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PRACA DOKTORSKA

Badania nad przełamaniem latencji podziałowej, rozwojem kolonii komórkowych oraz regeneracją roślin w kulturach protoplastów *Fagopyrum esculentum* Moench. oraz *Fagopyrum tataricum* (L.) Gaertn.

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Składam również podziękowania rodzinie za wsparcie, motywację oraz wyrozumiałość.

Spis treści

1. Wykaz użytych skrótów

2,4-D – kwas 2,4-dichlorofenoksyoctowy
AGP – białko arabinogalaktanowe (ang. *arabinogalactan protein*)
AIP – kwas 2-aminoindano-2-fosfonowy
B5 – pożywka wg Gamborg
BAP – benzyloaminopuryna
BBM – BABY BOOM
CCOMT – metylotransferaza caffeoyl-CoA (ang. *Caffeoyl-CoA O-methyltransferase*)
CLV3 – CLAVATA3
CPP – pożywka do protoplastów z ogonków liściowych marchwi (ang. *carrot petiole protoplast medium*)
CPPU – N-(2-chloro- 4-pirydynylo) -N′-fenylo- mocznik
CWP – białka ściany komórkowej (ang. *cell wall proteins*)
EK – embriogenny kalus
ENDO – endochitynaza
EP DC-8 – białko embriogenne typu DC8 (ang. *embryogenic protein DC8-like)*
EXT – ekstensyny
FLA – białko arabinogalaktanowe typu Fasciclin
FUS3 – FUSCA3
GalA – kwas galakturonowy
HG – homogalakturonan
KIN – kinetyna
KM – pozywka wg Kao i Michayluk
LEA – białko związane z późną embriogenezą (ang. *late embryogenesis abundant protein)*
LEC – LEAFY COTYLEDON
LMPA – agaroza o niskiej temperaturze topnienia (ang. *low melting point agarose*)
MK – morfogenny kalus
MS – pożywka wg Murashige i Skoog
NAA – kwas 1-naftylooctowy
NK – niemorfogenny kalus
OLEO – oleozyna
OLEO4 – oleozyna 4
PAL – liaza fenyloalaniny
PEKK – proembriogenne kompleksy komórkowe
PSK – fitosulfokina α
PUT – putrescyna
PVP – poliwinylopirolidon (ang. *polyvinylpyrrolidone*)
RGI – ramnogalakturonan I (ang. *rhamnogalactunonan* I)

RGII – ramnogalakturonan II (ang. *rhamnogalactunonan* II)
SBP – białko zawierające biotynę (ang. *seed biotin protein*)
SSPs – białka zapasowe nasion (ang. *seed storage proteins*)
TDZ – tidiazuron
TFs – czynniki transkrypcyjne (ang. *transsription factors*)
VIC – vicilina
WUS – WUSCHEL

2. Wykaz publikacji wchodzących w skład rozprawy doktorskiej

Publikacja 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**, 23, 385
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Publikacja 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
*równorzędny pierwszy autor

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

IF$_{2023}$: 2.3
Punkty MNiSW: 100

Publikacja 3 (P3):
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*ogy, **2025**, 25, 102
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IF$_{2023}$: 4.3
Punkty MNiSW: 140

3. Konferencje i wystąpienia

Wyniki otrzymane w rozprawie doktorskiej zaprezentowano na następujących konferencjach i spotkaniach naukowych:

1. **VI Polski Kongres Genetyki**, 27-30 czerwca 2022, Kraków

   Tytuł wystąpienia: Promotive effect of some growth factors and inhibitor of phenolic compounds on protoplasts development in *Fagopyrum tataricum*; **prezentacja ustna**

2. **9th Central European Congress of Life Sciences EuroBiotech**, 27-28 czerwca 2024, Kraków

   Tytuł wystąpienia: From single cell to plants: protoplasts of *Fagopyrum* as an efficient system for acquiring totipotency. Insight from the proteomics point of view; **poster**

3. **XVI Ogólnopolska Konferencja Kultur *In Vitro* i Biotechnologii Roślin**, 23-25 września 2024, Kraków

   Tytuł wystąpienia: Zmiany proteomiczne podczas przeprogramowania komórek *Fagopyrum esculentum* i *F. tataricum;* **prezentacja ustna**

4. **1st European Network for Protoplast regeneration and Microspore embryogenesis research**. Sympozjum zorganizowane przez Uniwersytet Gandawski, 9-10 września 2024, Gandawa, Belgia

   Tytuł wystąpienia: From single cell to plants: protoplasts of *Fagopyrum* as an efficient system for acquiring totipotency; **prezentacja ustna**

Praca doktorska została wykonana w ramach badań realizowanych w projekcie finansowanym przez Narodowe Centrum Nauki pt.: **Procesy reprogramowania komórek: analiza epigenetyczna i proteomiczna losów komórek gryki zwyczajnej i tatarki**
Nr grantu: 2020/37/B/NZ9/01499
Projekt w ramach konkursu OPUS 19, realizowany w latach 2021-2025
Kierownik projektu: dr hab. Alexander Betekhtin, prof. UŚ

W trakcie realizacji pracy doktorskiej odbyłam trzymiesięczny staż zagraniczny w ramach programu Erasmus+ na Université Toulouse III Paul Sabatier w Tuluzie (Francja) w Laboratorium Badawczym Nauk o Roślinach, pod kierownictwem dr Elisabeth Jamet. Podczas stażu zdobyłam teoretyczną wiedzę oraz praktyczne umiejętności z zakresu biologii molekularnej, przygotowania wektorów ekspresyjnych oraz ekstrakcji i separacji białek roślinnych, jak również rutynowo posługiwałam się systemem klonowania Golden Gate.

4. Streszczenie

Niniejsza praca koncentruje się na dwóch najczęściej uprawianych gatunkach gryki: gryce zwyczajnej (*Fagopyrum esculentum* Moench) oraz gryce tatarce (*F. tataricum* (L.) Gaertn). Rośliny te stanowią naturalne źródło biologicznie aktywnych substancji, ze względu na wysoką zawartość związków fenolowych, flawonoidów oraz białek. Jednak ich plonowanie odznacza się niską wydajnością i niestabilnością, co ogranicza uprawę gryki w Polsce i Europie. W celu zwiększenia plonu oraz poprawy wybranych cech gryki istotne znaczenie mają kultury *in vitro*. Kultura protoplastów stanowi użyteczne narzędzie, które w połączeniu w technikami inżynierii genetycznej może być wykorzystane w programach hodowlanych w celu poszerzenia zmienności genetycznej.

Pierwszym etapem badań prezentowanej rozprawy doktorskiej było przeprowadzenie szeregu eksperymentów mających na celu opracowanie warunków skutecznej kultury protoplastów w/w gatunków gryki. W związku z czym zbadano wpływ: 1) materiału donorowego do izolacji protoplastów, 2) podłoża do immobilizacji protoplastów, 3) fitosulfokiny, 4) auksyn i cytokinin oraz 5) absorbentów i inhibitorów związków fenolowych na aktywność podziałową w kulturze protoplastów. Wykazano między innymi, że kalus charakteryzujący się wysokim potencjałem regeneracyjnym jest lepszym materiałem donorowym w kulturach protoplastów w porównaniu do hipokotyli siewek. Podobnie, immobilizacja protoplastów w agarozie korzystnie wpływa na ich rozwój w porównaniu do immobilizacji w alginianie. Ponadto udowodniono, że suplementacja bazowej pożywki w fitosulfokinę skutecznie przełamuje latencję podziałową w kulturach protoplastów gryki. Natomiast zastosowanie odpowiedniego zestawu cytokinin do regeneracji kalusa otrzymanego z kultury protoplastów pozwoliło na regenerację roślin w stosunkowo krótkim czasie tj. 2-5 miesięcy od momentu regeneracji.

Zaobserwowane różnice w dynamice rozwoju kultury protoplastów oraz sposobie regeneracji stały się podstawą drugiej części badań ukierunkowanej na przeprowadzenie bardziej szczegółowych analiz. Analizie poddano trzy punkty czasowe kultury, podczas których obserwowano pierwsze podziały komórkowe, formowanie kolonii komórkowych oraz rozwój minikalusa. Celem tych badań było poznanie zmian jakie zachodzą w 1) rozmieszczeniu wybranych komponentów ściany komórkowej, 2) proteomie oraz 3) ekspresji wybranych genów i czynników transkrypcyjnych związanych z somatyczną embriogenezą podczas rozwoju kolonii komórkowych w kulturze protoplastów.

Analiza przestrzenno-czasowego rozmieszczenia składników ściany komórkowej ujawniła zmienną dystrybucję łańcuchów bocznych pektyn (ramnogalakturonanu I) jak również ekstensyn, co świadczy o różnicowaniu się kolonii komórkowych. Analiza proteomiczna wykazała wzrost akumulacji białek związanych z gromadzeniem substancji zapasowych jak również białek związanych z somatyczną embriogenezą, co może wskazywać na przygotowanie kultury do zdarzeń somatycznej embriogenezy.

Uzyskane wyniki, mogą pomóc w zrozumieniu kluczowych procesów zachodzących podczas rozwoju kultury protoplastów prowadzących w efekcie końcowym do regeneracji roślin. W szerszej perspektywie wyniki te mogą przyczynić się do rozwoju przyszłych programów hodowlanych gryki.

5. Summary

This study focuses on two of the most commonly cultivated buckwheat species: common buckwheat (*Fagopyrum esculentum* Moench) and Tartary buckwheat (*F. tataricum* (L.) Gaertn). These plants are a natural source of biologically active compounds due to their high phenolic compounds, flavonoids, and protein content. However, the yield of these plants is low and unstable, which limits the cultivation of buckwheat in Poland and Europe. *In vitro* cultures play a significant role in enhancing yield and improving selected traits of buckwheat. Protoplast culture is a valuable tool that, combined with genetic engineering techniques, can be used in breeding programs to expand genetic variability.

The first stage of the research presented in this doctoral dissertation involved conducting experiments to develop conditions for an effective protoplast culture of these two buckwheat species. Therefore, the influence of the following factors was examined (1) donor material for protoplast isolation, (2) immobilization medium for protoplasts, (3) phytosulfokines, (4) auxins and cytokinins, and (5) absorbents and inhibitors of phenolic compounds on cell division activity in protoplast cultures. The study demonstrated that callus with high regenerative potential is a more suitable donor material for protoplast cultures than seedling hypocotyls. Similarly, immobilization of protoplasts in agarose positively influenced their development compared to immobilization in alginate. Furthermore, it was shown that supplementation of the basal medium with phytosulfokine effectively breaks cell division latency in buckwheat protoplast cultures. Using an appropriate cytokinin combination for callus regeneration derived from protoplast cultures enabled plant regeneration within a relatively short period, ranging from 2 to 5 months.

Observed differences in the dynamics of protoplast culture development and regeneration pathways are the basis for the second part of the study, which focused on more detailed analyses. Three time points of culture development were examined: cell divisions, cell colony formation, and minicallus development. These investigations aimed to understand the changes occurring in (1) the spatial distribution of selected cell wall components, (2) proteome, and (3) the expression of selected genes and transcription factors associated with somatic embryogenesis during cell colony development in protoplast cultures.

The spatiotemporal analysis of cell wall component distribution revealed a variable localization of pectin (rhamnogalacturonan I) side chains and extensins, indicating differentiation within the cell colonies. Proteomic analysis showed an increased accumulation

of proteins involved in storage compound accumulation and proteins associated with somatic embryogenesis, suggesting that the culture was preparing for somatic embryogenesis events.

The obtained results may contribute to a better understanding of key processes occurring during the development of protoplast culture, ultimately leading to plant regeneration. From a broader perspective, these findings may support the advancement of future buckwheat breeding programs.

6. Wstęp

6.1. Gryka i jej znaczenie

Gryka jest rośliną dwuliścienną, należącą do rodzaju *Fagopyrum* (rodzina Polygonaceae), który obejmuje 22 gatunki występujące głównie na wyżynach Eurazji (Zhang *i inni*, 2015; Jha *i inni*, 2024). Szeroko uprawiane są tylko trzy gatunki, tj. gryka zwyczajna (*Fagopyrum esculentum* Moench), gryka tatarka (*Fagopyrum tataricum* Gaertn) i *Fagopyrum cymosum* (Trevir.) Meisn., spośród których *F. esculentum* jest jedynym gatunkiem powszechnie uprawianym i wykorzystywanym do spożycia w Polsce (Zarzecka, 2014). Ze względy na skład chemiczny nasion oraz agrotechnikę uprawy gryka zaliczana jest do pseudozbóż (Hornyak *i inni*, 2023). Wynika to przede wszystkim z wysokiej wartości odżywczej gryki związanej z dużą zawartością białka, flawonoidów, a także obecnością witamin o właściwościach antyoksydacyjnych, czy brakiem glutenu w nasionach (Zarzecka, 2014; Sytar *i inni*, 2018). Co więcej, gryka obfituje w różne związki fenolowe, w szczególności rutynę, kwercetynę i C-glikozyloflawony (orientynę, izoorientynę, witeksynę), które mają korzystne właściwości terapeutyczne i dietetyczne, sprzyjające zdrowiu człowieka (Joshi *i inni*, 2020). W porównaniu do gryki zwyczajnej, gryka tatarka zawiera więcej związków fenolowych, nie tylko w poszczególnych organach, ale także na różnych etapach wzrostu roślin (Gupta *i inni*, 2011). Z punktu widzenia wzrostu roślin, flawonoidy zmniejszają stres środowiskowy, a wysoki poziom rutyny i rutynozydazy prowadzi do powstawania kwercetyny, która odpowiada za silną gorycz nasion tym samym chroniąc rośliny przed spożyciem ze strony zwierząt (Kreft *i inni*, 2022). Jednak w kulturach *in vitro* związki fenolowe mogą wykazywać toksyczne działanie, ograniczając podziały komórkowe, hamując wzrost tkanek, a także prowadzić do brązowienia eksplantatów oraz pożywki (Cvikrová *i inni*, 2003). Głównym wyzwaniem w uprawie gryki zwyczajnej jest niski oraz niestabilny plon, który wynika z samoniezgodności, krótkiego czasu życia pojedynczego kwiatu (około jednego dnia), aborcji kwiatów, zarodków oraz nasion. Niejednorodność dojrzewania, pękanie oraz kiełkowanie nasion przed zbiorem, jak również wrażliwość na stresy biotyczne i abiotyczne to główne wady tych dwóch gatunków uprawnych, które obecnie stanowią wyzwanie w programach hodowli gryki (Jha *i inni*, 2024).

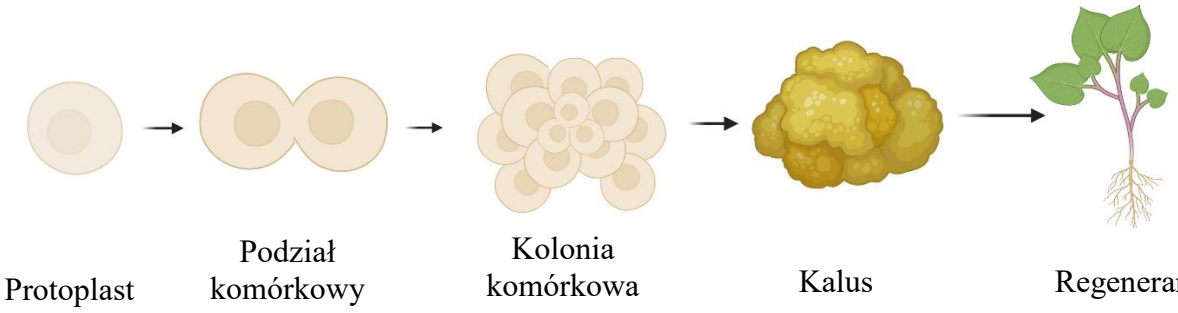
6.1.1. Kalus gryki

Kultury *in vitro* mogą być pomocne w procesach ulepszania roślin, selekcji genotypów o pożądanych cechach, propagacji materiału roślinnego oraz modyfikacjach genetycznych. Dzięki zoptymalizowanym i powtarzalnym protokołom prowadzenia kultur *in vitro* jak również posiadaniu stabilnego pod względem genetycznym i regeneracyjnym materiału roślinnego możliwe jest sprostanie wyżej wymienionym celom, jak również wspomnianym problemom gryki. Wykazano, że morfogenny kalus (MK) *F. tataricum*, charakteryzuje się stabilnym genomem jądrowym przez okres nawet dziesięciu lat prowadzenia kultury *in vitro* oraz wysokim potencjałem regeneracyjnym (Betekhtin *i inni*, 2017). Kalus ten składa się z proembriogennych kompleksów komórkowych (PEKK, Rycina 1a, czerwone strzałki) i „miękkiego" kalusa (Rycina 1a, białe strzałki), który pojawia się cyklicznie podczas rozwoju i dezintegracji PEKK, pełniąc funkcję „niańki". Według danych histologicznych ten typ kalusa zawiera trzy warstwy: (1) warstwę komórek bogatych w związki fenolowe, (2) warstwę komórek merystematycznych oraz (3) warstwę komórek parenchymatycznych. Zachodzące cykle endoreduplikacji w MK prawdopodobnie prowadzą do powstawania niemorfogennego kalusa (NK, Rycina 1b), który charakteryzuje się kruchą strukturą i jest niestabilny genetycznie (Betekhtin *i inni*, 2017). Embriogenny kalus (EK) *F. esculentum* różni się morfologią i strukturą od kalusa *F. tataricum* (Rycina 1c), jest mleczno-biały i globularny. Wykazano, że w centralnej części posiada komórki parenchymatyczne zawierające ziarna skrobi, które pokryte są kilkoma warstwami komórek merystematycznych. Według danych literaturowych kalus ten odznacza się najwyższą zdolnością do morfogenezy (Gumerova *i inni*, 2003; Rumyantseva *i inni*, 2005). Kalus obu gatunków, ze względu na wysoką zdolność regeneracyjną, może stanowić znakomity materiał wyjściowy w różnych systemach roślinnych kultur *in vitro*. M. in. może być wykorzystany w kulturach protoplastów, które stanowią użyteczne narzędzie zarówno w badaniach podstawowych jak i aplikacyjnych (Eeckhaut *i inni*, 2013; Jiang *i inni*, 2013).

Rycina 1 Morfogenny (a) i niemorfogenny kalus (b) *F. tataricum;* embriogenny kalus *F. esculentum* (c). Czerwone strzałki wskazują proembriogenne kompleksy komórkowe (PEKK), białe strzałki wskazują miękki kalus. Podziałka: 1 mm. Fotografie Magdalena Zaranek

6.2. Protoplasty roślinne

Protoplasty to żywe komórki roślinne, których ściana komórkowa została całkowicie lub częściowo usunięta za pomocą metod mechanicznych lub enzymatycznych, otoczone błoną komórkową (Jiang *i inni*, 2013). Teoretycznie protoplasty są totipotentne, co oznacza, że mają zdolność do odróżnicowania, odtworzenia ściany komórkowej, ponownego wejścia w cykl komórkowy, przejścia przez szereg podziałów mitotycznych, a następnie przekształcenia się w niezorganizowany tkankowo kalus, pojedynczy organ lub całą roślinę (Eeckhaut *i inni*, 2013); Rycina 2). Chociaż znane są protokoły regeneracji roślin z protoplastów dla ponad kilkuset gatunków, skuteczność aplikacji systemów protoplastowych w odniesieniu do wielu roślin uprawnych wciąż jest niska lub sporadyczna, zarówno w zakresie regeneracji całych roślin, jak i tworzenia kalusa (Davey *i inni*, 2005a; Davey *i inni*, 2005b). Do tej pory znanych było zaledwie kilka doniesień na temat kultur protoplastów w rodzaju *Fagopyrum*. W przypadku *F. esculentum* udało się otrzymać rośliny z kultury protoplastów hipokotylowych, jednakże charakteryzowały się one nieprawidłową morfologią (Adachi *i inni*, 1989) lub odnotowywano niski potencjał regeneracyjny kalusa uzyskanego po kulturze protoplastów (Gumerova, 2004). W przypadku *F. tataricum* do chwili obecnej nie odnotowano doniesień związanych z otrzymaniem roślin poprzez kulturę protoplastów. Publikacje stanowiące podstawę niniejszego doktoratu jako pierwsze opisują efektywną regenerację roślin gryki zwyczajnej oraz gryki tatarki poprzez kulturę protoplastów. Ostanie badania przeprowadzone przez Sala-Cholewa *i inni* (2024) dostarczają informacji na temat odbudowy ściany komórkowej podczas pierwszych 72 godzin kultury protoplastów w/w gatunków jak również ich mieszańców. Przedstawione wyniki wskazują na różnice w czasoprzestrzennym rozmieszczeniu poszczególnych epitopów ściany komórkowej.



Rycina 2 Model rozwoju kultury protoplastów. Schemat wykonany za pomocą platformy BioRender (https://app.biorender.com). Prawo do publikacji poświadczone licencją.

6.2.1. Kultura protoplastów

Na uzyskanie wydajnego i powtarzalnego narzędzia opartego na kulturze protoplastów wpływa wiele czynników, takich jak metoda izolacji protoplastów, wydajność i jakość wyizolowanych komórek, technika prowadzenia kultury oraz skład pożywki (Rahmani *i inni*, 2016). Wiele składowych wpływa na sam proces izolacji, wśród których można wyróżnić: materiał donorowy do izolacji protoplastów, skład mieszaniny enzymatycznej trawiącej ścianę komórkową, technikę izolacji jak również warunki prowadzenia wspomnianych etapów. Wszystkie te czynniki istotnie wpływają na wydajność izolacji, kondycję oraz żywotność komórek. Jeśli parametry izolacji dobrane są poprawnie, to średnio z grama świeżej masy powinno się uzyskać około $10^5 - 10^6$ protoplastów. Zwykle testowane są różne odmiany/genotypy oraz rodzaje materiału donorowego, aby wybrać ten o najlepszej odpowiedzi rozwojowej oraz największym potencjale regeneracyjnym (Davey *i inni*, 2005a; Grzebelus *i inni*, 2012a; Kiełkowska i Adamus, 2017; Kiełkowska i Adamus, 2019; Reed i Bargmann, 2021).

Stosowane są różne techniki prowadzenia kultury protoplastów od pożywek stałych po płynne, co ma istotne znaczenie dla rozwoju komórek. Jednakże immobilizacja protoplastów w zestalających się (agar, agaroza) lub polimeryzujących (alginian) podłożach, a następnie umieszczenie w płynnym medium zapobiega agregacji oraz obumieraniu komórek, zmniejsza produkcję polifenoli oraz zapewnia lepszą dyfuzję substancji odżywczych jak również szkodliwych metabolitów (Davey *i inni*, 2005a; Deryckere *i inni*, 2012). Wykazano stymulujące działanie alginianu na podziały mitotyczne w kulturach protoplastów *Brassica napus*, *Brassica oleracea*, *Beta vulgaris*, *Daucus carota* czy *Nicotiana tabacum* (Dovzhenko *i inni*, 1998; Grzebelus *i inni*, 2012a; Grzebelus *i inni*, 2012b; Kiełkowska i Adamus, 2012;

Mackowska *i inni*, 2014). Z kolei zastosowanie agarozy o niskiej temperaturze topnienia (LMPA, ang. *low melting point agarose*) do immobilizacji protoplastów *Cichorium*, *Ulmus americana* i *Artemisia judaica* okazało się być bardziej efektywne dla rozwoju kultury w porównaniu z zastosowaniem płynnej lub stałej pożywki (Pan *i inni*, 2003; Deryckere *i inni*, 2012; Jones *i inni*, 2015).

Istotnym czynnikiem stymulującym kulturę protoplastów jest pożywka, która dostarcza niezbędne substancje do wzrostu i rozwoju komórek. Dobranie odpowiednich składników odżywczych, suplementów czy regulatorów wzrostu jest niezbędne w celu indukcji podziałów mitotycznych, formowania kolonii komórkowych oraz ich dalszego różnicowania w rośliny (Davey *i inni*, 2005b). Auksyny i cytokininy mają szczególne znaczenie dla rozwoju i różnicowania komórek, jednak kluczowym aspektem często decydującym o powodzeniu kultury, jest odpowiedni dobór ich rodzaju, stężenia oraz wzajemnej proporcji (Davey *i inni*, 2005b; Reed i Bargmann, 2021). Większość pożywek do kultury protoplastów bazuje na składnikach pożywki Murashige i Skoog (1962; **MS**) lub Gamborg (1968; **B5**), choć często stosuje się bogatą w związki mineralne i organiczne formułę Kao i Michayluk (1975; **KM**). Przykładowo, po zastosowaniu pożywki opartej na formule KM w porównaniu z MS w kulturze protoplastów *Beta vulgaris* zaobserwowano wyższą żywotność protoplastów, a w kulturze protoplastów *Kalanchöe* odnotowano podziały komórek (Grzebelus *i inni*, 2012b; Cui *i inni*, 2019).

Powszechnym podejściem wspierającym podziały w kulturze protoplastów, rozwój kolonii komórkowych (ang. *cell colonies*) oraz formowanie minikalusa jest dodatek suplementów, takich jak peptydowe czynniki wzrostu, poliaminy czy substancje zdolne do absorpcji lub hamowania syntezy związków fenolowych. Doskonałym przykładem peptydowych czynników wzrostu jest fitosulfokina (PSK) – siarczanowany pentapeptyd, który promuje podziały komórkowe, wzrost i proliferację komórek, ale także stosowany jest w celu indukcji somatycznej embriogenezy u gatunków opornych do tworzenia zarodków somatycznych (Ochatt *i inni*, 2018; Hao *i inni*, 2023; Luo *i inni*, 2024). Jak wykazały badania, zastosowanie PSK umożliwiło przełamanie latencji podziałowej w kulturze protoplastów *Beta vulgaris* (Grzebelus *i inni*, 2012b), stymulowało podziały komórkowe oraz zwiększyło zdolności regeneracyjne protoplastów *B. oleracea* var. *capitata* L. (Kiełkowska i Adamus, 2017; Kiełkowska i Adamus, 2019) oraz wybranych gatunków w rodzaju *Daucus* (Mackowska *i inni*, 2014).

