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Application of biotechnological methods in studies on *Biscutella laevigata* L. ecotypes

Alina Wiszniewska[1*], Ewa Muszyńska[2], Barbara Tokarz[3], Ewa Hanus-Fajerska[4]

[1,3,4] *Department of Botany, Physiology and Plant Protection, Faculty of Biotechnology and Horticulture, University of Agriculture in Cracow, 29 Listopada 54 St., 31-425 Cracow*
[1] *e-mail: a.wiszniewska@urk.edu.pl, ORCID: 0000-0001-7737-819X*
[2] *Department of Botany, Institute of Biology, Warsaw University of Life Sciences-SGGW in Warsaw, 159 Nowoursynowska St, 02-776 Warsaw, e-mail: ewa_muszynska@sggw.edu.pl, ORCID: 0000-0003-0419-4597*
[3] *e-mail: b.piwowarczyk@urk.edu.pl, ORCID: 0000-0003-4131-8894*
[4] *e-mail: e.hanus-fajerska@urk.edu.pl, ORCID: 0000-0003-0566-0641*

Introduction

New directions of research that are useful in many fields of knowledge have been developed in laboratories, the results of which are frequently used in many areas of our life (Weber et al. 2006, Erpen-Dalla Corte et al. 2019, Xu et al. 2020). With regard to so-called green biotechnology (Da Silva 2004, Van Esse et al. 2020), methods are being improved using *in vitro* cultivation of plant material (Muszyńska et al. 2018, Nelofer et al. 2018, Hanus-Fajerska et al. 2019, Muszyńska et al. 2019). This methodical approach requires the propagation of cells, tissues, or plant organs by different techniques or isolated protoplast cultures (cells deprived of cell wall) using appropriate procedures (Jayaraman et al. 2016). Cultivation techniques may be indirect when the propagation of the material occurs via the callus tissue, that is meristematic tissue developing on the site of injury in wounded organs, on which organ formation is taking place. An alternative route is so-called direct regeneration, when organogenesis (shoot/root formation) takes place directly from explant tissues (fragments of tissues transferred onto culture medium) or by somatic embryogenesis, when embryos develop from somatic tissues (i.e. tissues other than those of the reproductive line) (Muszyńska and Hanus-Fajerska 2017, Park et al. 2017, Ramírez-Mosqueda and Iglesias-Andreu 2015, Wiszniewska et al. 2017). The plants obtained in the laboratory should then be acclimatized to *ex vitro* growing conditions (outside the culture vessels). Biotechnology also allows cryopreservation (long-term storage in below zero temperatures) of gene resources (Harding 2004, Engelmann 2004).

Due to the ability of the living plant cells to be totipotent (have the ability to differentiate into any type of cell) (Jayaraman et al.

