-author of the publication

Uniwersytet Śląski w Katowicach
Affiliation

STATEMENT

I declare that for the following work:

Pérez-Pérez, R.*, Piński, A.*, Zaranek, M., Beckmann, M., Mur, L., Nowak, K., Rojek-Jelonek, M., Kostecka-Gugała, A., Petryszak, P., Grzebelus, E., Betekhtin, A. (2025). Effect of potent inhibitors of phenylalanine ammonia-lyase and PVP on *in vitro* morphogenesis of *Fagopyrum tataricum*. BMC Plant Biology, 25 (469).

*Equal contribution

My contribution included obtaining project funding, planning and supervising the research, substantively supervising the research, interpreting the results, and editing all versions of the manuscript. I am the corresponding author.

Alexander Betekhtin

.....

Signature of the co-author of the publication

* applies to co-authors