Inne związki, takie jak poliaminy regulują procesy ekspresji genów (replikację, transkrypcję oraz translację) (Davey *i inni*, 2005b), a także pełnią funkcje ochronne przed

stresem oksydacyjnym w kulturach *in vitro* (Majewska-Sawka *i inni*, 1997; Mackowska *i inni*, 2014; Kiełkowska i Adamus, 2021). W kulturze protoplastów *Nicotiana tabacum* odnotowano, że putrescyna (PUT) zapobiega programowanej śmierci protoplastów i tym samym pozytywnie wpływa na żywotność komórek (Papadakis i Roubelakis-Angelakis, 2005). Wykazano pozytywne oddziaływanie PUT na podziały komórkowe oraz formowanie kolonii komórkowych w kulturze protoplastów *Alnus glutinosa* i *Alnus incana* (Huhtinen *i inni*, 1982), jak również na aktywność mitotyczną komórek oraz regenerację pędów w kulturze protoplastów *B. oleracea* (Kiełkowska i Adamus, 2021).

W celu poprawy rozwoju kultury protoplastów stosowane są również absorbenty lub inhibitory związków fenolowych. Powstające produkty utleniania związków fenolowych w kulturach *in vitro* stanowią duże wyzwanie ze względu na skutki uboczne jakie wywołują: hamują wzrost tkanek, prowadzą do brązowienia (obumierania) kultury, zmniejszają tempo regeneracji, jak również przyczyniają się do gromadzenia często szkodliwych substancji w podłożu hodowlanym (Jones i Saxena, 2013). Dlatego też, aby zmniejszyć zawartość związków fenolowych stosuje się absorbenty związków fenolowych lub inhibitory liazy fenyloalaniny (PAL, enzym szlaku syntezy związków fenolowych). Wykazano, że zastosowanie absorbentu poliwinylopirolidonu (PVP) w kulturze protoplastów *Cyamopsis tetragonoloba* i *Vitis* zredukowało brązowienie materiału roślinnego (Saxena i Gill, 1986; Reustle i Natter, 1994). Z kolei zastosowanie inhibitora PAL jakim jest kwas 2-aminoindano-2-fosfonowy (AIP) w kulturze protoplastów *Ulmus americana* wpłynęło pozytywnie na wydajność izolacji protoplastów, odbudowę ściany komórkowej, podziały komórek oraz zmniejszyło brązowienie zawiesiny i kultury kalusa (Johnson *i inni*, 2003; Jones *i inni*, 2012).

6.2.2. Podatność komórek roślinnych na przeprogramowanie

Dzięki temu, że komórki roślinne charakteryzują się wysoką plastycznością rozwojową oraz zdolnością do przeprogramowania, możliwe jest odtworzenie tkanek, organów, a nawet całego organizmu roślinnego (Pasternak *i inni*, 2020). Przeprogramowanie jest procesem, w którym zróżnicowane komórki lub tkanki przerywają swoją pierwotną drogę rozwojową, wchodzą w program odróżnicowania, by następnie odzyskać zdolność do różnicowania i odbudowy nowych tkanek (Sugimoto *i inni*, 2019). Podczas odróżnicowania komórki zwiększają swój potencjał rozwojowy, nabywają kompetencji do zmiany wzorca rozwojowego, jak również odzyskują zdolność do podziałów mitotycznych (Fehér, 2015). Proces odróżnicowania wiąże się z izolacją protoplastów, która zachodzi w wyniku zranienia tkanki, zakłócenia komunikacji

międzykomórkowej oraz poddania komórek działaniu czynników stresowych. Również formowanie kalusa jest efektem odróżnicowania zróżnicowanych wcześniej komórek (Fehér, 2015). Odróżnicowanie protoplastów przebiega poprzez indukcję procesu przeprogramowania, epigenetyczną przebudowę chromatyny oraz nabycie totipotencji rozwojowej. Uważa się, że powrót do cyklu komórkowego jak również odzyskanie zdolności podziałowej jest klasyfikowane jako jeden z etapów odróżnicowania komórek w tym protoplastów. Badania pokazały, że przywrócenie cyklu komórkowego związane jest z modyfikacją struktury i fizjologii komórek, procesów molekularnych, czy zmiany w architekturze wakuoli (Sheahan *i inni*, 2007; Pasternak *i inni*, 2020). Odróżnicowanie jak również regeneracja inicjowane są przez sygnały środowiskowe, takie jak uszkodzenie ściany komórkowej, stres osmotyczny czy bodźce fizyczne, co prowadzi do przerwania komunikacji międzykomórkowej (przez uszkodzenie plazmodezm lub śmierć sąsiednich komórek) i skutkuje przeprogramowaniem. Dodatkowo, endogenne i egzogenne hormony roślinne stosowane w kulturach *in vitro*, wpływają na zmiany w fazach cyklu komórkowego, jak również na stan zróżnicowania komórek (Sugimoto *i inni*, 2019). W przypadku roślin jednoliściennych, niektóre komórki mogą ponownie wejść w cykl komórkowy, jednak ich zdolności regeneracyjne są nietrwałe i zwykle szybko zanikają. U roślin jeno- jaki i dwuliściennych proces ten zależy od takich czynników jak genotyp, wiek komórki czy stan fizjologiczny (Pasternak *i inni*, 2020).

Zaobserwowano, że podczas przeprogramowania dochodzi do reorganizacji chromatyny, zmiany we wzorach ekspresji genów jak również zmian w składzie i architekturze ściany komórkowej (Potocka *i inni*, 2018). Jednakże mechanizmy będące podstawą tych procesów nie są do końca rozpoznane i zrozumiałe pomimo dużej liczby multidyscyplinarnych badań (Fehér, 2015). Źródła podają, że formowaniu embriogennego kalusa towarzyszą także zmiany w składzie oraz architekturze ściany komórkowej (Smertenko i Bozhkov, 2014; Elhiti i Stasolla, 2022; Hesami *i inni*, 2023).

6.3. Ściana komórkowa

Ściana komórkowa jest ważnym jak również dynamicznym kompartmentem komórek roślinnych, uczestniczącym w procesach rozwojowych (podziałach komórek, różnicowaniu) oraz w odpowiedzi na stresy biotyczne i abiotyczne. Głównymi komponentami pierwotnej ściany komórkowej odpowiadającymi za właściwości mechaniczne i warunkującymi jej strukturę są polisacharydy takie jak celuloza, pektyny i hemiceluloza. Z kolei białka ściany komórkowej odpowiadają za dynamikę oraz re-aranżację polisacharydów (San Clemente *i inni*, 2022). Celuloza jest głównym a zarazem najprostszym komponentem ściany komórkowej stanowiącym swego rodzaju rusztowanie do osadzania się pozostałych komponentów. Pektyny tworzą silnie usieciowioną żelową matrycę (ang. *gel-like matrix*), w której osadzone są pozostałe polimery, a ich podstawową jednostką jest kwas galakturonowy (GalA). Wśród pektyn wyróżnia się: homogalakturonan (HG) oraz ramnogalakturonan: ramnogalakturonan I (RG-I) oraz ramnogalakturonan II (RG-II). HG to liniowy homopolimer złożony z GalA, syntetyzowany w postaci zmetylowanej. Po demetylacji HG dochodzi do wiązania $Ca^{2+,}$ wskutek czego zmieniają się właściwości fizyczne ściany tj. następuje usztywnienie ściany, zwiększa się jej wytrzymałość, jak również dochodzi do zahamowania ekspansji komórek (Palin i Geitmann, 2012; Haas *i inni*, 2021). RG-I jest polisacharydem, którego szkielet główny zbudowany jest z powtarzającego się disacharydu GalA oraz ramnozy, do których dołączone są boczne łańcuchy galaktanu, arabinianu lub arabinogalaktanu - ich zmienna dystrybucja (obecność oraz przemiany) skorelowana jest z rozwojem oraz stanem zróżnicowania komórek (Srivastava *i inni*; Sala *i inni*, 2013; Potocka *i inni*, 2018). Arabiniany przeważają w młodych komórkach na wczesnych etapach ich rozwoju po podziale, podczas gdy komórki rozwijające się posiadają więcej galaktanów (Saffer, 2018). Wiąże się to z właściwościami arabinianów, które zapewniają elastyczność ścian komórkowych podczas podziałów oraz rozwoju komórek (Jones *i inni*, 2003; Moore *i inni*, 2013), z kolei występowanie łańcuchów bocznych galaktanów związane jest ze wzmacnianiem ścian. Struktura RG-II bazuje na szkielecie HG do którego przyłączone są rozgałęzione łańcuchy cukrowe. RG-II występuje w ścianie jako dimer kowalencyjnie połączony z borem, który warunkuje rozmiar porów w ścianie komórkowej oraz jej stabilność (Fleischer *i inni*, 1999). Hemiceluloza jest drugim po celulozie najliczniej występującym polisacharydem ściany komórkowej utrzymującym oraz regulującym jej strukturę poprzez łączenie ze sobą mikrofibryli celulozowych oraz regulowanie przestrzeni pomiędzy nimi. Wśród grupy hemiceluloz można wyróżnić ksyloglukany, ksylany, mannany

oraz kalozę. Ksyloglukany są głównymi komponentami pierwotnej ściany komórkowej roślin dwuliściennych, z kolei u roślin jednoliściennych występują ksylany (Hoch, 2007).

Białka ściany komórkowej (CWP, ang. *cell wall proteins*), odgrywają kluczową rolę w jej funkcjonowaniu. Uczestniczą w procesach modyfikacji struktury ściany, pełnią funkcje w przekazywaniu sygnałów międzykomórkowych oraz znacząco przyczyniają się do wzmacniania integralności mechanicznej całej struktury (Albenne *i inni*, 2013). Dominującymi CWP są bogate w hydroksyprolinę glikoproteiny takie jak ekstensyny (EXTs, ang. *extensins*) oraz białka arabinogalaktanowe (AGPs, ang. *arabinogalactan proteins*). EXT należą do białek strukturalnych, które odgrywają istotną rolę w procesie formowania ściany komórkowej, jak również zapewniają strukturalną integralność oraz wzmacniają jej strukturę (Albenne *i inni*, 2014). Wspomniane właściwości EXT związane są z kowalencyjnym sieciowaniem się sąsiednich łańcuchów EXT oraz wchodzeniem w interakcje z pektynami. Sieciowanie EXT jest niezbędne w celu utrzymania architektury oraz integralności ściany komórkowej jak również jej rozwoju (Mishler-Elmore *i inni*, 2021). Badania podają, że synteza EXT zachodzi na wczesnych etapach zakładania ściany komórkowej, ponieważ uczestniczą one w pierwszych podziałach komórkowych podczas embriogenezy będąc komponentami płytki komórkowej (Hall i Cannon, 2002; Cannon *i inni*, 2008). EXTs przypisuje się również rolę w modulowaniu ściany komórkowej w odpowiedzi na uszkodzenia mechaniczne, stres oraz infekcje patogenów (Mishler-Elmore *i inni*, 2021). Z kolei białkami sygnalizacyjnymi ściany komórkowej są wysoce glikozylowane AGPs zbudowane z polipeptydu bogatego w hydroksyprolinę, do którego przyłączone są długie oraz silnie rozgałęzione łańcuchy cukrowe oraz w przypadku części białek AGPs, kotwica glikozylofosfatydyloinozytolu umiejscawiająca białko na zewnętrznej powierzchni błony komórkowej. AGPs są kowalencyjnie związane ze ścianą komórkową, tworzą kompleksy z pektynami oraz ksylanami przez co stabilizują ścianę, jak również odgrywają rolę plastyfikatorów pektyn (ang. *pectic plasticizers*) rozluźniając sieć pektyn w warunkach stresowych (Rumyantseva, 2005; Albenne *i inni*, 2014; Leszczuk *i inni*, 2023). AGPs pełnią funkcję w różnych aspektach wzrostu i rozwoju komórek, reakcjach stresowych, morfogenezie, somatycznej embriogenezie oraz wzroście roślin (Leszczuk *i inni*, 2023). Do grupy białek sygnalizacyjnych należą również białka arabinogalaktanowe typu Fasciclin (FLAs, ang. *fasciclin-like arabinogalactan proteins*), które oprócz regionów glikozylowanych podobnych do AGP posiadają domeny o potencjale do interakcji białko-białko (domeny Fascyclinowe). Białka te pełnią funkcje strukturalne, sygnalizacyjne, regulują właściwości ściany komórkowej, ułatwiają adhezje komórek, co rozumie się przez interakcje komórka-komórka oraz komórka-macierz komórkowa (Johnson *i inni*, 2003; Johnson *i inni*,

2011). Zmiany w strukturze i składzie komponentów ściany komórkowej mogą być markerem zmian w kierunku różnicowania się komórek podczas procesu somatycznej embriogenezy, wzrostu rośliny oraz rozwoju. Oprócz tego ściana komórkowa może regulować procesy morfogenetyczne, jak również los komórek poprzez ustalanie i/lub utrzymywanie stanu zróżnicowania komórkowego (Potocka *i inni*, 2018).

6.4. Czynniki transkrypcyjne oraz białka związane z embriogenezą

Jak wspomniano powyżej rozwój kalusa wiąże się z przeprogramowaniem, co może skutkować złożonymi zmianami na poziomie ekspresji genów oraz proteomu (Tan *i inni*, 2013). Spośród różnych czynników kontrolujących przeprogramowanie komórek roślinnych i uczestniczących w nabywaniu lub przywracaniu kompetencji embriogennych, analizie poddawane są geny kodujące czynniki transkrypcyjne (TFs, ang. *transcription factors*) należące do grupy genów *LEAFY COTYLEDON* (*LEC*), która obejmuje geny: *LEC1, LEC2* i FUS 3 (*FUSCA3*). Geny te związane są z wieloma aspektami dotyczącymi embriogenezy roślin, nabywania oraz ustalania kompetencji embriogennych, jak również zaangażowane są w różnicowanie oraz rozwój zarodków somatycznych (Gaj *i inni*, 2005). Wspomniane geny regulują metabolizm cukrów, ekspresję genów kodujących białka zapasowe nasion (SSPs, ang. *seed storage proteins*), syntezę substancji zapasowych nasion (białek i lipidów), czy biosyntezę kwasów tłuszczowych (Gaj *i inni*, 2005; Kagaya *i inni*, 2005; Mu *i inni*, 2008; Nowak i Gaj, 2016). Geny z grupy *LEC* regulowane są przez gen *BABY BOOM* (*BBM*) będący czynnikiem transkrypcyjnym. *BBM* uczestniczy w biosyntezie auksyn i zaangażowany jest w kontrolę podziałów komórkowych, modyfikację ściany komórkowej, różnicowanie organów roślinnych, jak również nabywanie kompetencji embriogennych (Gaj *i inni*, 2005; Kulinska-Lukaszek *i inni*, 2012; Horstman *i inni*, 2017).

Warto również wspomnieć, że obecność substancji zapasowych może być wskaźnikiem potencjału embriogennego kultury, a co za tym idzie określać zdolności regeneracyjne. Sahara *i inni* (2023) wykazali zwiększoną regulację białek zapasowych podobnych do vicilin (ang. *vicillin-like proteins*) w kalusie charakteryzującym się wysokim poziomem embriogennym spekulując jednocześnie, że niska ich ekspresja w kalusie o niskim potencjale embriogennym może wiązać się z ograniczoną zdolnością do procesów embriogennych (Sahara *i inni*, 2023). Również Gliwicka *i inni* (2012) wykazała wyższą ekspresję genów kodujących oleozynę 4 (*OLEO4*) - białko otaczające oleosom, w embriogennej kulturze *Arabidopsis* w porównaniu do kultury nieembriogennej. Ponadto lipidy odgrywają ważną rolę podczas somatycznej

embriogenezy biorąc udział w tworzeniu i rozwoju struktur embriogennych, a zmiany w metabolizmie i składzie lipidów mogą wpływać na wydajność embriogenezy, czyniąc lipidy ważnymi regulatorami w tym procesie (Avjioglu i Knox, 1989; Dutta *i inni*, 1991).

Wiele białek zostało zaklasyfikowanych do grupy białek związanych z somatyczną embriogenezą jak np. grupa białek związanych z późną embriogenezą (LEA, ang. *late embryogenesis abundant protein*) oraz chitynazy (Gulzar *i inni*, 2020). Białka LEA na ogół akumulują się w odpowiedzi na stres biotyczny oraz abiotyczny, zapewniając funkcje ochronne (Amara Imen *i inni*, 2014). Stwierdzono, że ekspresja białka podobnego do DC-8 należącego do grupy LEA zachodzi w zarodkach somatycznych oraz zygotycznych w przeciwieństwie do zróżnicowanych tkanek (Hatzopoulos *i inni*, 1990). Z kolei endochitynazy należące do grupy chitynaz uwalniają cząsteczki sygnalne będące induktorami somatycznej embriogenezy (Hengel, 1998; Gulzar *i inni*, 2020).

7. Uzasadnienie podjęcia tematu badawczego

Gryka jest doskonałym naturalnym źródłem związków biologicznie aktywnych, co wynika z wysokiej zawartości związków fenolowych o działaniu antyoksydacyjnym (Joshi *i inni*, 2020). Jednakże nie jest powszechnie uprawiana w Polsce i Europie głównie ze względu na niską wydajność plonowania, przyzwyczajenia żywieniowe społeczeństwa oraz ograniczoną wiedzę na temat wartości odżywczych i korzystnych właściwości (Zhou *i inni*, 2018). Jednym z kluczowych problemów w uprawie gryki zwyczajnej jest niski i niestabilny plon, wynikający z samoniezgodności, krótkiej żywotności pojedynczego kwiatu, a także często występującej aborcji kwiatów, zarodków i nasion. W przypadku gryki tatarki jednym z głównych ograniczeń uprawy jest gorzki smak produktów uzyskiwanych po przetworzeniu, spowodowany wysoką zawartością rutyny. Dodatkowe trudności w uprawie obu gatunków to nierównomierne dojrzewanie, pękanie i przedwczesne kiełkowanie nasion przed zbiorem, a także duża podatność na stresy biotyczne i abiotyczne. Czynniki te stanowią istotne wyzwanie w programach hodowlanych gryki. Podejmowane próby krzyżowego zapylenia *F. homotropicum* z *F. tataricum* oraz *F. tataricum* z *F. esculentum*, mające na celu transfer genów zwiększających mrozoodporność i plonowanie, zakończyły się niepowodzeniem. Przyczyną były post-zygotyczne bariery biologiczne, które skutecznie uniemożliwiają krzyżowanie między tymi gatunkami (Shaikh *i inni*, 2001; Woo *i inni*, 2001).

Ze względu na szerokie możliwości zastosowania kultur *in vitro,* a w szczególności kultury protoplastów, nie tylko w badaniach podstawowych, ale również w badaniach aplikacyjnych uwzględniających m.in. poszerzenie zmienności genetycznej roślin uprawnych, istotnym może być opracowanie efektywnego systemu regeneracji roślin *F. esculentum* oraz *F.tataricum* w kulturze protoplastów. Dzięki dynamicznemu rozwojowi inżynierii genetycznej jak również poznaniu genów odpowiedzialnych za wymienione powyżej problemy spotykane w uprawie gryki możliwe jest wykorzystanie w przyszłości narzędzi bazujących na kulturze protoplastów do generowania roślin o zmienionych cechach, jak również regenerantów wolnych od obcego materiału genetycznego poprzez zastosowanie kompleksów rybonukleoproteinowych Cas9. Nieudane próby przeniesienia korzystnych cech pomiędzy gatunkami *Fagopyrum* z wykorzystaniem konwencjonalnych metod hodowli (krzyżowania generatywne), mogą zostać przezwyciężone poprzez fuzję protoplastów prowadzącą do wytworzenia międzygatunkowych mieszańców, z ominięciem barier pre- i post-zygotycznych. W związku z powyższym, brak skutecznego protokołu regeneracji protoplastów gryki stał się podstawą do podjęcia badań, które są przedmiotem niniejszej pracy doktorskiej.

Kultury protoplastów są również dobrym modelem badawczym w celu poznania mechanizmów rozwoju i różnicowania komórek. Jak do tej pory procesy i zmiany zachodzące podczas rozwoju kultury protoplastów, które prowadzą do formowania kalusa o potencjale regeneracyjnym są słabo poznane.

8. Hipotezy i cele badawcze

Szerokie możliwości zastosowania kultury protoplastów wraz z technikami inżynierii genetycznej, a także chęć zgłębienia kluczowych elementów kierujących jej rozwojem, stały się motywacją do podjęcia badań zaprezentowanych w niniejszej rozprawie doktorskiej. Badania te koncentrowały się na: 1) doborze właściwego materiału donorowego do kultury protoplastów, 2) procesach zachodzących od pierwszych podziałów komórkowych do formowania kolonii komórkowych oraz 3) powstawaniu minikalusa oraz regeneracji roślin.

Przed podjęciem badań przyjęto następujące hipotezy badawcze:

1. Morfogenny kalus *F. tataricum* oraz embriogenny kalus *F. esculentum*, ze względu na stabilność genetyczną i wysoki potencjał regeneracyjny, są właściwymi materiałami donorowymi do kultur protoplastów.

2. Rodzaj zastosowanego podłoża do immobilizacji protoplastów wpływa na ich zdolności rozwojowe.

3. Suplementacja pożywki do kultury protoplastów w fitosulfokinę oraz wybrane auksyny i cytokininy przełamuje latencję podziałową w kulturze protoplastów *Fagopyrum.*

4. Zastosowanie absorbentów i inhibitorów związków fenolowych wpływa pozytywnie na zdolności rozwojowe kultury protoplastów.

5. Wybrane cytokininy wpływają na przebieg somatycznej embriogenezy w kulturach gryki zwyczajnej.

6. Procesom rozwoju kolonii komórkowych w kulturze protoplastów towarzyszą zmiany dystrybucji komponentów ściany komórkowej, całkowitego proteomu oraz czynników

transkrypcyjnych, które mogą wskazywać na różnicowanie się komórek oraz odzyskiwanie kompetencji embriogennych.

Aby zweryfikować postawione hipotezy, wyznaczono następujące cele badawcze:

- Opracowanie wydajnego i szybkiego systemu regeneracji roślin *F. tataricum* oraz *F. esculentum* w kulturach protoplastów (**P1**, **P2**);

- Zbadanie wpływu: 1) sposobu immobilizacji protoplastów, 2) fitosulfokiny , 3) rodzaju i stężenia auksyn i cytokinin oraz 4) absorbentów i inhibitorów związków fenolowych na aktywność podziałową w kulturach protoplastów *F. tataricum* oraz *F. esculentum* (**P1**, **P2**);

- Dobór rodzaju oraz stężenia cytokinin promujących somatyczną embriogenezę w kulturach gryki zwyczajnej a tym samym regenerację roślin po kulturze protoplastów (**P1**, **P2**);

- Poznanie procesów i zmian, jakie zachodzą w dystrybucji komponentów ściany komórkowej, proteomie oraz ekspresji wybranych genów podczas rozwoju kolonii komórkowych w kulturze protoplastów (**P3**).

9. Materiały i metody

Materiał roślinny

W badaniach wykorzystano dwa gatunki gryki: komercyjnie dostępną odmianę gryki zwyczajnej (*F. esculentum*) Panda, pozyskaną z Małopolskiej Hodowli Roślin (Polska) oraz grykę tatarkę (*F. tataricum*), genotyp k-17, której nasiona otrzymano od Instytutu Roślinnych Zasobów Genetycznych im. N. I. Wawiłowa, Sankt Petersburg, Rosja.

Kalus EK i MK obu gatunków indukowany był z niedojrzałych zarodków zygotycznych, z kolei NK *F. tataricum* pojawił się na powierzchni MK po około dwóch latach hodowli, wówczas namnażany był oddzielnie (Betekhtin *i inni*, 2017; Betekhtin *i inni*, 2019). Dla pozyskania niedojrzałych zarodków zygotycznych, nasiona gryki wysiewano do doniczek z ziemią i wernikulitem w stosunku 3:1, i uprawiano w szklarni (25±1°C; fotoperiod: 16/8 h (światło/ciemność); oświetlenie emitujące światło białe o intensywności: 90 μmol m^{-2} s^{-1}). Następnie w odpowiednim momencie tj. gdy nasiona były miękkie i jasnozielone izolowano z nich niedojrzałe zarodki zygotyczne.

Do indukcji oraz namnażania kalusa obu gatunków zastosowano pożywkę RX (Betekhtin *i inni*, 2017) zawierającą: mikro- i makroelementy oraz witaminy według Gamborga (Gamborg *i inni*, 1968), 2 g L^{-1} N-Z aminę A (Sigma-Aldrich), 2 mg L^{-1} kwas 2,4-dichlorofenoksyoctowy (2,4-D, Sigma-Aldrich), 0,2 mg L^{-1} kinetynę (KIN, Sigma-Aldrich), 0,5 mg L^{-1} kwas indolilo-3-octowy (Sigma-Aldrich), 0,5 mg L^{-1} kwas 1-naftylooctowy (NAA, Sigma-Aldrich), 25 g L^{-1} sacharozę (Chempur) i 7 g L^{-1} fitoagar (Duchefa). Kultury kalusa prowadzono w szklanych szalkach Petriego w ciemności (26°C ±1). Kalus gryki zwyczajnej (EK) pasażowano na świeżą pożywkę co 2-3 tygodnie, z kolei kalus gryki tatarki (MK i NK) co dwa tygodnie.