* Corresponding author

2016, Fehér 2019), the use of micropropagation techniques allows a significant increase in the so-called multiplication coefficient compared to what specific populations can achieve when reproducing in natural conditions (Wiszniewska et al. 2017). Vegetative reproduction, not only under *in vitro* culture conditions, but also in greenhouse conditions, should be used especially when seeds harvested in the field have a reduced viability and germination capacity, or when seedlings have a low survival frequency in field conditions. In such circumstances, micropropagation methods may contribute to a more efficient vegetative reproduction of populations, which in natural conditions have limited possibilities of generative reproduction. Usually such problems result from disorders during sexual reproduction in the male and/or female line. In such a situation, the micropropagation methods developed may allow the introduction of plants obtained in laboratories to support the population of endangered species. This applies in particular to plants with unbalanced genomes – (auto)polyploids, hybrids, but also plants growing under stress conditions, including those inhabitating areas with a high degree of soil mineralization with calamine and serpentine, for example. Both genetic imbalance, and the negative effect of metals, which causes a genotoxicity effect, lead to the observed disturbances in the course of micro- and macrosporogenesis and in the formation of male and female gametophytes, embryo and endosperm (Izmaiłow et al. 2015, Bothe and Słomka 2017). The impact of environmental changes related to human activity also appears to be significant – broadly understood economic and industrial activities, expansion of the area occupied by agricultural crops and expansion of cities into open areas. This strong pressure on the natural environment that causes its transformation threatens the existence of certain plant populations, which may cause an irreversible loss of unique gene pools representing local populations (Pošcić et al. 2013, Babst-Kostecka et al. 2014, Wierzbicka et al. 2016). *In vitro* culture techniques may therefore play a key role in preserving resources and accelerating the growth of rare and valuable genotypes, for example calamine populations of *Biscutella laevigata* L., buckler mustard, (Wierzbicka et al. 2017, Bemowska-Kałabun et al. – Chapter 5 and 6 of this volume). Thus, the elaboration of protocols to the efficient micropropagation of given populations growing in the wild is of vital importance. The developed methods can also be applied in ecologically justified remediation, also referred to as assisted succession. Using native taxa adapted to local habitats (e.g. soil species contaminated with metals), it is possible to restore the natural values relatively quickly, even in extremely devastated areas (Ciarkowska and Hanus-Fajerska 2008, Suchkova et al. 2014, Muszyńska and Hanus-Fajerska 2017, Muszyńska et al. 2017, Hanus-Fajerska et al. 2019). Carrying out this type of remediation, however, requires the development of efficient production methods in *in vitro* cultures in a short time, and a large amount of aligned plant material ready for planting.

Regeneration abilities of *B. laevigata* calamine ecotype in *in vitro* cultures: micropropagation and acclimatization of microplants to *ex vitro* conditions

The possibility of increasing the vegetative propagation rate of *B. laevigata* calamine ecotype under *in vitro* conditions by direct organogenesis was demonstrated by Hanus-Fajerska and co-authors (2012). In the subsequent research carried out by authors of this chapter in the laboratory of the Department of

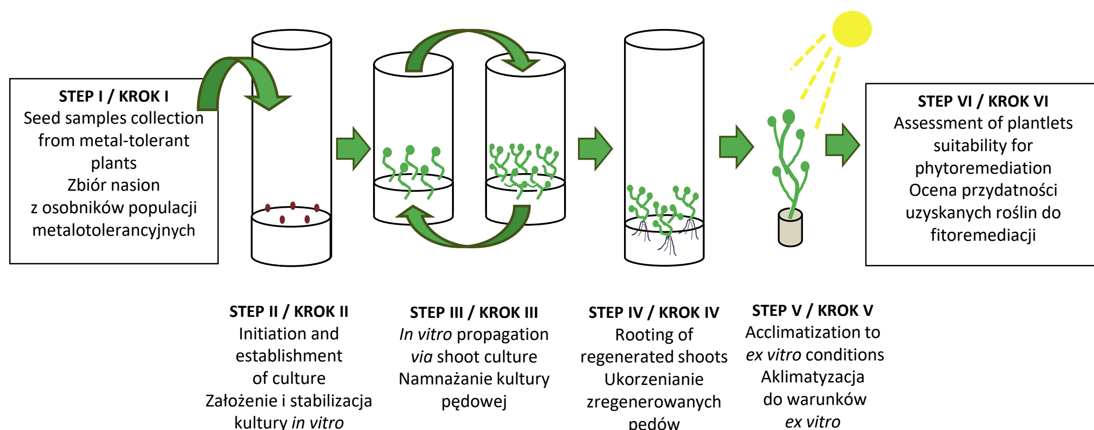


Fig. 1. The micropropagation scheme of plant material by shoot culture, which could be used for growing on polluted sites (according to Muszyńska and Hanus-Fajerska 2017, modified). Explanations in the text

Ryc. 1. Schemat mikrorozmnażania w kulturze pędowej materiału roślinnego, który może być wykorzystywany do uprawy na terenach zanieczyszczonych (według Muszyńska i Hanus-Fajerska 2017, zmienione). Objaśnienia w tekście