Metody badawcze

Badania prowadzono w oparciu o następujące techniki:

- izolacja i kultura protoplastów (**P1**, **P2** i **P3**);
- kultura i namnażanie kalusa oraz regeneracja roślin - wg zoptymalizowanego protokołu przedstawionego w publikacji **P1** i **P2**;
- utrwalanie materiałów roślinnych z wykorzystaniem paraformaldehydu, zatapianie w żywicy London Resin (LR White, Polysciences Inc.), krojenie preparatów z użyciem ultramikrotomu (Leica Biosystems) (**P1**, **P2**, **P3**);
- barwienie materiałów roślinnych do analiz histologicznych z zastosowaniem błękitu toluidyny O (**P1**, **P2**, **P3**);
- barwienia immunocytochemiczne, z wykorzystaniem przeciwciał do wykrywania epitopów ściany komórkowej (1) polisacharydów: pektyn (LM20, LM5, LM6), hemicelulozy (LM25) oraz (2) białek: AGPs (JIM13, JIM16), EXT (JIM20) (**P3**);
- barwienie lipidów z wykorzystaniem Sudanu III oraz Sudanu Czarnego (**P3**);
- izolacja białek całkowitych oraz analiza proteomiczna z wykorzystaniem techniki chromatografii cieczowej ze spektrometrią mas (LC-MS/MS) (**P3**);
- ilościowa reakcja łańcuchowa polimerazy w czasie rzeczywistym (RT-qPCR) do analizy ekspresji czynników transkrypcyjnych oraz genów kodujących: (1) białka związane z somatyczną embriogenezą, (2) białka zapasowe nasion oraz (3) białka ściany komórkowej (**P3**).

Obserwacje mikroskopowe prowadzono z wykorzystaniem:

- mikroskopu odwróconego Axiovert S100 (Carl Zeiss,Niemcy) (**P1**, **P2**)
- mikroskopu jasnego pola Olympus BX43F (Olympus LS, Japonia) (**P1**, **P2**, **P3**)
- mikroskopu epifluorescencyjnego Axio Imager Z2 Zeiss (Carl Zeiss) (**P3**)
- mikroskopu cyfrowego Keyence VHX-970F (Japonia) (**P1**, **P2**, **P3**)

Szczegółowy opis zastosowanych metod badawczych i eksperymentów, uzyskanych wyników oraz obserwacji znajduje się w publikacjach wchodzących w skład rozprawy doktorskiej oznaczonych jako **P1**, **P2**, **P3**. Publikacje umieszczono kolejno po każdym omówieniu badań. W celu uniknięcia autoplagiatu w niniejszej pracy doktorskiej ryciny zawarte w publikacjach oznaczone zostały jako Figures lub Tables.

10. Omówienie wyników prowadzonych badań

10.1. Publikacja 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, 23, 385 https://doi.org/10.1186/s12870-023-04402-9

Celem pierwszej publikacji było zidentyfikowanie czynników stymulujących rozwój protoplastów gryki tatarki oraz opracowanie systemu regeneracji roślin tego gatunku, opartego na kulturze protoplastów. Aby wybrać materiał donorowy o najlepszej zdolności do rozwoju oraz największym potencjale regeneracyjnym, protoplasty izolowano z NK (Figure 2a), czterech linii MK (linia: MK1, MK2, MK4, NL2018, Figure 2b-e) oraz hipokotyli siewek (Figure 2f). Linie MK różniły się wiekiem i miały 10-, 4- i 2- lata (odpowiednio MK1 i MK2; NL2018; MK4). Dodatkowo, kalus linii NL2018 miał bardziej miękką strukturę i drobniejsze PEKK (Figure 2e). Protoplasty izolowano wg protokołu Grzebelus *i inni* (2012a), który został zoptymalizowany pod kątem gryki. Procedura izolacji protoplastów polegała na wstępnym mechanicznym rozdrobnieniu materiału, preplazmolizie oraz enzymatycznym trawieniu ściany komórkowej. Kolejnym etapem była izolacja właściwa protoplastów ze zmacerowanej tkanki obejmująca: filtrowanie przez filtry nylonowe, wirowanie w gradiencie gęstości otrzymanego przesączu oraz kolejne tury wirowania w celu oczyszczenia wyizolowanych protoplastów. W początkowym etapie badań zoptymalizowano skład mieszaniny maceracyjnej do izolacji protoplastów oceniając wydajność izolacji w przeliczeniu na gram świeżej masy oraz żywotność komórek w dniu izolacji, jako miernik ich jakości, a tym samym wskaźnik dobrania optymalnej metody izolacji. Mieszanina maceracyjna zawierająca celulazę (1%) i pektoliazę (0,1%) pozwoliła na izolację protoplastów z kalusa. Średnia wartość wydajności izolacji dla NK wynosiła 0,43 × 10^6 protoplastów, a w przypadku linii MK wartość ta mieściła się w zakresie od 2,30 do 3,93 × 10^6 protoplastów (Table 1). Warto zauważyć, że najwięcej protoplastów uzyskano z linii NL2018 (3,93 × 10^6). Uwolnienie protoplastów z hipokotyli było możliwe po zastosowaniu mieszaniny maceracyjnej składającej się z celulazy (1%), macerozymy (0,6%) oraz driselazy (0,1; 0,15; 0,25%) otrzymując, w zależności od stężenia driselazy, od 0,39 do 0,71 × 10^6 wyizolowanych protoplastów (Table 2). Przy czym średnia wartość izolacji protoplastów z hipokotyli była pięć razy mniejsza w porównaniu do linii MK.

Ważnym czynnikiem warunkującym rozwój protoplastów i obejmującym kolejno odtworzenie ściany komórkowej, reinicjację podziałów komórkowych, formowanie kolonii komórkowych[1] oraz kalusa jest jakość uwolnionych protoplastów wyrażana poprzez określenie żywotności komórek bezpośrednio po ich izolacji. Parametr ten dla protoplastów kalusowych wynosił: 55% dla NK, od 64 do 78% dla linii MK (Table 1). Żywotność protoplastów hipokotylowych różniła się w zależności od zastosowanego stężenia driselazy w mieszaninie maceracyjnej. Największą wartością tego parametru (82%) odznaczały się komórki wyizolowane po zastosowaniu 0,25% driselazy (Table 2).

W przeprowadzonych badaniach rodzaj podłoża (agaroza, alginian) do immobilizacji protoplastów *Fagopyrum*, jak również zestaw regulatorów wzrostu (auksyn i cytokinin) zoptymalizowano bazując na symptomach rozwojowych protoplastów izolowanych z NK. W 10. dniu kultury protoplastów NK zliczano komórki wykazujące symptomy pre-mitotyczne takie jak: powiększenie komórek, zmiana kształtu komórek ze sferycznego na owalny, reorganizacja cytoplazmy. Obserwacje tych symptomów umożliwiły wytypowanie agarozy (LMPA) jako lepszego podłoża do immobilizacji protoplastów, dla której odnotowano dwukrotnie większą liczbą komórek o w/w symptomach w porównaniu do immobilizacji w alginianie (Figure 4a). W kontekście doboru rodzaju oraz stężenia auksyny i cytokininy, największą liczbę komórek (16%) z pozytywnymi symptomami odnotowano dla pożywki wzbogaconej w 0,2 mg L^{-1} KIN i 3,0 mg L^{-1} 2,4-D, natomiast najmniejszą (6,7%) dla pożywki zawierającej 0,2 mg L^{-1} KIN i 2,0 mg L^{-1} BAP (Figure 4b). W toku dalszych optymalizacji, na podstawie doświadczeń przeprowadzonych dla kultury protoplastów MK1 wytypowano 2,0 mg L^{-1} NAA i 1,0 mg L^{-1} BAP jako wzorcowy zestaw hormonów stanowiący uzupełnienie zmodyfikowanej pożywki CPP (ang. *carrot petiole protoplast medium*) (Dirks *i inni*, 1996) opartej na formule KM. Pożywka ta stanowiła pożywkę bazową do prowadzenia kultury protoplastów *Fagopyrum*, a w/w wzorcowy zestaw hormonów doprowadził do formowania minikalusa w kulturze protoplastów. Na etapie doboru linii kalusowej, do dalszych badań wskazano jedynie linię NL2018, dla której w w/w warunkach kultury odnotowano podziały komórkowe. Wzbogacenie pożywki bazowej do kultury protoplastów w PSK umożliwiło przełamanie latencji podziałowej protoplastów linii NL2018 oraz hipokotylowych.

[1] W publikacjach **P1** oraz **P2** dla kolonii komórkowych przyjęto termin agregaty komórkowe (ang. *cell aggregates*). W publikacji **P3**, uwzględniając sugestie recenzentów, dla określenia struktur powstających na skutek podziałów mitotycznych pojedynczej komórki, przyjęto termin kolonie komórkowe (ang. *cell colonies*).

Pierwsze podziały komórkowe obserwowano piątego dnia kultury, natomiast formowanie kolonii komórkowych odnotowano ósmego dnia (Figure 3d). W celu zwiększenia liczby tworzących się kolonii komórkowych testowano dodatkowe suplementy jak należącą do poliamin putrescynę (PUT) oraz syntetyczną cytokininę typu mocznika N-(2-chloro- 4-pirydynylo) -N′-fenylo- mocznik (CPPU). W celu absorpcji związków fenolowych i toksycznych metabolitów zastosowano PVP, a dla zahamowania syntezy związków fenolowych AIP. Wpływ testowanych związków oceniono na podstawie wydajności kultury (ang. *platting efficiency*) ocenianej 10. dnia kultury, zdefiniowanej jako liczba tworzących się kolonii komórkowych w stosunku do wszystkich obserwowanych obiektów (tj. komórek niepodzielonych oraz kolonii komórkowych). Wydajność kultury protoplastów MK w 10. dniu jej prowadzenia wynosiła od 14 do 18% (Figure 5) i zależała od zastosowania PUT oraz CPPU. Po suplementacji AIP wartość ta mieściła się w przedziale od 12 do 21%, jednak obserwowane różnice nie były statystycznie istotne (Figure 5). W przypadku kultury protoplastów hipokotylowych wydajność wahała się od 25 do 41% (Figure 6). Dodatek AIP i PVP nie wpłynął na wydajność kultury. Zaobserwowano natomiast dwukrotnie wyższą efektywność kultury protoplastów hipokotylowych w porównaniu do kalusowych.

W ośmiotygodniowej kulturze protoplastów na powierzchni kropel agarozowych (ang. *agarose beads*) zaobserwowano minikalus, a intensywność jego występowania zależała od materiału donorowego z którego pochodziły protoplasty. Intensywniejszy wzrost kalusa w kroplach agarozowych obserwowano w kulturach protoplastów hipokotylowych niż w kulturach protoplastów kalusowych (MK, NK). Zaobserwowano również, że zastosowanie PVP zredukowało unoszące się na powierzchni płynnej pożywki metabolity oraz brązowienie minikalusa.

Przeprowadzone analizy histologiczne ujawniły różnice w charakterze otrzymanego minikalusa zależnie od zastosowanego materiału donorowego do izolacji protoplastów. Materiał pochodzący z protoplastów NK składał się z cienkościennych komórek parenchymatycznych o zróżnicowanych rozmiarach (Figure 7a), z dużą wakuolą i nieregularnym jądrem zlokalizowanym przy ścianie komórkowej (Figure 7a, wstawka). W przypadku materiału z kultury protoplastów MK (linia NL2018) analiza histologiczna wskazała na obecność niejednorodnego kalusa, w którym wyróżniono kilka typów komórek (Figure 7b-d): na powierzchni komórki zawierające związki fenolowe (Figure 7b, czarna strzałka) charakteryzujące się dużą, centralnie położoną wakuolą w której występowały związki fenolowe (Figure 7b, wstawka, czarne strzałki); kolejną warstwę komórek stanowiły komórki merystematyczne (Figure 7b, czerwona gwiazdka), cechujące się obecnością kilku wakuol,

gęstą cytoplazmą i okrągłym jądrem z jednym bądź dwoma jąderkami (Figure 7c, czerwona strzałka), z kolei centralną cześć stanowiły komórki parenchymatyczne (Figure 7b,czarna gwiazdka). Odnotowano również występowanie komórek embriogennych o bardzo gęstej cytoplazmie, z licznymi drobnymi wakuolami i dużym, okrągłym jądrem zawierającym jedno jąderko (Figure 7d, czerwone strzałki). W materiale z kultury protoplastów hipokotylowych, analiza histologiczna ujawniła obecność licznych komórek parenchymatycznych (Figure 7e), wypełnionych wakuolą zajmującą prawie całą objętość komórki, jądro w tych komórkach miało nieregularny kształt, znajdowało się przyściennie w pobliżu błony komórkowej (Figure 7e, wstawka), zawierało przeważnie od jednego do trzech jąderek (Figure 7e, wstawka). Stwierdzono również obecność komórek gromadzących związki fenolowe w wakuoli (Figure 7e, czarne strzałki).

Wyniki analizy histologicznej jak również struktura kalusa uzyskanego po kulturze protoplastów pozwoliła wskazać, z których materiałów możliwe będzie uzyskanie regenerantów. Brak regeneracji odnotowano dla kalusa pochodzącego z protoplastów NK, charakteryzującego się kruchą strukturą (Figure 3g), zbudowanego z komórek z nieregularnymi, wielojąderkowymi jądrami, prawdopodobnie poliploidalnymi, co mogło być przyczyną braku zdolności do regeneracji. Podobne cechy budowy morfologicznej i histologicznej wykazywał materiał donorowy do izolacji protoplastów tj. NK (Betekhtin *i inni,* 2017). Podobnie, nie zregenerowano roślin z kultury protoplastów hipokotylowych - mogło być to także spowodowane zwiększeniem poliploidalności komórek budujących materiał poddany regeneracji. Jak wskazywali Pasternak *i inni* (2020) zastosowanie hipokotyli jako materiału donorowego do izolacji protoplastów wiąże się ze wzrostem poziomu ploidalności komórek, dodatkowo polisomatyczna natura gryki tatarki opisana przez Betekhtin *i inni* (2017) może tłumaczyć brak zdolności tej tkanki do regeneracji. W pełni wykształcone, o prawidłowej budowie rośliny zregenerowano w kulturze protoplastów MK, gdzie stwierdzono obecność PEKK, składających się z komórek embriogennych oraz obszarów zawierających komórki merystematyczne. Tkanka ta pod względem morfologicznym oraz histologicznym była podobna do materiału donorowego, który według Betekhtin *i inni* (2017) jest doskonałym przykładem utrzymania stabilności genetycznej oraz potencjału regeneracyjnego w długotrwałej hodowli.

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

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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

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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

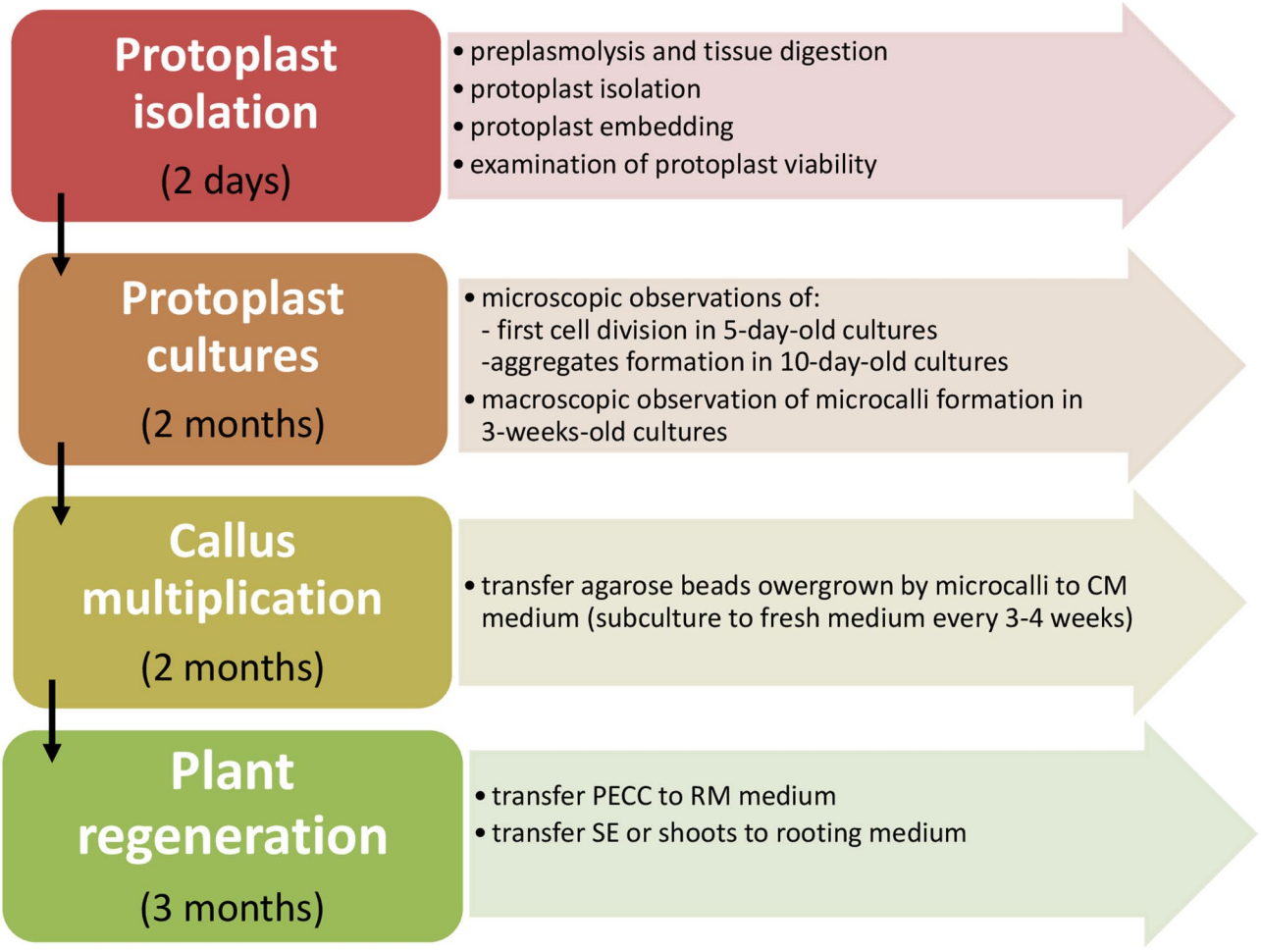

Fig. 1 Flow chart illustrating a step-by-step approach for plant regeneration via protoplast cultures of *Fagopyrum tataricum*. Details are described in the method section. *CM* callus multiplication medium; *PECC* pro-embryogenic cell complexes; *RM* regeneration medium; *SE* somatic embryos

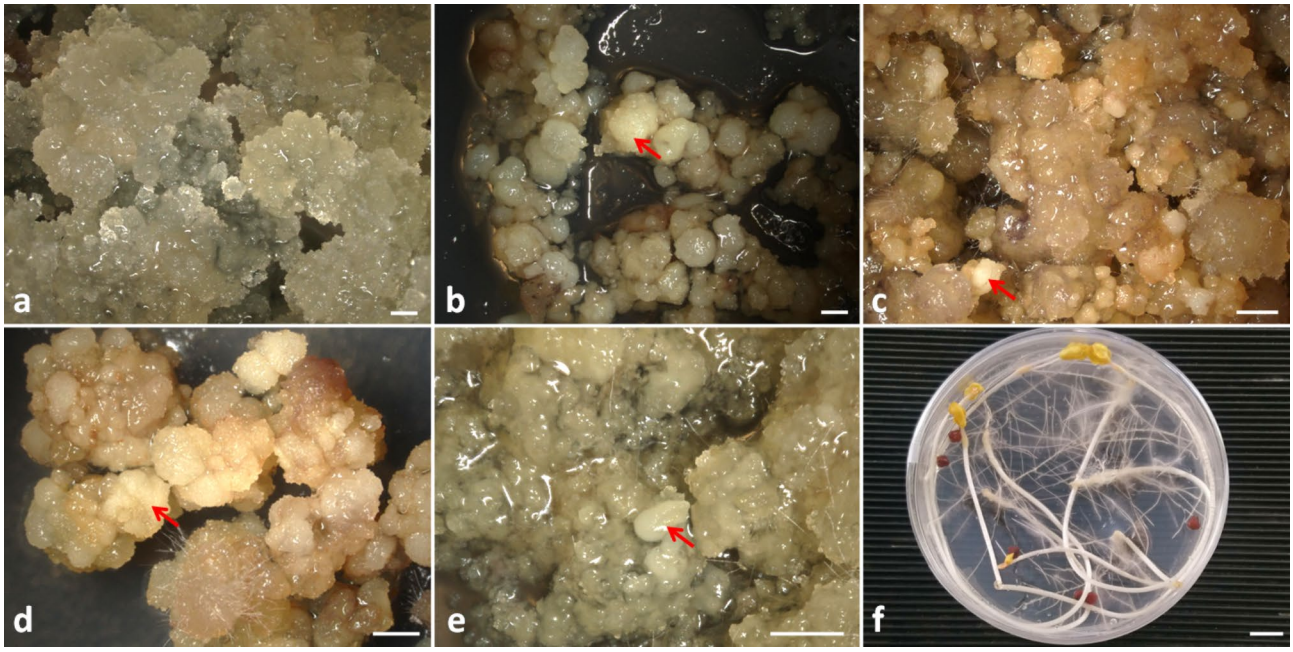

Fig. 2 Donor callus (**a-e**) and 10-day-old hypocotyls (**f**) of *Fagopyrum tataricum* used as source material for protoplast isolation. Morphology of 2-week-old callus lines: (**a**) non-morphogenic (NC) and morphogenic (MC) callus: (**b**) MC1, (**c**) MC2, (**d**) MC4, (**e**) NL2018. Arrows show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

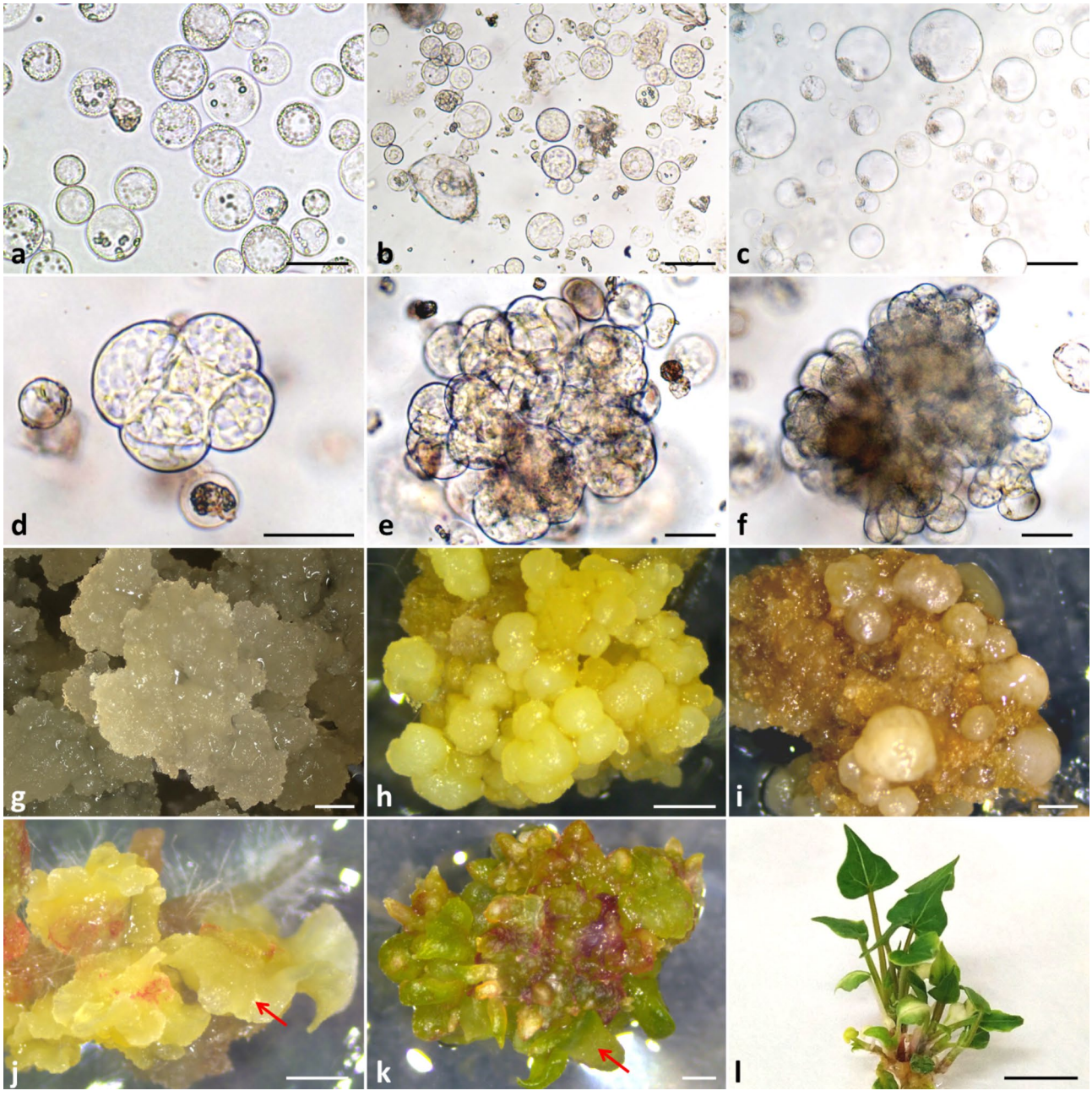

Fig. 3 Plant regeneration in protoplast cultures of *Fagopyrum tataricum*. Freshly isolated protoplasts from (**a**) non-morphogenic callus (NC), (**b**) morphogenic callus (MC) and (**c**) hypocotyls; multicellular aggregate in 8- (**d**), 10- (**e**), 20- (**f**) day-old protoplast cultures originating 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 $P \leq 0.05$

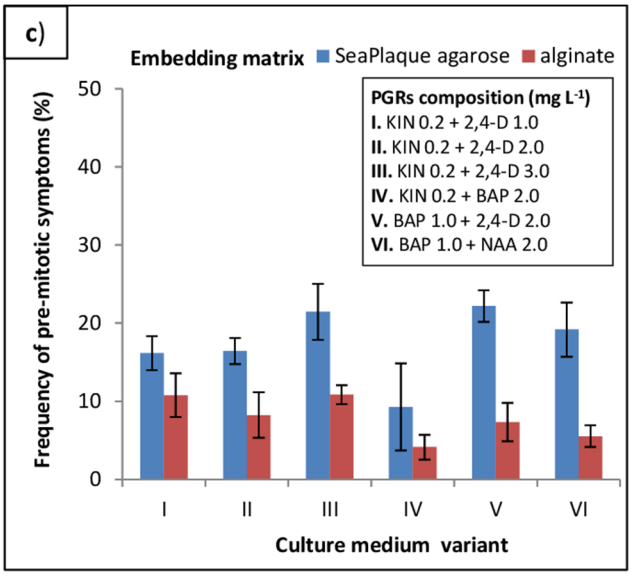

Fig. 4 Frequency of pre-mitotic symptoms in 10-day-old protoplast cultures originating from non-morphogenic callus of *Fagopyrum tataricum*. Effect of (**a**) embedding matrix, (**b**) plant growth regulators (PGRs) and (**c**) both treatments on culture development. *BAP* = 6-benzylaminopurine; *2,4-D* = 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

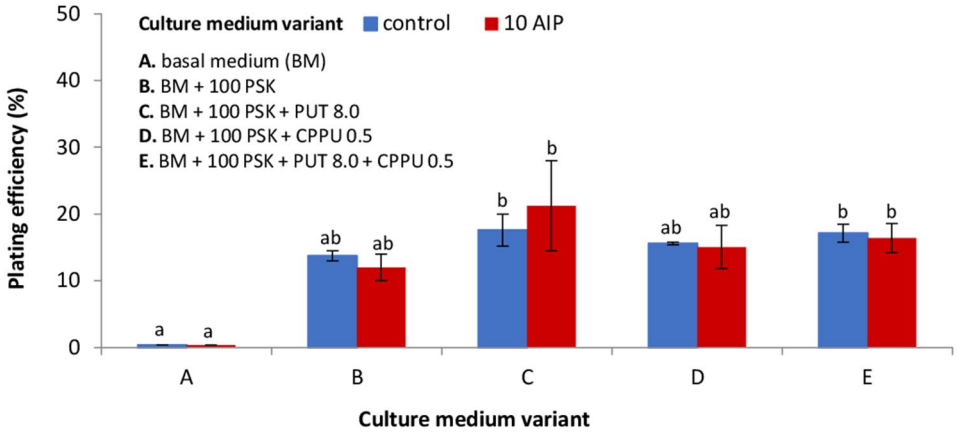

Fig. 5 Effect of plant growth regulators (PGRs) and AIP on plating efficiency in 10-day-old protoplast cultures originating from morphogenic callus (line NL2018) of *Fagopyrum tataricum*. PGRs composition in *BM* for protoplast cultures = 1.0 mg L^{-1} BAP (6-benzylaminopurine) + 2.0 mg L^{-1} NAA (α-naphthalene 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 $P \leq 0.05$

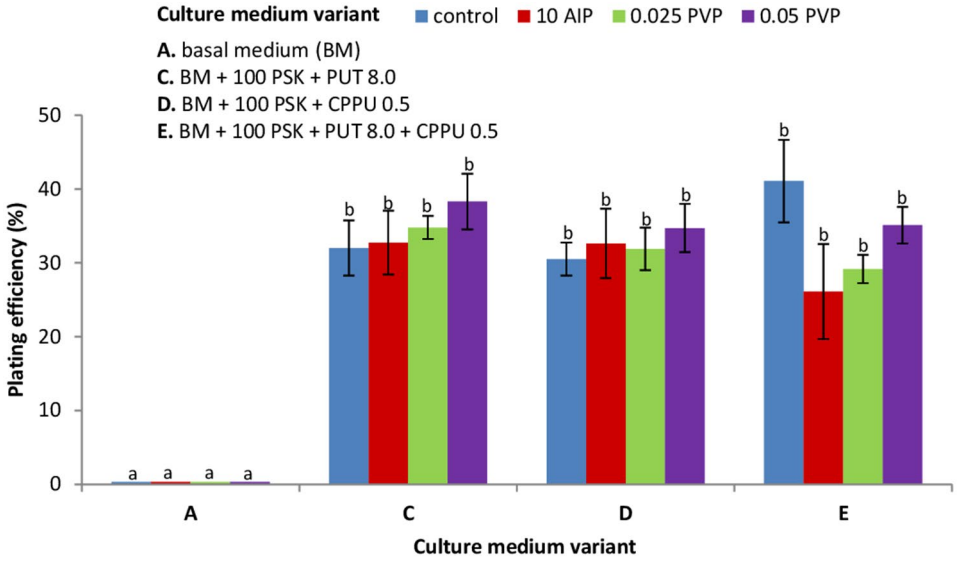

Fig. 6 Effect of plant growth regulators (PGRs) and compounds inhibiting (AIP) or absorbing (PVP) phenolics on plating efficiency in 10-day-old protoplast cultures originating from hypocotyls of *Fagopyrum tataricum*. PGRs composition in *BM* for protoplast cultures = 1.0 mg L^{-1} BAP (6-benzylaminopurine) + 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

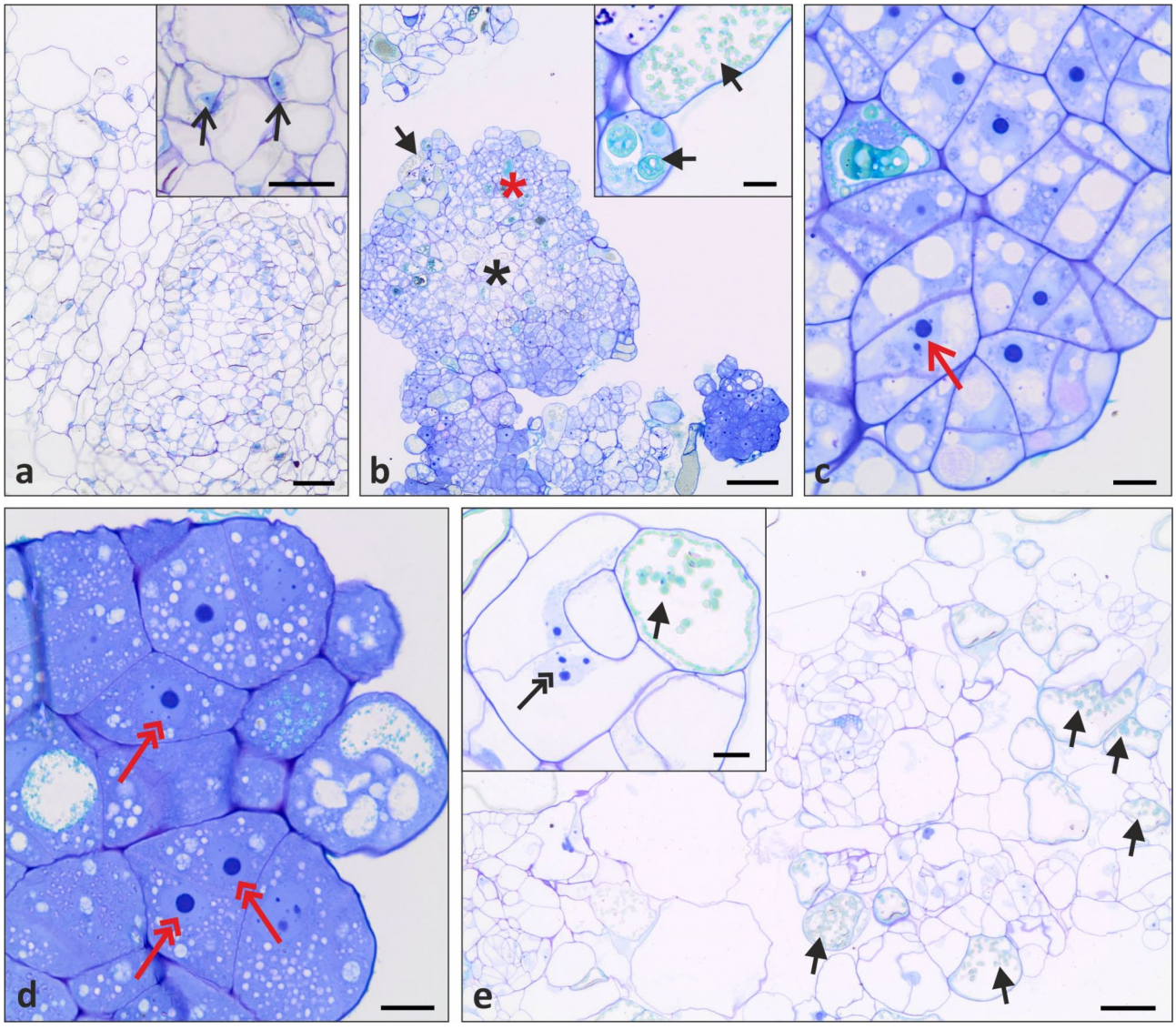

Fig. 7 Histological sections of protoplast-derived callus originating from: (**a**) non-morphogenic callus (NC), (**b-d**) morphogenic callus (MC; line NL2018) and (**e**) hypocotyls of *Fagopyrum tataricum*. Protoplast cultures from NC consisted of parenchymatous cells (**a**) with large vacuole and 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

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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.

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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.

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Publisher's Note

10.2. Publikacja 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. *Plant Cell, Tissue and Organ Culture*, 2023, 154, 673–687. https://doi.org/10.1007/s11240-023-02542-2
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Materiały uzupełniające dostępne online:

Supplementary file 1 Tabela 1 Testowane roztwory enzymatyczne użyte do izolacji protoplastów z hipokotyli *Fagopyrum esculentum*

W niniejszej pracy przedstawiono wydajny i szybki system regeneracji *in vitro* gryki zwyczajnej, oparty na zastosowaniu kultury protoplastów. Dla wybrania materiału donorowego o najlepszej odpowiedzi rozwojowej oraz o największym potencjale regeneracyjnym protoplasty izolowano z dwóch linii MK[2] *F. esculentum* (L1, NL2; Figure 1a, b) oraz hipokotyli siewek (Figure 2a). Średnia wydajność izolacji protoplastów z MK wynosiła od 0,8 do 1,5 × 10^6 protoplastów, w zależności od składu zastosowanej mieszaniny maceracyjnej (Table 3). Roztwór enzymatyczny zawierający celulazę (1,5%), pektoliazę (0,1%) oraz driselazę (0,15%) umożliwił uzyskanie dwukrotnie większej liczby protoplastów w porównaniu z roztworem zawierającym jedynie celulazę (1%) i pektoliazę (0,1%) (Table 3). Efekt ten można przypisać szerokiemu zakresowi aktywności enzymatycznej driselazy, który obejmuje rozkład ksylanów, laminaryn oraz celulozy (Ning *i inni*, 2022). Podobnie, w przypadku izolacji protoplastów z hipokotyli, użycie mieszaniny maceracyjnej z dodatkiem driselazy pozwoliło na osiągnięcie wystarczającej do założenia kultury liczby protoplastów tj. około 0,4 × 10^6 protoplastów z grama świeżej masy (Table 4).

W przeprowadzonych badaniach żywotność protoplastów była wysoka i wynosiła średnio od 74 do 81% dla protoplastów kalusowych i od 69 do 81% dla protoplastów hipokotylowych (Table 3, 4).

[2] W publikacji **P2** dla kalusa *F. esculentum* przyjęto określenie morfogenny kalus (MC, ang. *morphogenic callus*). W publikacji **P3**, dzięki lepszemu poznaniu procesu regeneracji kalusa poprzez somatyczną embriogenezę, jak również tożsamość komórek go tworzących, doprecyzowano nazewnictwo, wprowadzając termin embriogenny kalus (EK).

Na podstawie wyników doświadczeń z kulturami protoplastów gryki tatarki, protoplasty gryki zwyczajnej immobilizowane były w agarozie (LMPA), zastosowano bazową pożywkę do kultury protoplastów (zmodyfikowaną pożywkę CPP uzupełnioną o regulatory wzrostu NAA i BAP). Rozwój protoplastów odnotowano tylko w przypadku suplementacji w/w pożywki w PSK (Figure 3). Wydajność kultury protoplastów kalusowych, w zależności od linii kalusa, wynosiła od 21 do 35% (Figure 3a, b), z kolei kultura protoplastów hipokotylowych charakteryzowała się wydajnością na poziomie około 21% (Figure 3c). Zastosowanie dodatkowych suplementów tj. CPPU i PUT dało większe ale statystycznie nieistotne wartości wydajności kultury. Nie odnotowano również stymulującego efektu PVP na rozwój kultury.

Kolonie komórkowe (Figure 1e-f, 2e) kontynuowały wzrost, prowadząc do formowania minikalusa. Po 30 dniach krople agarozowe w kulturze protoplastów MK (linia NL2) przerosły minikalusem, obserwowano także powstawanie masy proembriogennej. Natomiast w kulturze protoplastów MK linii L1 oraz hipokotylowych proces formowania minikalusa wydłużył się do 60 dni. Następnie minikalus namnażany był na pożywce proliferacyjnej. Po trzech miesiącach od przeniesienia namnożonego kalusa z kultury protoplastów MK (linia L1) na pożywkę regeneracyjną obserwowano formowanie struktur pędo-podobnych (ang. *shoot-like structures*), a po (w sumie) czterech miesiącach odnotowano w pełni wykształcone rośliny (Figure 2h). Z kolei w przypadku kultury protoplastów linii NL2, formowanie zarodków somatycznych odnotowano już w pierwszym tygodniu regeneracji kalusa (Figure 1g), w 15. dniu regeneracji wykształcanie się struktur pędo-podobnych (Figure 1h), a po 2 miesiącach otrzymano zregenerowane rośliny (Figure 1i, Table 5). Kultura protoplastów hipokotylowych regenerowała poprzez somatyczną embriogenezę (Figure 2f) oraz organogenezę (Figure 2g), pojedyncze w pełni zregenerowane rośliny zaobserwowano po trzech miesiącach od momentu wyłożenia kalusa na pożywkę regeneracyjną (Figure 2h, Table 5). Warto zaznaczyć, że zastosowanie tidiazuronu (TDZ) w pożywce regeneracyjnej efektywnie stymulowało somatyczną embriogenezę w materiale otrzymanym z kultury protoplastów MK (linia NL2).

Przeprowadzone badania uzupełnione zostały o analizę histologiczną minikalusa uzyskanego po kulturze protoplastów w celu poznania charakteru oraz potencjału regeneracyjnego otrzymanego materiału. Analizy wykazały, że materiał z kultury protoplastów kalusowych (linia L1) zawierał komórki parenchymatyczne (Figure 4a, czarna gwiazdka) z dużą wakuolą i nieregularnie ukształtowanym jądrem z jednym lub dwoma jąderkami (Figure 4a, wstawka 1,), na powierzchni masy zidentyfikowano komórki zawierające związki fenolowe (Figure 4a, wstawka 2). Materiał z kultury protoplastów linii NL2 charakteryzował się obecnością komórek merystematycznych (Figure 4b, czerwona gwiazdka) z gęstą cytoplazmą,

małymi wakuolami i okrągłym jądrem z jednym lub dwoma jąderkami (Figure 4b, wstawka 1), a na powierzchni masy zaobserwowano pojedyncze komórki zawierające związki fenolowe (Figure 4b, wstawka 2). Kalus otrzymany z kultury protoplastów hipokotylowych również zawierał na powierzchni komórki bogate w związki fenolowe (Figure 4c, wstawka 1), podczas gdy wewnętrzne warstwy tworzyły w dużej mierze komórki parenchymatyczne (Figure 4c, czarna gwiazdka) z nieregularnie ukształtowanym jądrem zlokalizowanym w pobliżu ściany komórkowej (Figure 4c, wstawka 2). Zaobserwowano także małe obszary komórek podobnych do komórek merystematycznych (Figure 4c, czerwona gwiazdka) z dwoma lub trzema jąderkami i małymi wakuolami (Figure 4c, wstawka 3).

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ORIGINAL ARTICLE

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

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Abstract

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

Key message

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

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

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
BM	Basal medium
CM	Callus multiplication medium
CPPU	*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

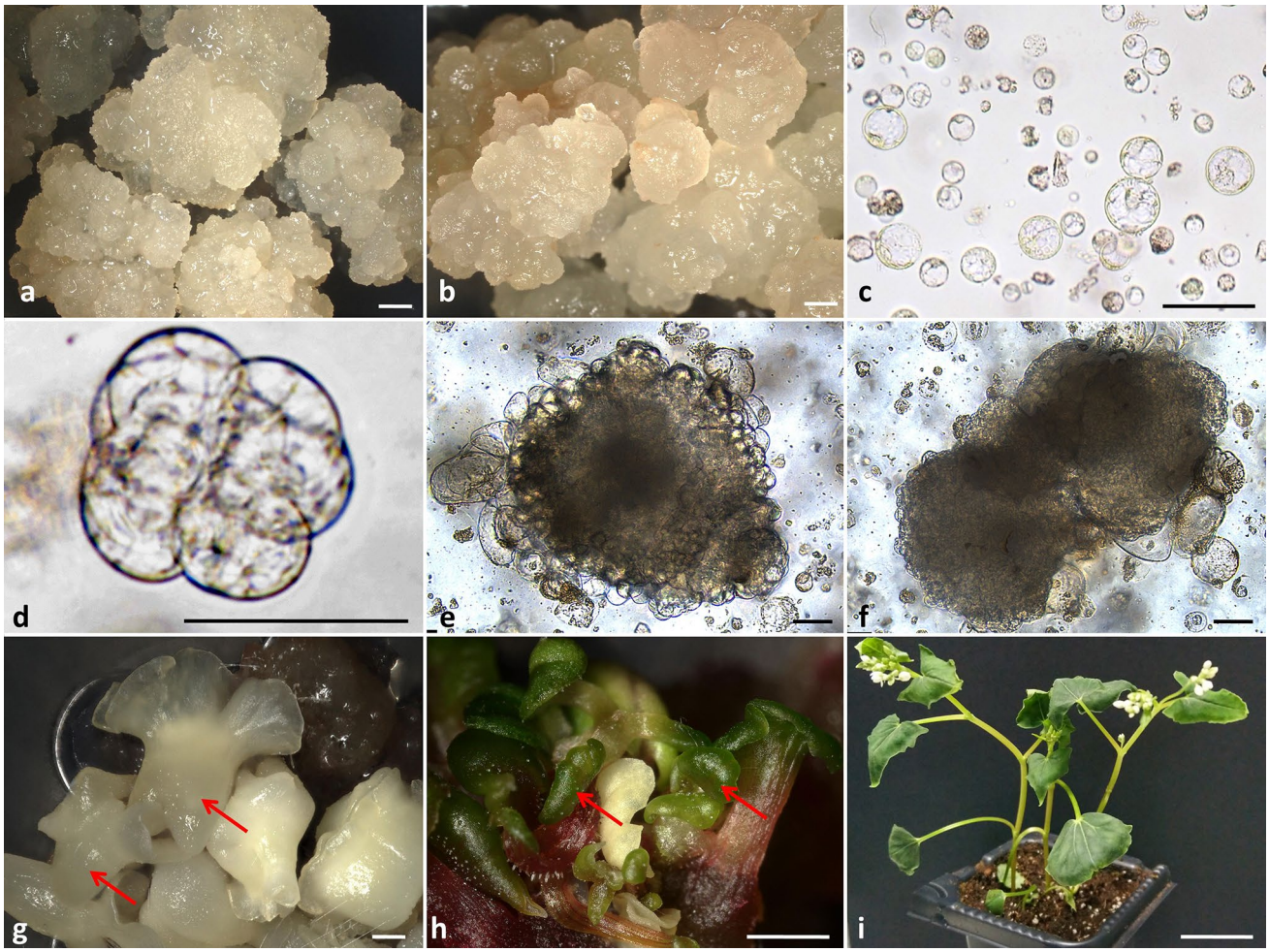

Fig. 1 Plant regeneration from morphogenic callus (MC)-derived protoplasts in *Fagopyrum esculentum*. Lines of MC used for protoplast isolation: **a** L1 and **b** NL2; **c** freshly MC-derived protoplasts; multicellular aggregate in **d** 6-, **e** 20- and **f** 30-day-old protoplast cultures; subsequent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

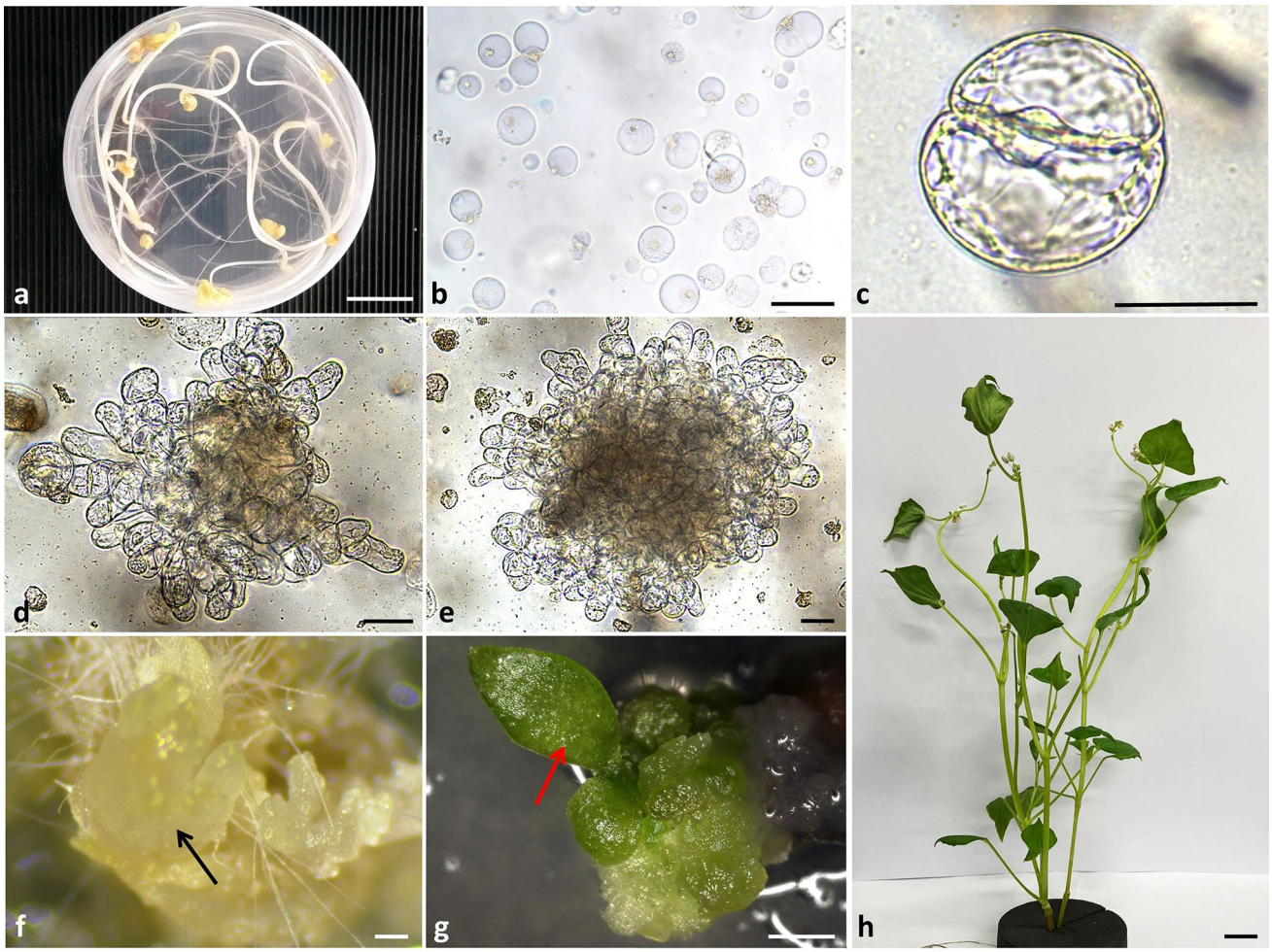

Fig. 2 Plant regeneration from hypocotyl-derived protoplasts of *Fagopyrum esculentum*. **a** 10-day-old etiolated hypocotyls used for protoplast isolation; **b** freshly hypocotyl-derived protoplasts; **c** 2-cell aggregate after the first mitotic division of the protoplast-derived cell; multicellular 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