Botany, Physiology and Plant Protection at the University of Agriculture in Cracow (UA), the conditions for initiating and conducting *in vitro* cultures of *B. laevigata* calamine ecotype were elaborated in detail. Particular attention was paid to the development of a micropropagation protocol for this species (Fig. 1). The starting material used for the initiation of aseptic cultures was the seeds obtained at the end of June of 2010 from individuals representing the calamine population of the Olkusz region (Fig. 2A). The germination capacity of seeds under *in vitro* conditions was about 70% (Muszyńska and Hanus-Fajerska 2012). Aseptically obtained seedlings were used as primary explants to start the stabilized shoot culture. The developed protocol for *in vitro* propagation includes the stages shown schematically in Figure 1: shoot culture stabilization from apical fragments of seedlings obtained from seeds, the efficient regeneration of microrosettes on modified MS medium (Murashige and Skoog 1962) (Fig. 2B), the rooting of rosettes on half-strength MS medium without growth regulators (Fig. 2C), followed by effective plant acclimation to *ex vitro* conditions. Acclimatized plants were successfully grown under greenhouse conditions (Fig. 2D) and on the experimental plots described in Chapter 7 of this volume, along with other species that tolerate heavy metals (Fig. 2E) (Hanus-Fajerska et al. 2012, Muszyńska et al. 2017).

Studies were also carried out to improve the efficiency of buckler mustard micropropagation. Enrichment of the medium with various types of organic compounds, including pineapple extract, coconut water, and conditioned medium after the cultivation of green algae *Desmodesmus subspicatus* (Chodat) E. Hegewald & A. W. F. Schmidt, were tested (Hanus-Fajerska et al. 2012). The conditioned medium is a spent medium obtained after culturing cells and tissues of different plants or microorganisms. In such a culture, not only the nutrients are consumed, but also explants can secrete biologically active substances. In this study, the conditioned medium after green algae cultivation contained compounds of autoinductive activity that increased the rate of cell division (Grabski and Tukaj 2008). The obtained results of *B. laevigata* clonal propagation on