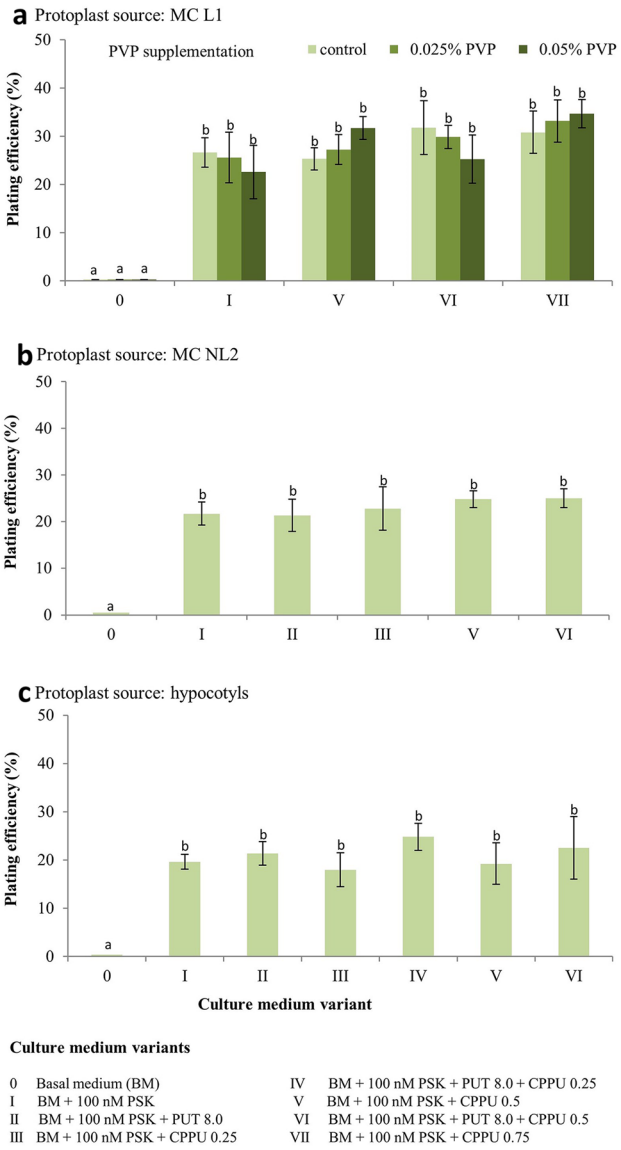

Fig. 3 Effect of different medium variants on plating efficiency in *Fagopyrum esculentum* 10-day-old protoplast cultures. Plant growth regulator composition in *BM* = BAP 1.0 mg L^{-1} + NAA 2.0 mg L^{-1}; *BAP* = 6-Benzylaminopurine; *NAA* = naphthylacetic acid; *100 PSK* = 100 nM phytosulfokine; *CPPU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

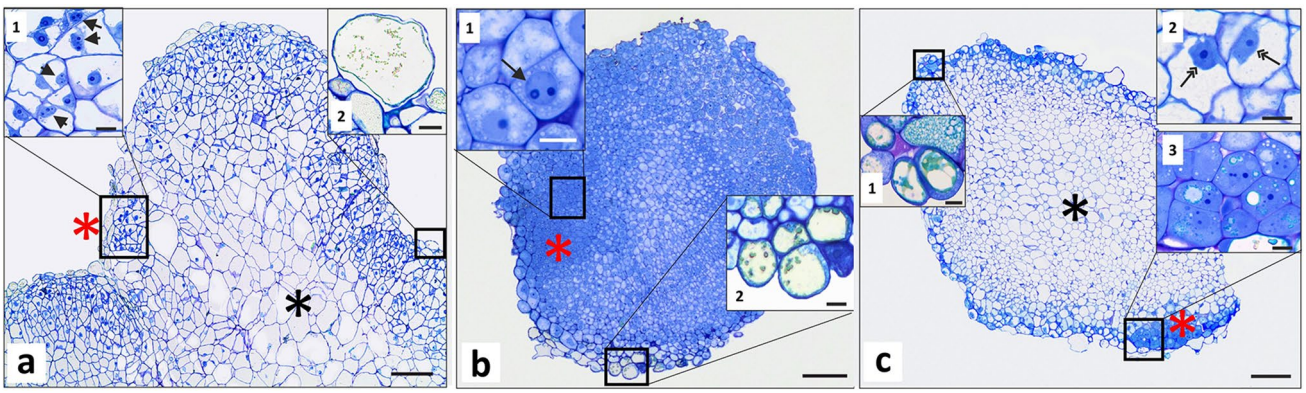

Fig. 4 Histological sections of protoplast-derived callus originating from morphogenic callus lines L1 (**a**), NL2 (**b**) and hypocotyls (**c**). **a** Calli originating from L1-derived protoplasts was composed chiefly of parenchymatous cells (black asterisk) with a big vacuole and an irregularly shaped 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.

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

Declarations

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

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

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10.3. Publikacja 3 (P3)

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Materiały uzupełniające dostępne online:

Supplementary table S1: Przeciwciała stosowane do immunobarwienia kultur protoplastów i rozpoznawane przez nie epitopy w ścianie komórkowej oraz odpowiednie odniesienia.

Supplementary table S2: Analiza statystyczna danych proteomicznych.

Supplementary figure S1-S19
https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-025-06119-3/MediaObjects/12870_2025_6119_MOESM3_ESM.pdf

Celem badań przedstawionych w trzeciej publikacji było opisanie oraz lepsze zrozumienie procesów zachodzących podczas rozwoju kolonii komórkowych w kulturze protoplastów *F. tataricum* oraz *F. esculentum* na poziomie proteomu, komponentów ściany komórkowej oraz ekspresji wybranych genów. Na podstawie obserwacji rozwoju kultury protoplastów opisanych w **P1** oraz **P2** stwierdzono różnice w czasie potrzebnym do rozwoju minikalusa, jak również czasie i sposobie regeneracji kultury protoplastów pomiędzy tymi dwoma gatunkami. Kultura protoplastów *F. tataricum* charakteryzowała się dłuższym czasem rozwoju prowadzącym do uformowania minikalusa (50 dni), w porównaniu do kultury *F. esculentum* (30 dni). Ponadto regeneracja roślin w kulturze protoplastów *F. tataricum* zachodziła poprzez organogenezę oraz somatyczną embriogenezę, z kolei w przypadku *F. esculentum* jedynie na drodze somatycznej embriogenezy.

W **P3** materiał badawczy stanowiła kultura protoplastów EK *F. esculentum* oraz MK *F. tataricum.* Analizie poddano trzy punkty czasowe kultury, podczas których obserwowano pierwsze podziały komórkowe (piąty dzień kultury), formowanie kolonii komórkowych (15. dzień) oraz rozwój minikalusa (30. dzień dla *F. esculentum*, 50. dzień dla *F. tataricum).*

Obserwacje histologiczne rozwoju kolonii komórkowych pozwoliły lepiej zrozumieć naturę komórek je tworzących, określić charakter komórek (merystematyczny, embriogenny) na podstawie Verdeil *i inni* (2007), a co za tym idzie wskazać na ich potencjał do regeneracji w rośliny. Pięciodniowa kultura protoplastów *F. tataricum* cechowała się kilkukomórkowymi koloniami komórkowymi zawierającymi komórki parenchymatyczne (Figure 1B-C, niebieskie strzałki), jak również komórki merystematyczne (Figure 1B-C, czerwone strzałki). Dnia 15. kolonie komórkowe składały się z cienkościennych, zwakuolizowanych komórek z dużym jądrem zawierającym kilka jąderek (Figure 1D-E, żółte strzałki), odnotowano również występowanie komórek merystematycznych (Figure 1D-E, czerwone strzałki). Dnia 50. minikalus zbudowany był z kilku typów komórek: komórek merystematycznych (Figure 1F-H, czerwona ramka, czerwone strzałki), komórek parenchymatycznych (Figure 1F-H, zielone strzałki) oraz komórek gromadzących związki fenolowe (Figure 1F-H, czarne strzałki), występujących w centralnej części jak również na powierzchni analizowanej struktury. Pięciodniowa kultura protoplastów *F. esculentum* odznaczała się obecnością struktur po pierwszym podziale mitotycznym, zawierających zwakuolizowane komórki z dużym jądrem (Figure 2B). Dnia 15. kolonie komórkowe w centralnej części zawierały komórki embriogenne (Figure 2C, pomarańczowe strzałki), z kolei na powierzchni komórki gromadzące związki fenolowe (Figure 2C, czarne strzałki). Struktury 30. dniowej kultury protoplastów otoczone były komórkami gromadzącymi związki fenolowe (Figure 2D, czarne strzałki), w centralnej części stwierdzono występowanie komórek merystematycznych (Figure 2D, czerwone strzałki), jak również parenchymatycznych (Figure 2D, żółte strzałki).

Wyniki analizy immunocytochemicznej ścian komórek kolonii komórkowych wykazały brak istotnych różnic w przestrzenno-czasowym rozmieszczeniu hemicelulozy (ksyloglukanów) oraz pektyn (homogalakturonanów), co może być związane z pełnionymi przez nie podstawowymi funkcjami w ścianie komórkowej, jak również wskazywać na prawidłowe osadzenie się tych komponentów podczas odtworzenia ściany komórkowej (Supplementary Figure S1-4). Zaobserwowano zróżnicowane występowanie łańcuchów bocznych ramnogalaktanów (RGI) tj. arabinianów i galaktanów, rozpoznawanych odpowiednio przez przeciwciało LM6 i LM5, pomiędzy analizowanymi punktami czasowymi kultury. W 15. dniowej kulturze epitop LM5 obecny był w wewnętrznych ścianach komórkowych kolonii *F. tataricum*, w przeciwieństwie do kultury *F. esculentum*, gdzie został zidentyfikowany w zewnętrznych, peryklinalnych ścianach komórkowych (Supplementary Figure S5B’ i S6B’). Występowanie galaktanów w ścianach komórkowych związane jest ze wzmacnianiem tych struktur (McCartney i Knox, 2002), występowaniem domen embriogennych (Potocka *i inni*,

2018) oraz różnicowaniem się i potencjałem do regeneracji (Wiśniewska i Majewska-Sawka, 2008), co koreluje z wynikami tej pracy. Ciągłą dystrybucję arabinianów w ścianach komórkowych odnotowano piątego oraz 30. i 50. dnia kultury, w przeciwieństwie do 15. dnia (Supplementary Figure S7 i S8). Analiza dystrybucji AGPs wykrywanych przez przeciwciało JIM13 ujawniła ich występowanie w ścianie komórkowej oraz kompartmentach cytoplazmatycznych w każdym z analizowanych punktów czasowych, zaobserwowano również niespecyficzne wiązanie JIM13 ze związkami fenolowymi (Supplementary Figure S9 C' i S10 C', fioletowe strzałki). W przypadku AGPs rozpoznawanych przez przeciwciało JIM16 odnotowano ich obecność w kompartmentach cytoplazmatycznych i wakuoli w kulturach *F. tataricum*, podczas gdy w kulturach *F. esculentum* nie stwierdzono obecności tego epitopu (Supplementary Figure S11 i S12). Epitop EXTs wykrywany przez przeciwciało JIM20, obecny był w ścianie komórkowej pięciodniowych kultur obu gatunków oraz na powierzchni zewnętrznych, peryklinalnych ścian komórkowych przeważnie w kulturze *F. esculentum* (Supplementary Figure S13 i S14).

Wyniki analizy proteomicznej wykazały obecność 3664 białek dla kultury *F. esculentum* oraz 3811 dla kultury *F. tataricum*, z czego największa liczba białek została zidentyfikowana w 50. dniu kultury *F. tataricum* (3046 białek) i 30. dniu dla *F. esculentum* (2933 białek). Największy wzrost akumulacji białka w kulturze *F. esculentum* odnotowano dla enzymu rozszczepiającego sacharozę (ang. *sucrose-cleaving enzyme*), z kolei znaczący spadek akumulacji dla białka embriogennego typu DC-8 (EP DC-8, ang. *embryogenic protein DC8-like*). W przypadku kultury *F. tataricum* największy wzrost akumulacji stwierdzono dla białka zawierającego biotynę (SBP, ang. *seed biotin containing protein*), a najbardziej znaczący spadek dla metylotransferazy caffeoyl-CoA (CCOMT, *ang. Caffeoyl-CoA O-methyltransferase*). Analizując dane proteomiczne, zaobserwowano, że wraz z czasem trwania kultury dochodzi do wzrostu akumulacji białek zapasowych nasion (SSPs, ang. *seed storage proteins*), szczególnie vicilin (VIC), oleozyn (OLEO) oraz SBP (Table 2), co może wskazywać na stopniową akumulację substancji zapasowych niezbędnych do rozwoju zarodków somatycznych. Stwierdzono również obecność białek należących wg. Gulzar *i inni* (2020) oraz Helleboid *i inni* (2000) do grupy białek związanych z somatyczną embriogenezą (ang. *somatic embryogenesis related proteins*) jak endochitynaz (ENDO) czy białek należących do grupy białek związanych z późną embriogenezą (LEA) takich jak EP DC-8 i SBP. Skonkludowano, że białka zapasowe nasion mogą być związane z aktywacją czynników transkrypcyjnych, jak również odnotowano korelację pomiędzy nimi co wnikliwie zostało przedyskutowane w części *Discussion* publikacji.

Badania zostały uzupełnione analizą ekspresji (1) czynników transkrypcyjnych związanych z regeneracją roślin (*LEC1, BBM, FUS3, WUS, CLV3*), (2) wybranych genów związanych z somatyczną embriogenezą (*LEA, EP DC-8, ENDO*), jak również (3) genów kodujących białka zapasowe nasion, dla których dane proteomiczne wskazywały na wysoki wzrost akumulacji w czasie trwania kultury (*VIC, OLEO,* Figure 3A-C). Na podstawie wyników analiz proteomicznych przeprowadzono także analizę ekspresji (4) genów związanych ze ścianą komórkową t.j. *FLAs, CCOMT* oraz *EXT* (Figure 3D).

Zaobserwowano różnice we wzorze ekspresji analizowanych genów i ich poziomie pomiędzy analizowanymi punktami czasowymi i gatunkami. Analiza ekspresji czynników transkrypcyjnych związanych z somatyczną embriogenezą wykazała masywne zwiększenie ekspresji *LEC1* 50. dnia kultury *F. tataricum*, z kolei w przypadku ekspresji *BBM* stwierdzono jej spadek wraz z czasem trwania kultury obu gatunków (Figure 3A). Największy wzrost ekspresji odnotowano dla *EXT* piątego dnia kultury *F. esculentum*, gdzie odnotowano 200 razy wyższą ekspresję niż w kulturze *F. tataricum* (Figure 3D).

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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*

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Abstract

Background Due to the totipotency of plant cells, which allows them to reprogram from a differentiated to a dedifferentiated state, plants exhibit a remarkable regenerative capacity, including under in vitro culture conditions. When exposed to plant hormones, primarily auxins and cytokinins, explant cells cultured in vitro can undergo differentiation through callus formation. Protoplast culture serves as a valuable research model for studying these processes in detail. This knowledge is particularly relevant for improving common and Tartary buckwheat species. To gain deeper insights into the stages of cell development from protoplasts—such as cell division, cell colony formation, and microcalli development—we focused on analyzing proteomes, cell wall composition, and changes in the expression profiles of selected genes in *Fagopyrum* protoplast cultures.

Results The results demonstrate a significant accumulation of somatic embryogenesis-related proteins like late embryogenesis abundant proteins (embryogenic protein-DC-8-like, seed biotin-containing protein) and endochitinases during the developmental path of protoplast-derived cultures. Additionally, we noted an extensive increase in seed storage proteins like vicilin, oleosins, and seed biotin-containing proteins during the culture. Investigation of somatic embryogenesis-associated transcription factors revealed massive up-regulation of *LEAFY COTYLEDON1* for the 50th day of *F. tataricum* protoplast-derived cultures. However, for *BABY BOOM*, the transcription factor was noted to be down-regulated during the development of cell colonies. Furthermore, we demonstrated the

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variable distribution of cell wall components like pectin side chains, arabinogalactan proteins (AGPs) and extensins (EXTs), indicating the reorganisation of cell wall composition during the culture period.

Conclusions This study revealed changes correlating with regaining embryogenic competence during the development of *Fagopyrum* protoplast-derived cell colonies. Our findings revealed variable expression levels of genes and proteins associated with somatic embryogenesis. This analysis identified an increase in seed storage proteins that play a significant role in the somatic somatic embryogenesis pathway of regeneration. Furthermore, the relationship between transcription factors and these processes seems to be connected with regaining somatic cells' totipotency and promoting embryogenic competence of protoplast-derived cell colonies. Additionally, we observed dynamic changes in cell wall composition during the development of the protoplast-derived cultures.

Clinical trial number Not applicable.

Keywords Differentiation, *Fagopyrum*, Oleosins, Protoplast, Somatic embryogenesis, Transcription factors, Vicilins

Introduction

Buckwheat is an important under-used crop plant despite its noteworthy pharmaceutical and nutritional qualities. The most cultivated ones are common (*Fagopyrum esculentum*) and Tartary buckwheat (*Fagopyrum tataricum*), distinguished by the presence of phenolic compounds, minerals, dietary fibre and high-quality proteins with a well-balanced amino acid profile [1–3]. The main challenge in cultivating common buckwheat is their low and inconsistent yield, which results from self-incompatibility, short flower lifespan, embryo, seed, and flower abortion. The heterogeneity of maturation, seed shattering, preharvest sprouting, and sensitivity to biotic and abiotic stresses are the main cons of these two species, which are a challenge in buckwheat breeding programs [4]. An efficient Agrobacterium-mediated transformation and genome editing of *F. tataricum* was recently established, allowing for the improvement of agronomical traits [5]. We have also demonstrated protoplast-to-plant regeneration ability in cultures of common [6] and Tartary buckwheat [7]. This was achieved after supplementing a culture medium with phytosulfokine (PSK) and embedding protoplast in an agarose matrix. Additionally, the time needed for plant regeneration via protoplast cultures of buckwheat compared with other plants was faster, taking three to five months (common and Tartary buckwheat, respectively). Protoplast-to-plant regeneration opens the possibility of protoplast transfection and genetic modifications, especially by delivery of a ribonucleoprotein complex of Cas9-gRNA instead of a vector, resulting in transgene-free and homogenous plant [8], since regenerated plants originate from a single protoplast. However, researchers face challenges in achieving successful plant regeneration from single cells. The efficient process initially requires selecting the appropriate genotype and tissue to provide protoplasts capable of further development and regeneration. Choosing the proper protoplast culture conditions (liquid or semi-solid medium), hormones, and additional substances is crucial for overcoming the division latency of protoplasts. These factors also influence the culture's embryogenic competence and regenerative potential [9].

Plant tissue plasticity enables cells to gain pluri- or totipotency, allowing their development into plants from single cells [10]. However, little is known about what happens and how differentiated cells reprogram and regenerate [11]. Protoplasts are an excellent example of gaining totipotency as differentiated cells undergo enzymatic cell wall removal and stress conditions, leading to cell dedifferentiation processes. Protoplasts are susceptible to surrounding conditions, such as hormones, that can create their development path. Enriched liquid medium for protoplast culture enables *de novo* cell wall synthesis and reinitiating of cell division [12]. Protoplast cell wall reconstruction is the main point of interest for many researchers [13–17]. The scaffold of cellulose microfibrils, cross-linked by hemicelluloses and embedded in the matrix of pectic polysaccharides, provides cell wall structure and physical properties [18]. Additionally, hydroxyproline-rich glycoproteins such as AGPs and EXTs are responsible for cell wall dynamics and mechanical properties [19]. Besides these, a cell wall can regulate morphogenetic processes and maintain differentiated cellular fate. Moreover, changes in the chemical composition of cell walls could be a marker of changes in the differentiation direction during the somatic embryogenesis process and plant growth [20]. Fagopyrum's protoplast cell wall reconstruction pattern was recently shown [13].

Lipids are crucial in in vitro cultures, particularly in somatic embryogenesis. They are essential components of cell membranes, signalling molecules, and energy reserves. During somatic embryogenesis, lipids are involved in the formation and development of embryonic structures. The accumulation of lipid bodies often marks embryo development. Moreover, alterations in lipid metabolism and composition can influence the efficiency of embryogenesis, making lipids important regulators in the process. Studying lipid dynamics provides valuable insights into in vitro culture systems and can improve embryogenesis outcomes [21, 22].

However, far too little attention has been paid to the processes occurring during the middle and late stages of protoplast-derived cell colonies. Most studies focus on gene expression and proteome analysis of the first hours or the first week of culture. Characterisation of the changes in the transcript profile during the early steps of dedifferentiation and reentry into the cell division process was reported by Chupeau, et al. 2013 [23]. Gene expression in culture of mesophyll-derived protoplasts was shown by Xu, et al. 2021 [11]. Wang, et al. 2017 [24] provide information about proteomic analysis of developmental reprogramming in protoplast-derived cultures of moss *Physcomitrella patens*. de Jong, et al. 2007 [25] also showed a proteome study of the first days of *Medicago truncatula* protoplasts, focusing on molecular changes during protoplast proliferation.

Our recent investigations into the morphogenic and embryogenic callus of *F. tataricum* and *F. esculentum* revealed distinct differences in the development of protoplast-derived cultures [6, 7]. We observed variations in the time required for microcallus formation and plant regeneration. Specifically, protoplast cultures of *F. tataricum* took a longer period (50 days) for microcallus formation compared to *F. esculentum* (30 days). Additionally, our previous study confirmed that plant regeneration from protoplast-derived cultures in *F. tataricum* occurs through both organogenesis and somatic embryogenesis, while in *F. esculentum*, it occurs solely via somatic embryogenesis. Until now, information about the processes occurring in culture's middle and late periods derived from protoplasts is scarce. Therefore, our goal was to describe and better understand what happens during the formation of cell colonies (day 5th, 15th and 30/50th) on the level of proteomes, cell wall composition, and gene expression profile starting from protoplast cultures of *F. tataricum* and *F. esculentum*.

Results

Morphology of the callus used as a material source for protoplast isolation

For protoplast cultures, the cells were isolated from the morphogenic callus (MC; Fig. 1A) of *F. tataricum* consisting of small pro-embryogenic cell complexes (PECCs; Fig. 1A white arrows) and 'soft' callus cells. In the embryogenic callus (EC) of *F. esculentum*, dense globular milky-white structures can be distinguished due to the accumulation of starch grains in the storage cells (Fig. 2A) [6, 7, 26]. The calli of both species differed in age: five-year-old for *F. tataricum* and two-year-old *F. esculentum* were used.

For the analysis conducted in this study, three important time points of protoplast-derived cultures were selected. The events representing first cell division (5th day), cell colonies (15th day), and microcalli formation (30th for *F. esculentum* / 50th day for *F. tataricum*). The structures varied in size, measuring approximately 70 µm, 200 µm, and 0.6 mm, respectively (Figs. 3 and 4).

Histological observations of protoplast-derived cell colonies

The histological observations of the culture at three time points (5th, 15th and 30/50th days) revealed differences in the size and number of cells that formed cell colonies (Fig. 1B-H). On the 5th day of *F. tataricum* culture, the structure consisted of parenchymatous-like cells with vacuoles of different sizes and numbers (Fig. 1B-C; blue arrows). However, some cell colonies cells exhibited certain meristematic cell features such as dense cytoplasm and a round-shaped, large nucleus with one or two nucleoli and smaller vacuoles (Fig. 1B, C; red arrows). On the 15th day, the cell colonies were composed mainly of thin-walled cells with numerous small vacuoles and large nuclei with one to three nucleoli (Fig. 1D-E; yellow arrows). Also, the occurrence of meristematic cells was noted (Fig. 1E; red arrows). Those cells were characterized by numerous small vacuoles and a large, round nucleus with one or more nucleoli. On the 50th day, the microcalli comprised various types of cells (Fig. 1F). Regions rich in meristematic cells were observed within the microcalli surface (Fig. 1F; red frames and Fig. 1G-H; red arrows) as well as phenolic-containing cells (PCCs) (Fig. 1F and H; black arrows). Parenchymatous cells with small vacuoles and starch grains (Fig. 1F and H; green arrows) and PCCs constituted microcallus central and most prominent part.

On the 5th day of *F. esculentum* culture, the cell colonies consisted mostly of cells after the first cell division (Fig. 2B). These cells had one or numerous vacuoles and sizeable nuclei. On the 15th and 30th day, the cell colonies and microcalli were characterized by different cells distributions than *F. tataricum* culture (Fig. 2C-D). On the 15th day, the structure consisted of embryogenic cells in the central part (Fig. 2C; orange arrows) and PCCs on the surface (Fig. 2C; black arrows), while *F. tataricum* culture did not contain PCCs at this time point (compare Fig. 1D-E, and Fig. 2C). On the surface of the 30th day microcall, PCCs with small phenolic droplets occurred (Fig. 2D; black arrows). The central part of microcalli was composed of meristematic cells, characterised by dense cytoplasm and round-shaped nuclei with one to three nucleoli (Fig. 2D; red arrows) and parenchymatous cells (Fig. 2D; yellow arrows).

Immunocytochemical analysis

An immunocytochemical assay was performed to analyse the spatiotemporal distribution of selected cell wall components. Studied cell wall components included cellulose (visualised by FB28 staining), hemicellulose (xyloglucan,

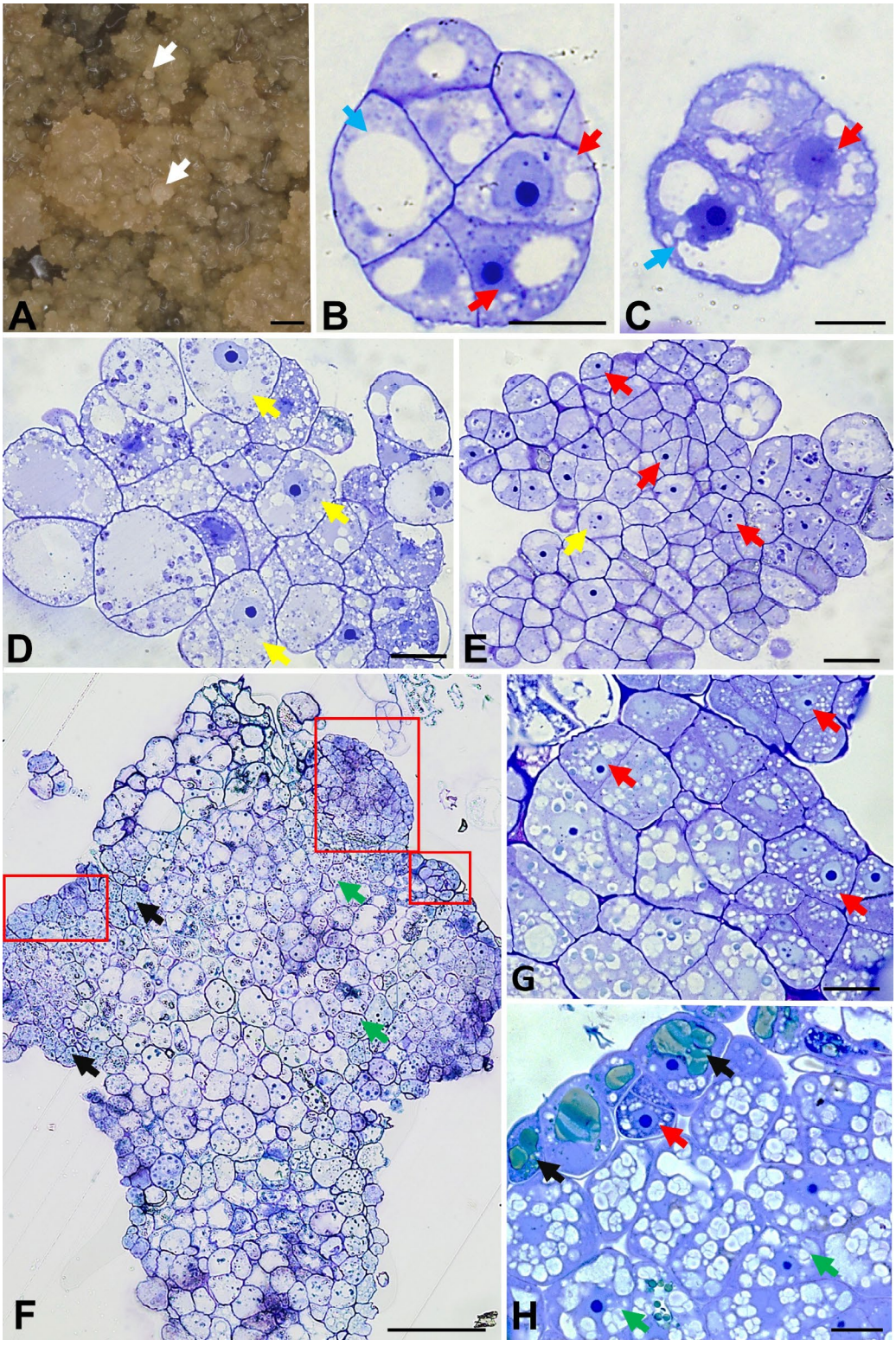

Fig. 1 Morphology of one-week-old callus (**A**) and histological sections of the morphogenic callus-derived protoplast cultures of *F. tataricum* on the 5th (**B-C**), 15th (**D-E**) and 50th (**F-H**) day of the culture. White arrows (**A**) indicate pro-embryogenic cell complexes, blue arrows (**B**-**C**) point to vacuolated cells, red arrows (**B**, **C**, **E**, **G**, **H**) indicate meristematic cells; yellow arrows (**D**, **E**) show cells with multiple vacuoles and large nuclei; black arrows (**F**, **H**) indicate phenolic-containing cells; green arrows (**F**, **H**) demonstrate parenchymatous cells with small vacuoles and starch grains; red frame (**F**) marks regions rich in meristematic cells. Scale bars: 1 mm (**A**); 20 µm (**B-E**, **G-H**); 100 µm (**F**)

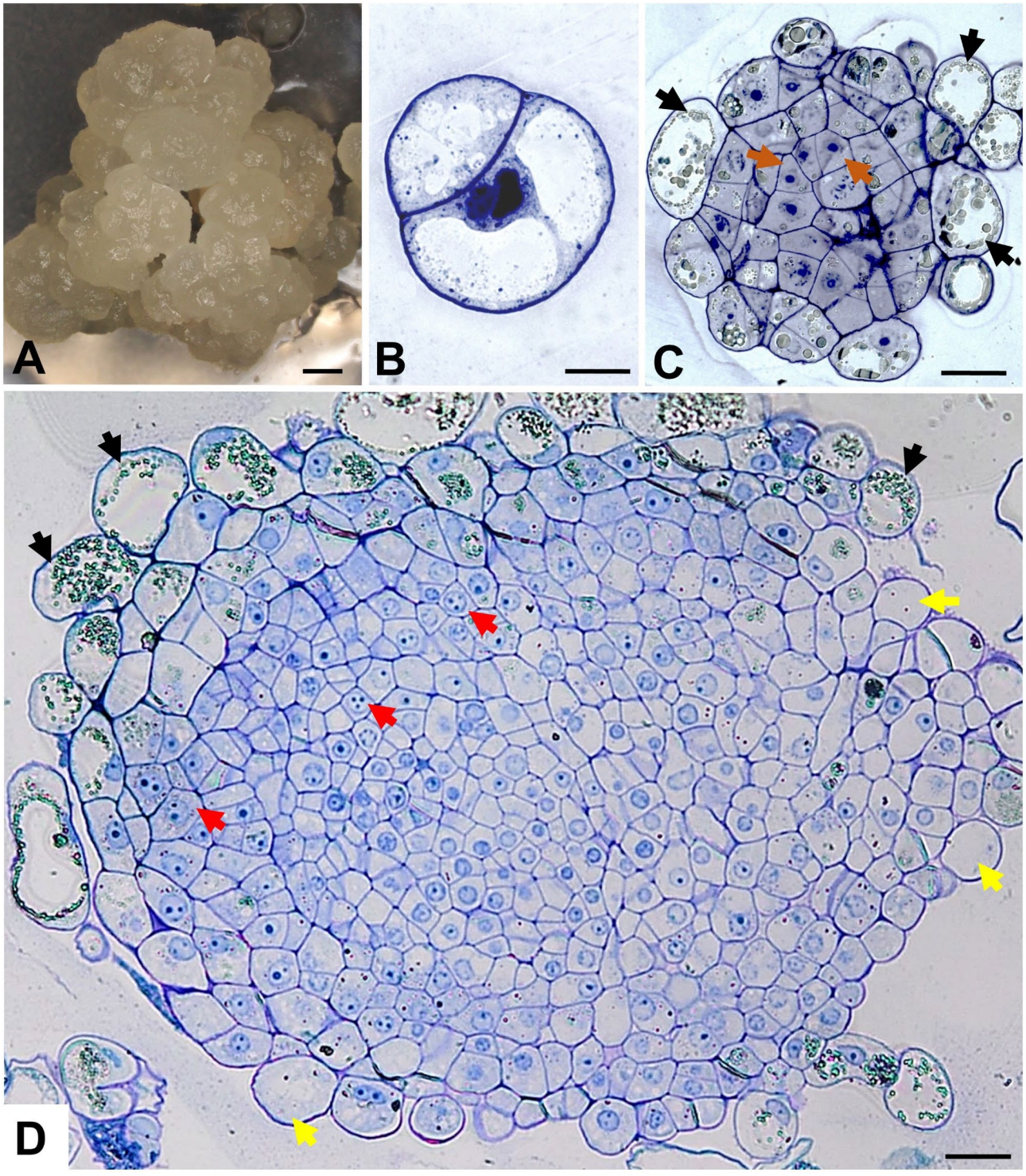

Fig. 2 Morphology of two-week-old callus (**A**) and histological sections of the embryogenic callus-derived protoplast cultures of *F. esculentum* on the 5th (**B**), 15th (**C**) and 30th (**D**) day of the culture. Black arrows indicate phenolic-containing cells (**C-D**); orange arrows show embryogenic cells (**C**), red arrows point to meristematic cells (**D**); yellow arrows note the parenchymatous cells (**D**). Scale bars: 1 mm (**A**), 10 µm (**B**), 20 µm (**C-D**)

recognised by LM25 antibody; Supplementary Table S1), pectins (galactan, arabinan and homogalacturonan, recognized by LM5, LM6 and LM20 antibody, respectively; Supplementary Table S1) and hydroxyproline-rich glycoproteins such as AGPs (recognized by JIM13 and JIM16 antibody; Supplementary Table S1) and EXT (recognized by JIM20 antibody; Supplementary Table S1). The epitopes were selected based on previous work

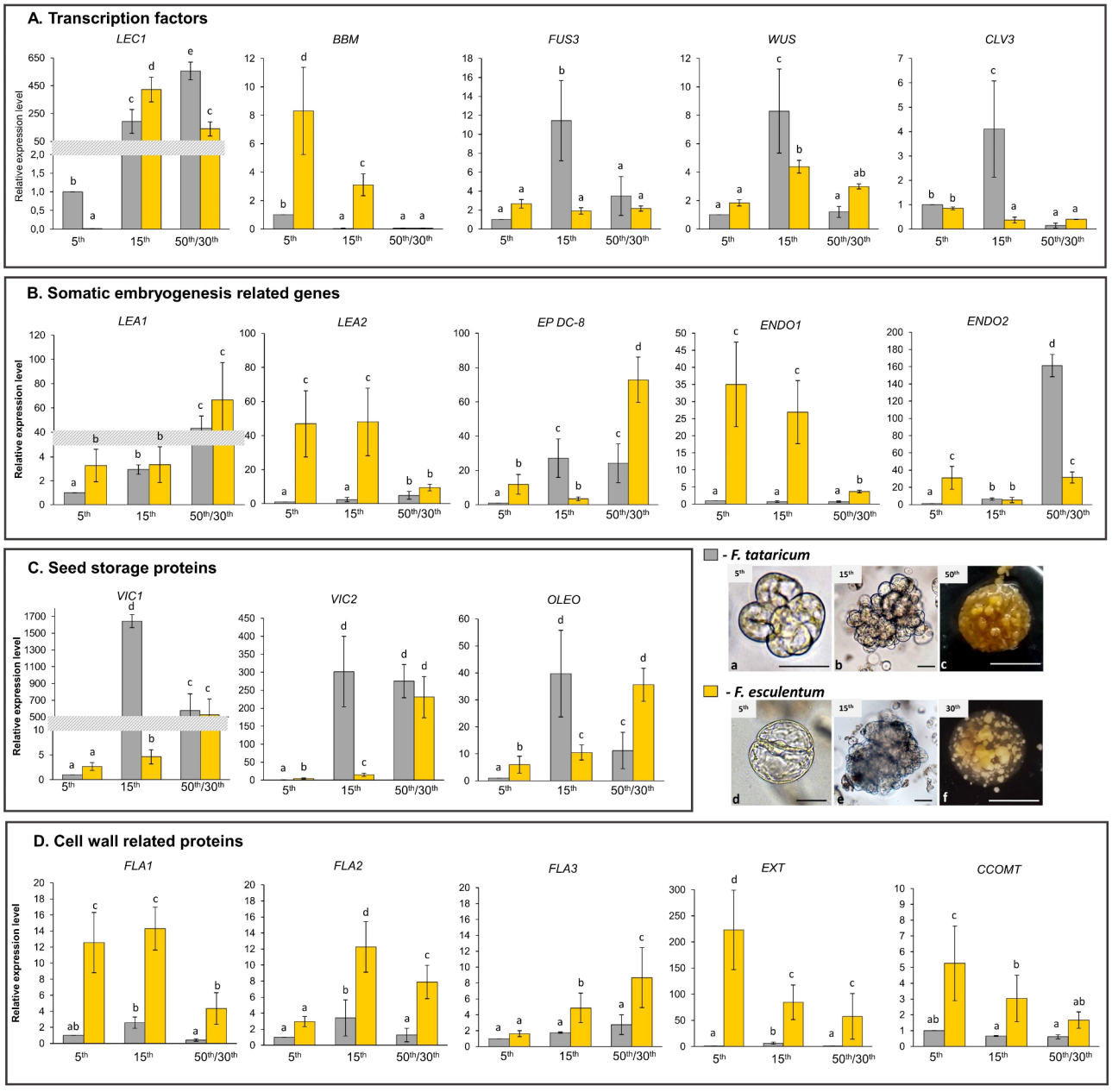

Fig. 3 The expression level of (**A**) transcription factors, (**B**) somatic embryogenesis related genes, (**C**) genes coding seed storage proteins and (**D**) cell wall related proteins in the protoplast cultures of *F. tataricum* (day 5th, 15th, 50th ; gray bar charts) and *F. esculentum* (day 5th, 15th, 30th ; yellow bar charts). The expression level of genes was calibrated to expression at 5th culture of the *F. tataricum*. Different letters indicate a significant difference between time points and species according to Tukey's HSD test ($p < 0.05$; $n = 3$; means ± SD are given). *LEAFY COTYLEDON* (*LEC1*); *BABY BOOM* (*BBM*); *FUSCA3* (*FUS3*); *WUSCHEL* (*WUS*); *CLAVATA3* (*CLV3*); *LATE EMBRYOGENESIS ABUNDANT PROTEIN* (*LEA*); *EMBRYOGENIC PROTEIN* DC-8-like (*EP DC-8*); *ENDOCHITYNASE* (*ENDO*); *VICILIN* (*VIC*); *OLEOSIN* (*OLEO*); *FASCICLIN-LIKE ARABONOGALACTAN PROTEIN* (*FLA*); *EXTENSIN* (*EXT*); *CAFFEOYL-COA O-METHYLTRANSFERASE* (*CCOMT*). The photo panel show the analysed time points of *F. tataricum* (**a**-**c**) and *F. esculentum* (**d**-**f**) protoplast cultures. Scale bars 50 µm (**a**-**b**, **d**-**e**), 5 mm (**c**, **f**)

concerning rebuilding cell walls in protoplast cultures of these two buckwheat species [13]. Additionally, the localisation of selected AGPs and EXT was analysed in the MC of *F. tataricum*, which was used in our research as the donor material for protoplast isolation [26]. The results of epitopes distribution were summarised in Table 1 based on photographs presented in Supplementary Fig. S1-S14.

Xyloglucan epitope was mainly distributed in the cell walls of *F. esculentum* and *F. tataricum* culture at each analysed time point (Supplementary Fig. S1, S2). The fluorescence signal was also present in cytoplasmic compartments (Supplementary Fig. S1A'-A"; red arrows) as well on the surface of outer periclinal walls of the peripheral cells (Supplementary Fig. S1C'-C"; brown arrow) in *F.*

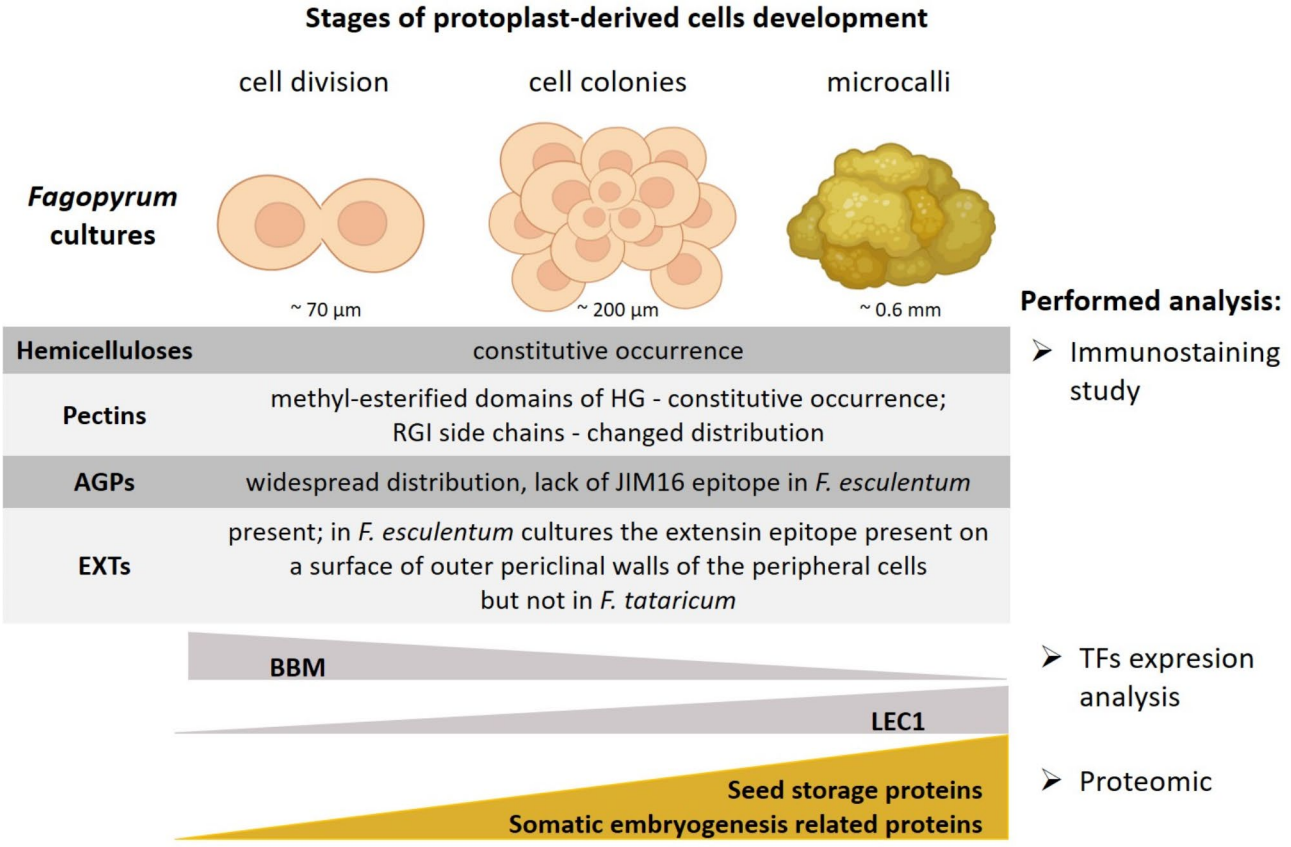

Fig. 4 General overview on the analysed time points of the protoplast-derived cultures of *F. tataricum* and *F. esculentum*. The main results of the performed analysis are shown. Arabinogalactan protein (*AGPs*); *BABY BOOM* (*BBM*); extensins *(EXTs);* homogalacturonan *(HG); LEAFY COTYLEDON* (*LEC1*); rhamnogalacturonan I (*RGI*). This figure was created with http://BioRender.com. BioRender certificate confirming the publication rights is available upon request from the authors. The relative size of the stages of protoplast-derived cells is an approximation

tataricum. Methyl-esterified homogalacturonan domains were localised in the cell wall at the analysed steps in both species cultured (Supplementary Fig. S3, S4). Additionally, the LM25 epitope was noted on the surface of the outer periclinal walls of the peripheral cells (Supplementary Fig. S3C'-C"; brown arrow) in *F. tataricum*.

LM5 epitope (β-1,4-galactan in side chains of rhamnogalacturonan I (RGI)) was detected differently (Supplementary Fig. S5, S6). On the 15th day, the LM5 epitope was distributed only in some inner cell walls of the *F. tataricum* cell colonies (Supplementary Fig. S5B-B"; yellow arrows) in contrast to *F. esculentum*, in which the epitope was localised mainly in the outer periclinal cell walls (Supplementary Fig. S6B'-B", blue arrows). LM6 epitope (α-1,5-arabinan from side chains of RGI) occurred in cell walls (Supplementary Fig. S7, S8) as well as in cytoplasmic compartments (Supplementary Fig. S7A'-A" and S8A'-A").

AGPs epitope detected by the JIM13 antibody was localised in the cell walls and cytoplasmic compartments at each of the analysed time points (Supplementary Fig. S9-S10). Nonspecific binding to phenolics, localised in phenolic-containing cells, was noted (Supplementary Fig. S9C'-C", S10C'; purple arrows) as well as the presence of AGPs in the vacuole of *F. esculentum* culture occurred (Supplementary Fig. S10A', B'; white arrows). The JIM16 epitope was mainly detected in the cytoplasmic compartments (Supplementary Fig. S11; red arrows) and vacuoles (Supplementary Fig. S11A'; white arrow) of *F. tataricum* in comparison to *F. esculentum* where the epitope was absent (Supplementary Fig. S12).

Extensin epitope, detected by JIM20 antibody, was present in the cell walls on the 5th day of *F. tataricum* (Supplementary Fig. S13A'-A") and *F. esculentum* (Supplementary Fig. S14A'-A") cultures in a dotted manner. As well, the distribution of the JIM20 epitope was restricted to the surface of the outer periclinal walls of the peripheral cells of *F. tataricum* (Supplementary Fig. S13C'; brown arrow) and *F. esculentum* (Supplementary Fig. S14B' and C'; brown arrows).

Lipid staining

The occurrence of lipid substances was demonstrated by Sudan Black and Sudan III staining, as both are used for

Table 1 Summary of the immunocytochemical detection of selected antigens in protoplast cultures of *F. tataricum* and *F. esculentum*

Antibody	Day of culture	Presence (+) or absence (-) of the analysed epitope		Signal localisation			
				Cell wall		Cytoplasmic compartments	
		Ft	*Fe*	*Ft*	*Fe*	*Ft*	*Fe*
Hemicelluloses							
LM25	5th	+	+	+	+	+	-
	15th	+	+	+	+	-	-
	50th /30th	+	+	+*	+	+	-
Pectins							
LM20	5th	+	+	+	+	-	-
	15th	+	+	+	+	-	-
	50th /30th	+	+	+*	+	-	-
LM5	5th	+	+	-	+	+	-
	15th	+	+	+	+	-	-
	50th /30th	+	+	+	+	-	-
LM6	5th	+	+	+*	+	+	+
	15th	+	+	+	+	-	-
	50th /30th	+	+	+	+	-	-
Arabinogalactan proteins							
JIM13	5th	+	+	+	+	+	+
	15th	+	+	+	+	+	+
	50th /30th	+	+	+	+	+	+
JIM16	5th	+	-	-	-	+	-
	15th	+	-	-	-	+	-
	50th /30th	+	-	-	-	+	-
Extensin							
JIM20	5th	+	+	+	+	-	-
	15th	+	+	+	-*	-	-
	50th /30th	+	+	+*	-*	-	-

*epitopes were noted on a surface of outer periclinal walls of the peripheral cells

Ft - F. tataricum; Fe - F. esculentum

staining triglycerides, lipids, and lipoproteins. However, Sudan Black can additionally stain phospholipids. A positive reaction is indicated by the orange colour for Sudan III and black or blue for Sudan Black [27].

In *F. tataricum* protoplast-derived cultures, the occurrence of lipids at each of the analysed time points was observed. Six hours after protoplast isolation, small lipid droplets were revealed in the cytoplasm compartments (Supplementary Fig. S15A-B; red and black arrows). On the 5th and 15th day of the culture, the lipid droplets had different sizes, either easily detectable (Supplementary Fig. S15C-F) or very small and dispersed throughout the cytoplasm (Supplementary Fig. S15C'-D'). On the 50th day of culture, the lipid droplets were often noted near the cell wall (Supplementary Fig. S16A, C). Staining by Sudan Black showed a higher amount of blue-colored lipid droplets (Supplementary Fig. S16D-E; red arrows) compared to the application of Sudan III, possibly due to the affinity of Sudan Black to phospholipid fraction.

In *F. esculentum* protoplast cultures, the staining of small lipid droplets was noted in protoplasts after isolation (Supplementary Fig. S17A-B). However, in some cells, bigger droplets were detected, and those were located near the plasma membrane (Supplementary Fig. S17B'). On the 5th day of culture, most of the cell colony were intensely coloured by Sudan Black (Supplementary Fig. S17C); however, in some, only a few lipid droplets were distributed near the cell wall (Supplementary Fig. S17C'). On the 15th day of culture, the lipids were present in the cytoplasm and the cell walls (Supplementary Fig. S17E; red arrows). The dark black color of the cell colonies indicated its dense cytoplasm. For Sudan III staining, only some orange droplets were noted in the cells of cell colonies (Supplementary Fig. S17F; black arrows). On the 30th day of culture, lipid storage was pointed out, especially in peripheral cells of the microcalli (Supplementary Fig. S18A-C), which was demonstrated by Sudan III staining. Sudan Black staining revealed the presence of more lipid droplets (Supplementary Fig. S18D-F) distributed throughout the cell's cytoplasm and localised in cell walls (Supplementary Fig. S18D).