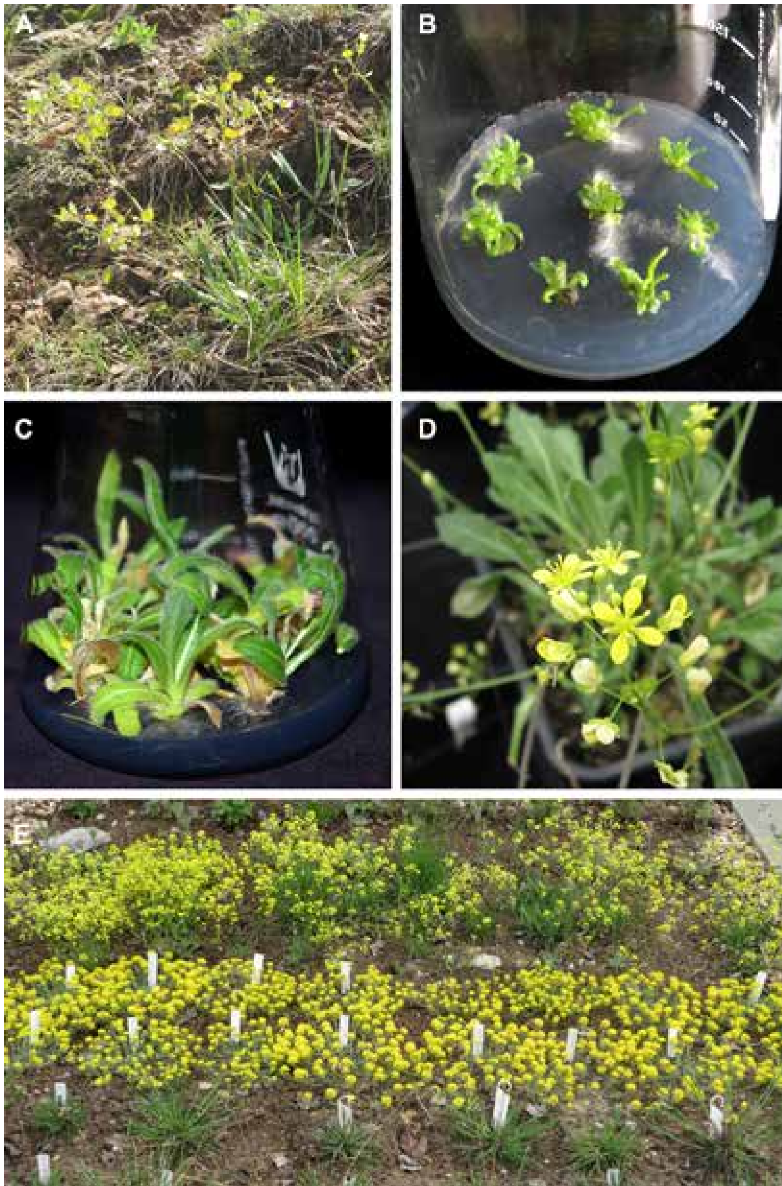


Fig. 2. *Biscutella laevigata* calamine ecotype from the Olkusz region. A – plant habitat. B, C – successive stages of leaf microrosette proliferation obtained from aseptic seedlings on modified MS medium supplemented with plant growth regulators: 1.0 mg/l cytokinin isopentenyloadenine (2iP) and 0.1 mg/l auxin naphthaleneacetic acid (NAA) (B) and without plant growth regulators (C). D – blooming specimens after acclimatization to greenhouse conditions. E – Plants growing on an experimental plot in the field. Explanations in the text

Ryc. 2. Ekotyp galmanowy *Biscutella laevigata* z rejonu olkuskiego. A – siedlisko roślin. B, C – kolejne stadia namnażania mikrorozet liściowych z fragmentów sterylnie uzyskanych siewek na zmodyfikowanej pożywce MS wzbogaconej w regulatory wzrostu roślin: 1,0 mg/l cytokininy – izopentenyloadeniny (2iP) oraz 0,1 mg/l auksyny – kwasu naftylooctowego (NAA) (B) oraz bez roślinnych regulatorów wzrostu (C). D – kwitnące okazy po aklimatyzacji do warunków szklarniowych. E – wzrost roślin na poletku doświadczalnym w warunkach polowych. Objaśnienia w tekście

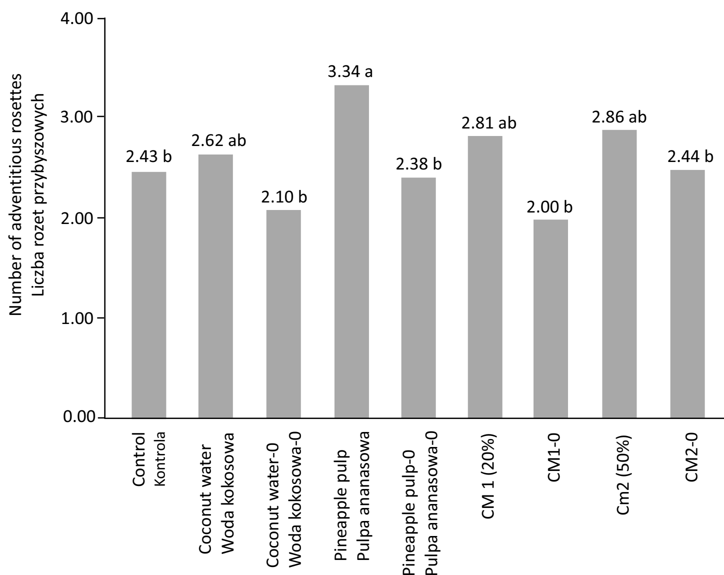


Fig. 3. The influence of organic additives on *Biscutella laevigata* micropropagation efficiency on control MS medium, the same MS medium supplemented with 10 ml/l of coconut water, 10 ml/l of pineapple pulp or conditioned medium at two concentrations 20% (CM1) or 50% (CM2) as well as on MS medium with supplements but deprived of plant growth regulators (described as coconut water-0, pineapple pulp-0, CM1-0, CM2-0, respectively) (according to Hanus-Fajerska et al. 2012). Mean values obtained in three replications (total 15 flasks per treatment) were presented, the same letters indicate that means do not differ statistically