Proteomics analysis

The proteomic analysis performed at three time points of protoplast-derived cultures allowed us to identify 3664 proteins in *F. esculentum* and 3811 proteins in *F. tataricum* (Supplementary Fig. S19A). In *F. tataricum*, on the 50th day of culture, we identified the highest number of proteins, 3046, followed by 2646 on the 5th day and 2344 on the 15th day. The 1622 proteins were shared between all timepoints in the culture of *F. tataricum*. Similarly, in *F. esculentum*, the highest number of proteins were identified on the 30th day of culture, with 2933 different proteins being identified, followed by 2947 proteins on the 5th day and 2321 proteins on the 15th day, with 1846 proteins shared between all time points. The comparative analysis revealed significantly over-accumulated and under-accumulated proteins between the days of culture in both species (Supplementary Fig. S19B), showing the most pronounces changes in the protein accumulation between the 5th and 30th /50th days of culture (for *F. tataricum*: 503 over-accumulated and 657 under-accumulated, for *F. esculentum*: 541 over-accumulated and 655 under-accumulated) and the smallest between the 15th and 30th /50th days of culture (for *F. tataricum*: 189 over-accumulated and 287 under-accumulated, for *F. esculentum*: 139 over-accumulated and 193 under-accumulated) (Supplementary Fig. S19B and C). In *F. esculentum*, the highest increase in protein accumulation was observed for the sucrose-cleaving enzyme (33.3-times in comparison of 15th vs. 5th, 188.7-times for 30th vs. 15th) (Supplementary Table S2, sheet 1) and the most significant decrease for embryonic protein DC-8-like (EP DC-8) (22.6-times in comparison of 15th vs. 5th) (Supplementary Table S2, sheet 2 and Table 2). In *F. tataricum*, the highest increase in protein accumulation was observed for seed biotin containing protein (SBP) (94.1 times in comparison of 50th vs. 5th, 163 times for 50th vs. 15th) (Supplementary Table S2, sheet 1) and the most significant decrease for caffeoyl-CoA O-methyltransferase (CCOMT) (35.1-times in comparison of 15th vs. 5th) (Supplementary Table S2, sheet 2 and Table 2). The Gene Ontology (GO) term enrichment of proteins present in both species at analysed timepoints showed 61 enriched terms shared by all analysed samples (12 molecular functions (MF), 22 cellular compartments (CC), and 27 biological processes (BF)), among them GO terms related to cell wall (CC, GO:0005618), plasmodesma (CC, GO:0009506), and response to cytokinin (BP, GO:0009735) (Supplementary Table S2, sheet 3 and 4). On the 5th day, two enriched GO terms were shared by both species: vesicle organisation (BP, GO:0016050) and TBP-class protein binding (MF, GO:0017025). On the 15th day, three enriched GO terms were shared by *F. esculentum* and *F. tataricum*: response to anoxia (BP, GO:0034059), intracellular protein-containing

Table 2 Differentially accumulated proteins (DAPs) of *F. tataricum* and *F. esculentum* protoplast cultures

Majority protein IDs	Name	Abbreviation	*F. tataricum*			*F. esculentum*		
Ft/Fe			15th vs. 5th	50th vs. 5th	50th vs. 15th	15th vs. 5th	30th vs. 5th	30th vs. 15th
Somatic embryogenesis related proteins								
GWHPBJBL008574/ GWHPBJBK010836	Embryogenic protein DC-8-like	EP DC-8	NS	NS	28.5	-22.6	-1.8	12.6
GWHPBJBL018466/ GWHPBJBK015397	Endochitinase	ENDO1	NS	NS	NS	NS	NS	NS
GWHPBJBL018472/ GWHPBJBK015393		ENDO2	NS	NS	NS	NS	NS	NS
Seed storage proteins								
GWHPBJBL016675/ GWHPBJBK015259	Vicilin	VIC1	NS	136.1	100.8	NS	35.7	41.4
GWHPBJBL032170/ GWHPBJBK037628		VIC2	NS	84	101.6	NS	71.7	88.7
GWHPBJBL013999/ GWHPBJBK017428	Oleosin	OLEO	NS	24.3	6.0	NS	9.5	25.4
Cell wall related proteins								
GWHPBJBL002808/ GWHPBJBK004524	Fasciclin-like arabinogalactan protein	FLA1	NS	NS	NS	NS	NS	NS
GWHPBJBL002736/ GWHPBJBK004425		FLA2	NS	NS	NS	NS	3.9	4.7
GWHPBJBL013649/ GWHPBJBK018791		FLA3	NS	4.7	4.9	NS	NS	4.3
GWHPBJBL027662/ GWHPBJBK030169	Extensin	EXT	NS	NS	NS	-2.9	-2.3	NS
GWHPBJBL024749/ GWHPBJBK027741	Caffeoyl-CoA O-methyltransferase	CCOMT	-35.1	NS	NS	-4.1	NS	NS

NS – non significant

complex (CC, GO:0140535), transporter complex (CC, GO:1990351). For the 30th /50th day of culture, 12 GO terms were enriched, among others: response to desiccation (BP, GO:0009269), cellular oxidant detoxification (BP, GO:0098869), and peroxisome (CC, GO:0005777).

Genes expression analysis

We analysed the expression level of genes related to plant regeneration: *LEAFY COTYLEDON1* (*LEC1*), *BABY BOOM* (*BBM*), *FUSCA 3* (*FUS3*), *WUSCHEL* (*WUS*), *CLAVATA* (*CLV3*) and genes encoding proteins that are accumulated at a high level during protoplast-derived cultures, such as *LATE EMBRYOGENESIS ABUNDANT PROTEIN* (*LEA1, LEA2*), *EP DC-8*, *ENDOCHITYNASES* (*ENDO1*, *ENDO2*), *VICILIN* (*VIC1*, *VIC2*), *OLEOSIN* (*OLEO*), *FASCICLIN-LIKE ARABINOGALACTAN PROTEINS* (*FLAs*), *EXTENSINE* (*EXT*), *CCOMT*. The expression of all analysed genes was calibrated to the 5th day of *F. tataricum* culture to reveal the difference in expression level between time points of the culture and species (Fig. 3). We observed the higher expression of *BBM*, *LEA2*, *ENDO1*, *CCOMT*, *FLA1*, *2*, *3*, *EXT* genes at almost all analysed time points of the *F. esculentum* culture compared *to F. tataricum*. The most intense upregulation of expression was observed for the *EXT* gene on the 5th day of *F. esculentum* culture, where the expression was over 200 times higher than at *F. tataricum* culture. In contrast, lower expression of *VIC1* and *VIC2* genes was observed during the *F. esculentum* culture, and transcription of both genes was strongly stimulated on the 15th day of the *F. tataricum* culture. The differences in expression of other analysed genes, such as *FUS3*, *WUS*, *CLV3*, *LEA1*, *EP DC-8*, *ENDO2*, *and OLEO*, between the species were indicated at time points depending on genes. In summary, the expression pattern and level of analysed genes differed between species and time points of the culture.

Discussion

Plant cells devoid of cell walls are characterised by acquiring or regaining totipotency due to the possibility of reprogramming from a differentiated to a dedifferentiated state and high regeneration ability [28]. Last year, we confirmed the ability of common and Tartary buckwheat protoplasts for regeneration [6, 7]. Differences in the time required for microcalli formation and plant regeneration were noted. Therefore, we focused on determining whether differences, shared aspects, or potentially universal processes are involved. Research has focused chiefly on transcriptome and proteomic analysis of developmental reprogramming in protoplast-derived cultures [11, 23–25], while the fate of protoplast during development and gaining totipotency remained unexplored. Analysis of proteome, cell wall composition, and changes in the expression profile of the selected genes in *Fagopyrum* protoplast-derived cultures brought the missed information about plant cells' lack of cell wall ability to develop and form higher structures like cell colonies, microcalli and plants.

Transcription factors and proteins related to somatic embryogenesis

Among various factors that control plant cell reprogramming and are involved in gaining embryogenic competence, the major genes encoding transcription factors (TFs) like *LECs*, *BBM*, *WUS*, *CLV3*, and *FUS3* were studied [10, 29, 30]. The *LEC1* gene is essential for in vitro somatic embryogenesis induction and acquisition of embryogenic competence and controls many aspects of plant embryogenesis [31–33]. The present work provides a relation between *LEC1* expression and induction of somatic embryogenesis. Also, it explains events that occur during protoplast reprogramming at three time points (day 5th, 15th and 30th /50th) of *Fagopyrum* protoplast-derived cultures.

LEC1 is a sugar metabolism regulator by activating *SUCROSE SYNTHASE 2* (*SUS2*) [34]. The non-hormonal inducers of embryo development belong to sucrose concentration [35]; thus, SUS2 control somatic embryogenesis, which was proposed by Rolland, et al. 2002 [36]. Our proteomic results indicate a significant accumulation of SUCROSE SYNTHASE (SUS) on the 15th day of *F. esculentum* culture, which correlates with high expression of *LEC1* (Fig. 3A). According to Stein, et al. 2019 [37] and Ruan 2007 [38] *SUS* is required for the turgor build-up necessary for cell elongation, cellulose synthesis for cell walls and deposition of callose in the plasmodesmata. We can speculate that the identified enzyme can be significant for sugar metabolism in relation to the development of protoplast cultures that go through dynamic metabolic changes. Additionally, *LEC1* controls the expression of seed storage proteins (SSPs) genes by regulation of *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) and *FUS3* genes [39]. Interestingly, we observed a greatly increased expression of VIC1 and VIC2 with cultivation time (Table 2), belonging to a storage protein that correlates with *LEC1* activity (compare Fig. 3A with C). In oil palm, vicilin-like proteins were upregulated in high-embryogenic ortets and were suggested as markers of embryogenic tissue. Sahara, et al. 2023 [40] proposed that low expression of VICs in the oil palm callus can result in low-embryogenic induction. Interestingly, we observed a high increase in the expression of *VIC1* and *VIC2* along with cultivation time (Fig. 3C). Therefore, we can suspect that increasing expression of *VIC* genes in the *Fagopyrum* cultures can be related to somatic embryo formation. Moreover, overexpression of *LEC1* with *ABI3* and *FUS3* leads to globally higher expression of fatty acid synthesis genes and significantly increased levels of fatty

acid compounds in *Arabidopsis* [41]. We noted a high accumulation of OLEOSIN proteins (structural proteins of oil bodies) on the 15th day of *F. tataricum* and the 30th day of *F. escilentum* cultures (Table 2), which correlates with high expression of *LEC1* (compare Fig. 3A with C). Gliwicka, et al. 2012 [42] showed that higher expression of the *OLEOSIN4* (*OLEO4*) gene in embryogenic cultures of *Arabidopsis* compared to non-embryogenic ones and inactivation of *OLEO4* gene resulted in impaired somatic embryogenesis induction. The researchers suggested that *OLEO4* genes may support the tissue's perception of embryogenic signals, leading to embryogenic development. Dutta, et al. 1991 [22] revealed a higher proportion of lipid body area in somatic embryos than in callus and suspension culture cells. The high presence of OLEO proteins in *Fagopyrum* protoplast cultures can indicate an increase in oil bodies, thus leading to preparation for somatic embryogenesis events. Additionally, Potocka, et al. 2012 [43] showed that staining *Arabidopsis* explants with lipophilic dyes revealed lipid lamellae within some cell walls, which correlate with lipid transfer protein 1 distribution, pointing to morphogenic events occurring during somatic embryogenesis. In our investigation, lipids staining did not show any significant differences between the analysed time points, and we noted abundant phospholipids occurrence in *Fagopyrum* protoplast cultures.

The *LEC1* activity can also be connected with processes occurring during protoplast cultures, like cell elongation. Junker, et al. 2012 [44] demonstrated that *LEC1* targets cell-wall modification- and hormone- related genes leading to hypocotyls elongation of *Arabidopsis*. It was revealed that *LEC1* targets genes coding enzymes that modify cell walls, like xyloglucan hydrolase and expansins, which are involved in cell wall modification; thus, cell expansion processes can be observed during the development of protoplast-derived cultures.

Another TF belonging to the LECs group of genes is FUS3, which plays a key role in seed development, somatic embryos and plant lateral organ formation [45]. Moreover, *FUS3* can enhance the competence for somatic embryogenesis by decreasing the level of gibberellin, ethylene, and activation of *YUC* genes responsible for auxin biosynthesis [31]. *FUS3* is also a key regulator in accumulating SSPs and other reserve materials like lipids and carbohydrates [45]. We observed significantly increased expression of *FUS3* on the 15th day of *F. tataricum* culture, which correlated with the highest accumulation of *OLEO* and *VICs* (compare Fig. 3A with C).

BBM carries important functions in proliferation and re-differentiation during embryogenesis, participating in regulating genes involved in controlling cell signalling, cell wall biosynthesis and modification [31, 46, 47]. Kulinska-Lukaszek, et al. 2012 [46] noted that the GUS gene expression under the BBM promoter in immature zygotic embryos of *Arabidopsis* was restricted to dividing cells and cell clusters. Authors postulated that *BBM* promotes cell proliferation and defines an undifferentiated state [48]. In our study, we observed the highest level of *BBM* expression among examined time points on the 5th day for *F. tataricum*, the 5th and 15th day of *F. esculentum* (Fig. 3A), which are significant time points for the first cell division events in *Fagopyrum* protoplasts-derived cultures [6, 7]. Thus, we can speculate that *BBM* activity was related to cell proliferation, cells re-entering the cell cycle and dividing cells. Moreover, the abovementioned processes relate to changes in the structure of the cell wall that can be regulated through genes and proteins targeted by *BBM*, such as actin DEPOLYMERIZING FACTOR 9, AGPs, and proteins participating in synthesis or modification of cell wall polysaccharides as shown for *Brassica napus* [48]. Interestingly, after the activation of the *BBM* gene in *Arabidopsis* mesophyll cells, the actin cytoskeleton's reorganisation was observed, forming dense actin networks [48]. Such changes are characteristic of cells reorganising their cytoplasm before division (which occurs in protoplasts) or elongation. Among other genes regulated by *BBM*, Horstman, et al. 2017 [49] noted *LECs* genes, which show a relation between the *BBM*- and *LEC*-mediated somatic embryogenesis pathways. Therefore, we presume that the high activity of *BBM* on the 5th day of the cultures was associated with *BBM*-positive somatic embryogenesis regulations that next are taken over by *LEC1* (compare Fig. 3A). Moreover, *LEC1* and *BBM* are essential genes for embryo differentiation and maturation [47]. Together, this relation can suggests that *BBM* activity in *Fagopyrum* protoplast cultures may be related to different developmental pathways that occur close to the time of the first cell division and influence further embryo formation.

WUS and *CLV3* are meristem central regulators playing a pivotal role in determining stem cell fate in shoot apical meristem based on antagonistic function [50]. Furthermore, WUS prominent role is to maintain totipotent, embryogenic cell potential and to prevent their differentiation. Importantly, *WUS* expression is relevant for determining cell ability to become pluripotent or totipotent [30, 51]. Deyhle, et al. 2007 [52] observed that *WUS* regulates cell differentiation due to early expression during anther development and terminated expression during cell differentiation. Results of Zuo, et al. 2002 [53] show that *WUS* is a key factor in maintaining or inducing embryogenic potential compared to *LEC1*, which is a driving force for embryo cell differentiation. In the *Fagopyrum* cultures, the highest level of *WUS* on the 15th day (Fig. 3A) was observed, which may relate to cells' totipotent character after the second step of dedifferentiation (cell division). For many species, increased expression

of *WUS* increases the ability to form somatic embryos and regeneration capacity [30, 47]. On the other hand, *WUS* activates the expression of *CLV3*, which inhibits its expression, causing the differentiation of meristematic cells [51]. In our results, we observed a correlation in the level of *WUS* and *CLV3* (significantly high level for *WUS* and *CLV* expression) on the 15th day of *F. tataricum* culture (compare Fig. 3A), which can indicate the feedback loop between these genes. According to Elhiti 2010 [54], genes repressing meristematic cell characters like CLV3 strongly reduce somatic embryogenesis; therefore, the low level of *CLV3* in *Fagopyrum* cultures correlates with the high regeneration ability.

Somatic embryogenesis-related proteins

Among the most common proteins reported as potential markers of somatic embryogenesis [55] in *Fagopyrum* protoplast cultures, we distinguished the late embryogenesis abundant (LEA) proteins group. LEA proteins generally accumulate in response to biotic and abiotic stresses and provide a protective function. Conditions of *in vitro* culture and reactive oxygen species generate significant oxidative stress effects during the whole developmental period of protoplast cultures. These factors can correlate with increased levels of LEAs proteins like EP DC-8 and SBP (also belong to LEA group), which are highly accumulated in *Fagopyrum* cultures. We can suspect that LEA and SSPs can play a protective function and increase tolerance to stress factors in protoplast cultures. The dicot LEA protein DC8-like is known to be expressed in somatic or zygotic embryos but not in mature tissue; thus, according to Hatzopoulos, et al. 1990 [56], it is an embryo-specific gene. Additionally, many reports have shown that the accumulation of LEA and storage proteins is associated with embryogenic transition, leading to the induction of somatic embryogenesis [57]. Likely, the occurrence of LEAs, SSP, SBP and VIC in *Fagopyrum* cultures can coincide with events leading to somatic embryogenesis.

Endochitinases may influence somatic embryo development by releasing signal molecules [55]. In *Fagopyrum* protoplast cultures, we noted significantly increased expression of the endochitinases gene on the 50th day for *F. tataricum* (Fig. 3B). Hengel 1998 [58] demonstrated that endochitinases and AGPs can promote the formation of somatic embryos in protoplast cultures of carrot, which is related to the presence of endochitinases cleavage sites in AGPs, based on chitinase-like protein secretion in embryogenic and non-embryogenic cultures of *Dactylis glomerate* L. Tchorbadjieva, et al. 2006 [59] distinguished these proteins as marker of embryogenic potential. In accordance with this, we can suggest that signal molecules generated by endochitinases may affected somatic embryos formation in *Fagopyrum* cultures.

Changes in the cell wall

Removal of the cell wall, which is the first step of protoplast isolation, leads to cell dedifferentiation; after that, wall re-establishment is the most prominent step for protoplasts to divide and develop into callus. The wall components such as cellulose, hemicellulose, and pectin are crucial for establishing and maintaining the cellular differentiation status by maintaining cell-cell communication [12, 20]. As it was postulated by Majewska-Sawka, et al. 2003 [15] and Wiśniewska, et al. 2008 [16], cell wall composition is one of the important factors controlling plant regeneration *via* the protoplast cultures. For *Fagopyrum* protoplasts-derived cultures, Sala-Cholewa, et al. 2024 [13] described the mechanism of cell wall re-synthesis and pointed out differences in the spatio-temporal appearance or disappearance of individual epitopes during the first 72 h of protoplast cultures. Therefore, our goal was to investigate the cell wall composition and distribution of their components in the reconstituted cell wall within the path of protoplast cultures. Selected polysaccharides, such as hemicellulose (xyloglucan) and pectins (homogalacturonan, galactan, and arabinan), as well as proteins, were investigated due to their well-known involvement in various developmental processes, ranging from cell proliferation, differentiation, and expansion to somatic embryogenesis and plant growth [13, 16, 60, 61] (Fig. 4).

We noted a constitutive occurrence of hemicelluloses (galactosylated xyloglucan) and pectins (homogalacturonan methyl-esterified domains) in the walls of regenerating protoplasts and protoplast-derived cells. However, our results showed a changed distribution of RGI side chains, arabinan and galactan, in the analysed *Fagopyrum* species (Supplementary Fig. S5-S8). Many studies confirm that RGI side chain composition changes are related to cell differentiation status [20, 62]. The occurrence of galactan in the cell walls relates to their strengthening in some studied plant systems [63]. The appearance of the LM5 epitope on the 5th day of *Fagopyrum* cultures, just after cell division, could point to the strengthening of newly produced cell walls. Additionally, Potocka, et al. 2018 [20] noted the occurrence of galactan within the embryogenic domain of the *Arabidopsis* explant in cells presumably competent for somatic embryo formation. Therefore, we can suppose that the occurrence of galactans in inner cell walls on the 15th day of *F. tataricum* cultures can mark embryogenic domains within the cell colonies (Supplementary Fig. S5). Our results are also consistent with observations related to galactan-rich tobacco protoplasts that differentiated and were able to form new organs, compared to sugar beet protoplasts,

which were poor in galactan and were not able to regeneration [16].

Arabinan side chains provide elasticity of cell walls, thus ensuring flexibility for intensively dividing cells [64], which could correlate with the presence of the LM6 epitope on the 5th day of *Fagopyrum* cultures (Supplementary Fig. S7-S8). Moreover, arabinan is important in regulating the water content during desiccation and in salt-tolerant species, preventing the irreversible aggregation of cell wall polymers [65]. Protoplast cultures constantly go through intensive development related to cell division, growth or elongation; therefore, the occurrence of arabinans ensures that elasticity is necessary. Additionally, the culture is susceptible to desiccation when the callus overgrows agarose beads at the end of the culture. It can explain the occurrence of the LM6 epitope on the 30th and 50th day of *F. esculentum* and *F. tataricum* cultures, respectively. So far, the occurrence of arabinans was noted in guard- and mesophyll-derived protoplasts of tobacco and only in protoplast-derived callus of sugar beet [15, 16]. In general, both galactan and arabinan are essential for proper protoplast regeneration.

Hydroxyproline-rich proteins such as highly glycosylated AGPs are involved in different developmental processes, from cell proliferation, differentiation and expansion to somatic embryogenesis and plant growth [66]. Because of the multifunctionality of AGPs, many studies regarding protoplast cultures detected AGPs during cell wall re-building and during protoplast development [13–16]. In our research, widespread occurrence of AGPs recognised by JIM13 antibody was detected in the cell wall and internal cell compartments (Table 1), which may indicate their role in many cellular processes. Our results are in accordance with reports noted for sugar beet, tobacco and carrot protoplast cultures where the abundant distribution of JIM13 antibody was noticed [14–16]. Interestingly, the abundant occurrence of AGPs in cytoplasmic compartments, e.g. vacuoles, can indicate carbohydrate turnover. Butowt, et al. 1999 [67] detected AGPs in the vacuoles of sugar beet protoplast cultures, which shows these components' degradation. On the other hand, the localisation of AGPs detected by the JIM16 antibody differed between the analysed *Fagopyrum* species and the time points. In *F. tataricum* cultures, the JIM16 epitope was observed mainly in internal cell compartments until the 50th day, when the signal was undetected. For *F. esculentum* protoplast cultures, AGPs recognised by the JIM16 antibody were not localised at any point in the study. The noted differences are in accordance with the observed disappearance and enhanced expression of AGPs in some species undergoing cell differentiation [20, 68]. Godel-Jędrychowska, et al. 2019 [14] observed differences in AGPs expression in different carrot species pointing to cell-, tissue- and species specific AGPs presence.

Among AGP subclasses, fasciclin-like arabinogalactan proteins (FLAs) containing AGP-like glycated domains and fasciclin domains were detected by proteomic analysis. FLAs are proposed to play structural and signalling functions by organising cell wall components, regulating cell wall properties and affecting cell-to-cell interactions. Additionally, a relation between FLAs accumulation and cells' embryogenic potential and involvement in competence acquisition during shoot organogenesis in tissue culture of *Arabidopsis* was reported [69]. In our research, the occurrence of FLAs between *F. esculentum* and *F. tataricum* varied (in *F. esculentum* cultures, an increase over time was observed for *F. tataricum* – a decrease; Fig. 3D). The higher FLAs accumulation on the 5th day of *F. tataricum* culture, as followed by a decrease in their accumulation, can point to the role of FLAs in controlling cell expansion and cell-cell interaction during cell colony formation. It suggests that FLAs modulate the organisation of cell wall polysaccharides during cell division events.

Extensins are moderately glycosylated proteins that participate in cell extension and provide a stabilising and reinforcing role in the wall of cells that have stopped elongating [70]. Lee, et al. 2013 [71] demonstrated both the presence of EXTs on the surface cells of embryogenic callus and protocorm-like bodies of *Phalaenopsis* and their absence in non-embryogenic callus cells, leading to reduced regeneration potential. Interestingly, protocorm-like bodies showed mainly epidermal localisation of these proteins. EXTs epitopes were also found in *Mussa* spp. AAA embryogenic cells, proembryos and globular embryos, especially in their cell walls and extracellular matrix [72]. Our results concerning EXTs presence on the surface of outer periclinal walls of the peripheral cells of *F. esculentum* protoplast-derived cultures (Supplementary Fig. S14) are in accordance with the examples mentioned above. EXTs presence was observed between the 10th and 20th day of culture in carrot protoplast cultures but disappeared at the end of the cultures [14]. However, in our research, we noted on the 5th day EXTs presence, which can explain EXTs participation in cell plate formation during cell division as it was reported for Arabidopsis *root-*, *shoot*, *hypocotyl-defective* (*rsh*) mutant [73, 74]. Like in carrot protoplast cultures, we also observed the disappearance of EXTs in *F. tataricum* cultures.

CCOMT is involved in reinforcing plant cell walls by participating in the lignin biosynthesis pathway, especially in forming cell wall ferulic esters [75]. In *F. esculentum* cultures, we noted a decrease in *CCOMT* expression among the analysed time points (Fig. 3D). Sharifi, et al. 2012 [76] showed a significant decrease in CCOMT accumulation in embryogenic callus compared to

non-embryogenic callus of *Crocus sativus.* Authors suggest that higher expression of CCOMT in non-embryogenic callus may inhibit somatic embryogenesis by increasing cell wall lignification, thus strengthening the cell wall. In alfalfa plants, Guo, et al. 2001 [77] noted that down-regulation of *CCOMT* led to reduced lignin levels and accumulation of soluble caffeic acid β-D-glucoside. We can suspect that during the development of protoplast cultures, the cell wall is enriched in caffeic β-D-glucoside because CCOMT downregulation enables cell wall stretching.

Materials and methods

Protoplast isolation

Protoplasts were isolated from the EC of *F. esculentum* and the MC of *F. tataricum.* The callus lines were obtained from immature zygotic embryo for both species and maintained on RX medium according to Betekhtin, et al. 2017 [68] at 26 ± 1°C in the dark. The callus was subcultured every three weeks for *F. esculentum* and every two weeks for *F. tataricum*. The calli of both species exhibited different regeneration modes. EC of *F. esculentum* regenerated exclusively through somatic embryogenesis, whereas MC of *F. tataricum* regenerated via both organogenesis and somatic embryogenesis. All protoplast isolation and culture steps were performed according to the established protocol for *F. esculentum* [6] and *F. tataricum* [7].

At three time points of the culture: 5th, 10th and 30th or 50th day for *F. esculentum* and *F. tataricum*, respectively, the agarose beads were frozen in 5 ml Eppendorf's and kept at -80°C to use as a material for proteomics and RT-qPCR. Fresh material was used for lipid staining. The material was fixed at the respective three-time points for immune- and histological analyses.

Histological and immunostaining procedure

At each of the three time points representing characteristic cellular events, the beads of protoplast cultures were prepared according to the procedure described by Betekhtin, et al. 2019 [26] for histological and immunostaining study. The material was placed in a fixative mixture of 4% paraformaldehyde (PFA, POCH) and 1% glutaraldehyde (GA, POCH) in phosphate buffer saline (PBS, pH = 7.2), deaerated and incubated overnight at 4°C. Then the samples were washed three times with PBS (15 min each), dehydrated in increasing ethanol concentrations (10%, 30%, 50%, 70%, 90%, 100% *v/v*) two times for each concentration (30 min each) and gradually embedded in London Resin (LR White resin, Polysciences Inc.) according to Milewska-Hendel, et al. 2024 [78]. After that, the samples were cut into 1.5 µm thick sections using an EM UC6 ultramicrotome (Leica Biosystems) and put on glass slides coated with poly-L-lysine (Gerhard Menzel). For histology analysis, the slides were stained with 0.05% Toluidine Blue O (prepared based on water, Sigma-Aldrich) for 5 min, washed with distilled water and viewed in a brightfield microscope (AxioImager Z2 epifluorescence microscope, Zeiss).

Immunostaining started with applying blocking buffer (BB; 2% fetal calf serum and 2% bovine serum albumin in PBS) to the sections for 30 min at RT. Then, primary monoclonal antibodies (diluted 1:20 in BB; Plant Probes) were applied on the slides, and overnight incubation at 4°C was performed. The antibodies used for the current study are listed in the Supplementary Table S1. After that, the samples were washed three times in BB and incubated with the secondary antibody (AlexaFluor 488 goat anti-rat IgG, Jackson ImmunoResearch Laboratories; diluted 1:100 in the BB) for 1.5 h at RT. After that time, the slides were washed thrice in the BB and PBS, 5 min each. Next, fluorescent brightener 28 (FB28; 0.01%; diluted in PBS; Sigma-Aldrich) was applied for 5 min at RT to visualise cellulose in the cell wall. After that, the slides were washed thrice in the PBS and distilled ultrapure H2O for 5 min each. Slides were sealed by application of the Fluoromont mounting medium (Sigma-Aldrich) and stored at 4°C.

All of the photographs and observations were performed using an AxioImager Z2 epifluorescence microscope equipped with an AxioCam Mrm monochromatic camera (Zeiss) equipped with narrow-band filters for visualisation of AlexaFluor 488 and FB28 fluorescence.

Lipid staining

The occurrence of lipid substances was detected by application of Sudan Black B and Sudan III (Sigma-Aldrich). Before staining, the material was fixed overnight at 4°C in a mixture of PFA and GA as described above and washed three times in PBS (5 min each) prior to lipid staining. For Sudan Black staining, the material was incubated in a 1% solution of Sudan Black B in 70% ethanol for 20 min in RT. In the case of Sudan III, the material was immersed in 0.5% staining solution for 2 h in RT. After staining, the material was washed in 50% ethanol and distilled water (three times, 5 min each). Observations were carried out with the use of a brightfield microscope (Zeiss).

Total proteins isolation and LC-MS/MS analysis

The total proteins extraction was performed from material frozen at -80 °C. The isolation and further analysis were performed for four biological replications. The material was ground in liquid nitrogen with the addition of 100 mg/per sample of polyvinylpyrrolidone (PVP, average mol wt 40,000, Sigma-Aldrich). The proteins were isolated from grounded material according to the protocol by Wu, et al. 2014 [79] following the tissue disruption (step 1), trichloroacetic acid/acetone precipitation

(steps 2–3) and sodium dodecyl sulfate extraction of proteins (steps 9–11). The protein extract was further purified and precipitated using the chloroform/methanol precipitation approach, as described by Wessel, et al. 1984 [80]. The proteins were resuspended in buffer (7 M urea, 2 M thiourea, 1.7% PMSF, 50 mM DTT). The proteins concentration was measured using Bradford Reagent (Sigma-Aldrich, B6916-500 M) with Albumin Standard (Thermo Scientific) for standard curve preparation. The analysis of protein samples using liquid chromatography tandem-mass spectrometry (LC-MS/MS) was performed according to Pinski, et al. 2021 [81]. Briefly, the samples were prepared using the FASP protocol, digested with trypsin and peptides were used for LC-MS/MS analysis. The obtained data were processed using the MaxQuantsoftware package (version 1.5.8.3) and Perseus platform. The protein sequences were downloaded from the Chinese National Genomics Data Center database (https://bigd.big.ac.cn/) for *F. esculentum* "Pintian4" (GWHBJBK00000000) and *F. tataricum* "Pinku1" (GWHBJBL00000000) references genomes [82]. Gene ontology analysis was performed by annotating the genes with eggNOG 5.0 [83] and GO Enrichment version 2.0.1 implemented in Galaxy Australia version 21.09 [84].

RNA isolation and real-time qPCR

Total RNA was isolated from the material of *F. esculentum* and *F. tataricum* on the 5th, 15th, and 30th (for *F. esculentum*) and 5th, 15th, and 50th (for *F. tataricum*) day of protoplast-derived cultures. Total RNA was isolated using a FastPure Plant Total TNA Isolation Kit - Polysaccharides and polyphenolics-rich (Vazyme Biotech). RNA concentrations were measured using a NanoDrop ND-1000 (NanoDrop Technologies). The DNA was removed from the RNA samples by digesting them with an RNase-free DNase Set (Qiagen). Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and oligo-dT primers were used to produce the cDNA. The cDNA was diluted four-fold with water and used in a qPCR reaction (2.0 µl). Analyses were performed using a LightCycler® 480 SYBR Green I Master (Roche) according to reaction conditions described in Sala-Cholewa, et al. 2024 [85]. The primers were designed based on *F. esculentum* "Pintian4" and *F. tataricum* "Pinku1" references genomes with Primer3Plus (Supplementary Table S2, sheet 7). The control genes (SAND, ACTIN) had a constant expression level in all tissue samples. The Ct values were calculated using LinRegPCR software (version 11, Academic Medical Centre). The tissues for the Real-Time qPCR analysis were produced in three biological repetitions, and two technical replicates of each repetition were analysed. The relative expression level was calculated 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 calculate any significant differences between the experimental combinations. The graphs (Fig. 3) show the average values with the standard deviation (SD).

Conclusions

This study provides a comprehensive analysis of the development of *Fagopyrum* protoplast-derived cultures, tracing the process from the initial cell division through to cell colony formation and the development of microcalli. Insights into the cell wall composition and expression profile of selected genes revealed changes that can correlate with regaining embryogenic competence during cell colony development. Proteomic analysis revealed an accumulation of storage and embryogenesis-related proteins. We demonstrated varied expressions of somatic embryogenesis-related genes and proteins. Based on this analysis, we distinguished seed storage proteins like VIC, OLEO, and SBP, which may play an important role in the somatic embryogenesis pathway of regeneration. The seed storage proteins seems to be connected with activation of TFs. Additionally, we confirmed changes in the cell wall composition during the development of cell colonies, indicating ongoing differentiation processes.

Abbreviations

AGPs	Arabinogalactan proteins
BB	Blocking buffer
BBM	BABY BOOM
BM	Basal medium
CCOMT	Caffeoyl-CoA O-methyltransferase
CLV3	CLAVATA
EC	Embryogenic callus
ENDO	Endochitynases
EP DC-8	Embryogenic protein DC-8-like
EXTs	Extensins
FLAs	Fasciclin-like arabinogalactan proteins
GA	Glutaraldehyde
LEA	Late embryogenesis abundant protein
LEC1	LEAFY COTYLEDON
MC	Morphogenic callus
OLEO	Oleosin
PBS	Phosphate buffer saline
PCCs	Phenolics-containing cells
PECCs	Proembryogenic cell complexes
PFA	Parafolmaldehyde
PSK	Phytosulfokine-α
RGI	Rhamnogalacturonan I
RT	Room temperature
SBP	Seed biotin-containing protein
SSPs	Seed storage proteins
SUS	SUCROSE SYNTHASE
TFs	Transcription factors
VIC	Vicilin
WUS	WUSCHEL

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06119-3.

Supplementary Material 1: **Supplementary table S1**: Antibodies used for immunostaining of protoplast cultures and the epitopes they recognise in the cell wall and relevant references.

Supplementary Material 2: **Supplementary table S2**: The statistical analysis of proteomic data.

Supplementary Material 3: **Supplementary figure S1**: Immunolocalisation of LM25 epitope in *F. tataricum* protoplast cultures on the 5th (A-A″), 15th (B-B″), and 50th (C-C″) day of the culture. A′ red arrows point to the presence of epitope in cytoplasmic compartments; C′ and C″ brown arrow points to a fluorescence signal detected on a surface of outer periclinal walls of the peripheral cells. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S2**: Immunolocalisation of LM25 epitope in *F. esculentum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 30th (C-C″) day of the culture. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S3**: Immunolocalisation of LM20 epitope in *F. tataricum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 50th (C-C″) day of the culture. C′ and C″ brown arrows point to the presence of epitope on a surface of outer periclinal walls of the peripheral cells. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S4**: Immunolocalisation of LM20 epitope in *F. esculentum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 30th (C-C″) day of the culture. B″ yellow arrows point signal in the internal cell walls. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S5**: Immunolocalisation of LM5 epitope in *F. tataricum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 50th (C-C″) day of the culture. B′ yellow arrows indicate a signal in the internal walls of the cell colonies. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S6**: Immunolocalisation of LM5 epitope in *F. esculentum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 30th (C-C″) day of the culture. B′ blue arrows indicate the outer periclinal cell wall. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S7**: Immunolocalisation of LM6 epitope in *F. tataricum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 50th (C-C″) day of the culture. B′ yellow arrows indicate the presence of the epitope in cell walls. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S8**: Immunolocalisation of LM6 epitope in *F. esculentum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 30th (C-C″) day of the culture. C′ yellow arrows indicate the presence of the epitope in cell walls. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S9**: Immunolocalisation of JIM13 AGPs epitope in *F. tataricum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 50th (C-C″) day of the culture. C′ purple arrows indicate nonspecific binding of the antibody to phenolics. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S10**: Immunolocalisation of JIM13 AGPs epitope in *F. esculentum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 30th (C-C″) day of the culture. A′ and B′ white arrows indicate localisation of the epitope in the vacuole; C′ purple arrows indicate nonspecific binding of the antibody to phenolics; C′ yellow arrows point to fluorescence signal in the cell wall of peripheral cells. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S11**: Immunolocalisation of JIM16 AGPs epitope in *F. tataricum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 50th (C-C″) day of the culture. B′ white arrow indicates localisation of the epitope in the vacuole; B′ red arrows points to the fluorescence signal in cytoplasmic compartments. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S12**: Immunolocalisation of JIM16 AGPs epitope in *F. esculentum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 30th (C-C″) day of the culture. *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S13**: Immunolocalisation of JIM20 extensin epitope in *F. tataricum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 50th (C-C″) day of the culture. C′ red arrows indicate localisation of the epitope in the intercellular spaces; brown arrow point to the presence of epitope on the surface of the outer periclinal walls of the peripheral cells *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S14**: Immunolocalisation of JIM20 extensin epitope in *F. esculentum* protoplast cultures on the 5th (A-A″), 15th (B-B″) and 30th (C-C″) day of the culture. B′, C′ and C″ brown arrows point to the presence of epitope on a surface of outer periclinal walls of the peripheral cells; B′ purple arrows indicate the phenolics; *FB* fluorescent brightener. Scale bars 10 µm. **Supplementary figure S15**: Lipid staining in *F. tataricum* protoplast cultures during three times points: 6 h after protoplast isolation (A, B); on the 5th (C, C′, D, D′) and 15th (E, F) day of the culture. Lipid droplets were stained black or blue after Sudan Black staining (A, C, C′, E); orange color is a positive reaction after Sudan III staining (B, D, D′, F). Red and black arrows indicate lipid droplets. Scale bars 10 µm. **Supplementary figure S16**: Lipid staining in *F. tataricum* protoplast cultures on the 50th day of the culture. Lipid droplets stained orange after Sudan III staining (A-C); black or blue after Sudan Black staining (D, D′, E). Red and black arrows indicate lipid droplets. Scale bars 20 µm (A, D, E); 10 µm (B, C, D′). **Supplementary figure S17**: Lipid staining in *F. esculentum* protoplast cultures during four times points: 6 h after protoplast isolation (A, B); on the 5th (C, D) and 15th (E, F) day of the culture. Lipid droplets stained black or blue after Sudan Black staining (A, C, E); orange after Sudan III staining (B, D, F). Red and black arrows indicate lipid droplets. Scale bars 10 µm. **Supplementary figure S18**: Lipid staining in *F. esculentum* protoplast cultures on the 30th day of the culture. Lipid droplets stained orange after Sudan III staining (A-C); black or blue after Sudan Black staining (D-F). Red and black arrows indicate lipid droplets. Scale bars 10 µm (B, C, E, F); 20 µm (A, D). **Supplementary figure S19**: The summary of proteomics analysis results. The Venn diagram shows protein presence in *F. tataricum* and *F. esculentum* on different days of protoplast cultures. The protein was designated to be present in the treatment if protein was detected in at least three out of four biological replications (A). The count of differentially accumulated proteins (DAPs) in *F. tataricum* and *F. esculentum* protoplast cultures at different days (B). The cluster maps of treatments for proteomics data for *F. tataricum* and *F. esculentum* (C).

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Author contributions
Conceptualisation: AB, AP; Methodology: MZ, AP, KN, K S-CH, UJ, B S-R; Formal analysis: MZ, AP, K G-J, K S-CH, UJ, B S-R, EK, AB, EG; Investigation: MZ; Resources: AB; Writing—original draft: MZ, AP, K S-CH, KN; Writing—review & editing: all authors; Visualization: MZ, AP, K S-CH, KN; Supervision: AB, AP, EK, EG; Project administration: AB; Founding acquisition: AB. All authors have read and approved the final manuscript.

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Data availability
The mass spectrometry data were deposited at the ProteomeXchange Consortium via the MassIVE repository with the dataset identifier PXD055850.

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. *F. esculentum* cultivar Panda seeds are commercially available and purchased from the Malopolska Plant Breeding company (Poland).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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11. Podsumowanie

W trakcie realizacji niniejszej pracy doktorskiej opracowano system efektywnej i szybkiej regeneracji roślin poprzez kulturę protoplastów *F. esculentum* oraz *F. tataricum.* Przeprowadzone badania dostarczyły narzędzi umożliwiających wydajną i szybką regenerację roślin gryki zwyczajnej oraz gryki tatarki, które mogą być zastosowane w wielu aspektach. Jednym z nich jest modyfikacja genetyczna roślin poprzez transfekcję protoplastów przy wykorzystaniu kompleksów rybonukleoproteinowych Cas9 w celu otrzymania roślin wolnych od obcego materiału genetycznego (Woo *i inni*, 2015). Kolejnym istotnym kierunkiem jest hybrydyzacja somatyczna dla poszerzenia puli genetycznej poprzez tworzenie somatycznych mieszańców międzygatunkowych oraz transfer cech w obrębie rodzaju *Fagopyrum* (Xia, 2009). W/w działania mogą pomóc w przezwyciężeniu problemu samoniezgodności gryki zwyczajnej oraz innych czynników (wspomnianych we wstępnie pracy) wpływających na niestabilność plonowania, a także przyczynić się do poprawy cech agronomicznych obu gatunków. Przeprowadzone badania pozwoliły wytypować kluczowe czynniki warunkujące rozwój protoplastów. (1) Dowiedziono, że kalus o wysokich zdolnościach regeneracyjnych i potencjale embriogennym jest lepszym od hipokotyli siewek materiałem donorowym do izolacji protoplastów. Sprawiło to, że pod wpływem dodatkowych suplementów wspomagających rozwój, protoplasty (kalusowe) były zdolne do przywrócenia podziałów mitotycznych, formowania kolonii komórkowych, minikalusa, a w efekcie końcowym w pełni wykształconych i funkcjonalnych roślin. (2) Wykazano, iż immobilizacja protoplastów w niskotopliwej agarozie (LMPA) pozytywnie wpływa na rozwój kultury protoplastów, a (3) suplementacja bazowej pożywki do kultury protoplastów w fitosulfokinę, przełamuje latencję podziałową tej kultury. Wartym zauważenia jest również fakt, że czas niezbędny do regeneracji w pełni wykształconych roślin został skrócony do 2-5 miesięcy w porównaniu z innymi doniesieniami (Adachi *i inni,* 1989; Gumerova, 2004). Dodatkowo, przedstawiona praca po raz pierwszy pokazuje możliwość regeneracji gryki tatarki poprzez kulturę protoplastów, co otwiera szerokie możliwości jej aplikacji.

Zaobserwowane różnice w czasie rozwoju kultury protoplastów jak również sposobie regeneracji stanowiły podstawę do bardziej wnikliwych analiz. Przeprowadzone badania poszerzają aktualny stan wiedzy na temat procesów i zmian zachodzących podczas rozwoju kultury protoplastów, dostarczając nowych danych z zakresu architektury ściany komórkowej, proteomiki, ekspresji czynników transkrypcyjnych oraz wybranych genów związanych z somatyczną embriogenezą. Obserwowane zmiany w kulturze protoplastów *Fagopyrum*

korelowały z odzyskiwaniem kompetencji embriogennych podczas rozwoju kolonii komórkowych. Analiza proteomiczna ujawniła wzrost akumulacji białek związanych z magazynowaniem substancji zapasowych, które mogą odgrywać ważną rolę w regulacji szlaku somatycznej embriogenezy. Potwierdzono również zmiany w składzie ściany komórkowej, co świadczy o procesach różnicowania kolonii komórkowych. Efekty przeprowadzonych badań i osiągnięte rezultaty pogłębiają zrozumienie kluczowych procesów ważnych dla rozwoju kultur protoplastów.

12. Wnioski

Na podstawie przeprowadzonych badań wyciągnięto następujące wnioski:

1. Zastosowanie morfogennego kalusa *F. tataricum* oraz embriogennego kalusa *F. esculentum* jako materiału donorowego pozwoliło na efektywną regenerację roślin w kulturach protoplastów *Fagopyrum*.

2. Immobilizacja protoplastów w agarozie (LMPA) pozytywnie wpłynęła na rozwój kultury protoplastów *Fagopyrum*.

3. Skład pożywki do kultury protoplastów, a zwłaszcza rodzaj i stężenie auksyny i cytokininy korzystnie oddziaływało na rozwój kultury protoplastów. W szczególności wzbogacenie pożywki w fitosulfokinę przełamało latencję podziałową kultury protoplastów gryki.

4. Suplementacja pożywki bazowej do kultury protoplastów w AIP oraz PVP nie zwiększyła wydajności kultury protoplastów.

5. Zastosowanie TDZ podczas regeneracji kalusa pochodzącego z kultury protoplastów gryki zwyczajnej stymulowało somatyczną embriogenezę.

6. Zaobserwowane zmiany w dystrybucji komponentów ściany komórkowej tj. łańcuchy boczne RGI, czy EXT wskazują na procesy różnicowania się kolonii komórkowych.

7. Procesom rozwoju kolonii komórkowych w kulturze protoplastów towarzyszyły zmiany proteomu, ekspresji czynników transkrypcyjnych oraz genów związanych ze zdarzeniami somatycznej embriogenezy:
   - wzrost akumulacji białek związanych z gromadzeniem substancji zapasowych może wskazywać na przygotowanie kultury do zdarzeń somatycznej embriogenezy,
   - analiza ekspresji czynników transkrypcyjnych wykazała zmienny poziom ekspresji genów i białek związanych z somatyczną embriogenezą.

13. Uzupełnienie - pozostały dorobek naukowy

Zaranek, M., Pérez-Pérez, R., Malec, J., Grzebelus, E. (2024). Protoplast Isolation, Culture, and Regeneration in Common and Tartary Buckwheat. w: Betekhtin, A., Pinski, A. (edytorzy) Buckwheat. Methods in Molecular Biology, vol 2791. Humana, New York: Springer 2024: 45-56.
https://doi.org/10.1007/978-1-0716-3794-4_5
Punkty MNiSW: 20

Zastosowana w badaniach metodyka dotycząca izolacji, kultury oraz regeneracji protoplastów gryki tatarki oraz gryki zwyczajnej została opisana w rozdziale piątym monografii wydanej przez wydawnictwo Springer z serii *Methods in Molecular Biology* pt. *Buckwheat: Methods and Protocols.*

Rozdział zawiera szczegółowy protokół opisujący proces izolacji, kultury oraz regeneracji protoplastów kalusowych oraz hipokotylowych *F. tataricum* oraz *F. esculentum*. Izolacja protoplastów obejmuje wstępną plazmolizę komórek w tkance oraz całonocną maceracją w obecności enzymów trawiących ścianę komórkową, jak również oczyszczanie protoplastów poprzez wirowanie w gradiencie gęstości. Podstawowa mieszanina maceracyjna do izolacji protoplastów zawiera enzymy o aktywności celulazy i pektoliazy, natomiast w przypadku hipokotyli oraz MK *F. esculentum* także driselazę. Opisany, krok po kroku, protokół umożliwia wyizolowanie odpowiednio dużej do założenia kultury liczby protoplastów. Rozwój protoplastów warunkuje ich immobilizacja w LMPA oraz kultura w płynnej pożywce wzbogaconej o NAA, BAP oraz PSK. Rozdział dodatkowo zawiera: (1) opis procesu namnażania minikalusa, otrzymanego w efekcie rozwoju protoplastów, na pożywce wzbogaconej w 2,4-D, KIN oraz PSK; oraz (2) opis procesu inicjującego regenerację, gdzie dla kultury gryki tatarki efektywna okazała się pożywka Murashige i Skoog (1962) wzbogacona w BAP oraz KIN, z kolei dla gryki zwyczajnej w BAP i TDZ. Zaprezentowany protokół pozwala na szybką regenerację roślin tj. w okresie 2-5 miesięcy od momentu regeneracji. W rozdziale zawarto szczegółowy spis materiałów oraz sprzętu niezbędnego do przeprowadzenia doświadczeń; roztworów oraz pożywek.

Protokół pozwala na dokładne odtworzenie eksperymentu. Zawiera również pomocne wskazówki umieszczone w sekcji *Notes* pozwalające na uniknięcie błędów podczas przeprowadzania doświadczeń.

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15. Oświadczenia autorów publikacji wchodzących w skład rozprawy doktorskiej

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Oświadczam, że w pracy:

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, 23, 385.

Mój udział polegał na optymalizacji i wykonaniu badań dotyczących izolacji, kultury oraz regeneracji protoplastów pochodzących z morfogennego kalusa oraz hipokotyli; przygotowaniu materiału do badań histochemicznych; obserwacjach mikroskopowych; analizie statystycznej, analizie i interpretacji otrzymanych wyników; przygotowaniu pierwszej oraz ostatecznej wersji manuskryptu.

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My participation consisted of optimization of the medium for regeneration of *Fagopyrum tataricum* culture.

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My participation consisted of optimization and conducting studies concerning isolation, culture, and regeneration of protoplast derived from embryogenic callus; preparing samples for histological analyses after that observing using microscopic techniques; processing the results; preparing the final version of the manuscript.

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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*ogy, 2025, 25, 102.

Mój udział polegał na przygotowaniu materiału tj. zakładaniu kultury protoplastów oraz przeprowadzaniu eksperymentów związanych z: analizą histologiczną, immunohistochemiczną, barwieniem związków lipidowych; obserwacjach mikroskopowych wykonanych preparatów; analizie i interpretacji otrzymanych wyników; wykonaniu zdjęć preparatów. Przygotowałam również materiał do analizy ekspresji genów metodą RT-qPCR oraz analiz proteomicznych. Opracowałam otrzymane wyniki oraz przygotowałam pierwszą oraz ostateczną wersję manuskryptu.

Magdalena Zaranek

podpis

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OŚWIADCZENIE

WSPÓŁAUTORA OSOBY UBIEGAJĄEJ SIĘ O WŁASNYM WKŁADZIE W POWSTAWANIE PRACY

Katowice, 11.03.2025

dr Artur Piński

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OŚWIADCZENIE

Oświadczam, że w pracy:

Zaranek, M., Pinski, A., Skupien-Rabian, B., Jankowska, U., Godel-Jedrychowska, K., Sala-Cholewa, K., Nowak, K., Kurczyńska, E., Grzebelus, E., Betekhtin, A.

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Mój udział polegał na izolacji białek całkowitych do analiz proteomicznych; wyborze genów do analizy RT-qPCR; analizie, interpretacji i opracowaniu otrzymanych wyników, przygotowaniu ostatecznej wersji manuskryptu. Jestem autorem korespondencyjnym.

Artur Piński

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OŚWIADCZENIE

Oświadczam, że w pracy:
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*ogy, 2025, 25, 102.

Mój udział polegał na analizie wyników histologicznych oraz immunohistochemicznych; barwieniu związków lipidowych; analizie, interpretacji oraz opracowaniu wyników; przygotowaniu ostatecznej wersji manuskryptu.

Katarzyna Sala-Cholewa
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OŚWIADCZENIE

Oświadczam, że w pracy:

Zaranek, M., Pinski, A., Skupien-Rabian, B., Jankowska, U., Godel-Jedrychowska, K., Sala-Cholewa, K., Nowak, K., Kurczyńska, E., Grzebelus, E., Betekhtin, A.

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Mój udział polegał na planowaniu i opiece merytorycznej badań z zakresu izolacji i kultury protoplastów oraz edycji manuskryptu.

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dr hab. Alexander Betekhtin, prof. UŚ

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Oświadczam, że w pracy:

Zaranek, M., Pinski, A., Skupien-Rabian, B., Jankowska, U., Godel-Jedrychowska, K., Sala-Cholewa, K., Nowak, K., Kurczyńska, E., Grzebelus, E., Betekhtin, A.

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Mój udział polegał na pozyskaniu finansowania projektu, planowaniu i nadzorze badań, opracowaniu koncepcji badań, opiece merytorycznej badań, interpretacji wyników oraz edycji wszystkich wersji manuskryptu. Jestem autorem korespondencyjnym.

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