Ryc. 3. Wpływ dodatków organicznych na wydajność tworzenia przybyszowych rozet liściowych *Biscutella laevigata* na pożywce MS wzbogaconej o 10 ml/l wody kokosowej lub 10 ml/l pulpy ananasowej lub kondycjonowanej pożywki w dwóch stężeniach 20% (CM1) lub 50% (CM2), jak również na pożywce MS z dodatkami, ale pozbawionej regulatorów wzrostu roślin (opisane jako woda kokosowa-0, pulpa ananasowa-0, CM1-0, CM2-0, odpowiednio) (według Hanus-Fajerskiej i in. 2012). Przedstawiono średnie wartości z trzech powtórzeń doświadczenia (w sumie 15 kolb dla każdego traktowania), takie same litery oznaczają, że średnie nie różnią się statystycznie od siebie

media in the presence of various additives are presented in Fig. 3. The medium supplementation favorably influenced *B. laevigata* growth and enhanced multiplication efficiency, however a statistically significant increase in adventitious rosette regeneration was observed after the addition of pineapple pulp (Hanus-Fajerska et al. 2012).

Heavy metal tolerance

In vitro cultures are useful to create model systems to study the morphogenic, physiological and biochemical responses of plants in various growth conditions (Reinert and Bajaj 2013). Their obvious advantage is the ability to test the effects of single stressors and environmental factors, as well as their combinations, on plant organs, tissues, and cells, and the level of these stimuli can be easily controlled by the investigator. Compounds of interest, i.e. potential growth promoters, inhibitors, toxins, and mutagens, can be added to the culture medium in specific concentrations.

The cultures of *B. laevigata* have also been used as a model system to evaluate the plant's reactions to various combinations of cadmium ($CdCl_2$) and lead ($Pb(NO_3)_2$) salts. *In vitro* selection was performed using a propagation medium supplemented with 0.5 µM $CdCl_2$ and 0.1 mM $Pb(NO_3)_2$, or with the

Table 1. Parameters of *Biscutella laevigata* calamine ecotype micropropagation on two culture media containing various doses of cadmium (Cd) and lead (Pb) evaluated after 16 weeks of cultivation *in vitro*. Different letters indicate means that are statistically significant

Tabela 1. Parametry mikrorozmnażania ekotypu galmanowego *Biscutella laevigata* na dwóch pożywkach zawierających zróżnicowane dawki kadmu (Cd) i ołowiu (Pb) ocenione po 16 tygodniach kultury *in vitro*. Różne litery oznaczają różnice istotne statystycznie

Treatment Dawka	Leaf rosette Rozeta liściowa				Root Korzeń			
	MC*	Fresh weight Świeża masa	Dry matter content Zawartość suchej masy	Diameter Średnica	Number Liczba	Length Długość	Fresh weight Świeża masa	Dry matter content Zawartość suchej masy
		[g]	[%]	[mm]		[mm]	[g]	[%]
Control (without metals) Kontrola (bez metali)	2.67 ab	0.849 c	9.70 c	42.63 a	13.87 a	50.64 a	0.44 a	10.06 a
0.5 µM Cd + 0.1 mMPb**	2.20 b	1.242 a	14.22 a	31.74 b	12.33 ab	40.11 b	0.14 b	9.01 a
5.0 µM Cd + 0.5 mMPb	3.00 a	1.092 b	12.55 b	30.33 b	10.00 b	34.32 c	0.06 c	8.89 a

* MC – micropropagation coefficient calculated as the ratio of number of regenerated rosettes to the number of primary explants.

* MC – współczynnik mikrorozmnażania czyli stosunek liczby zregenerowanych przybyszowych rozet liściowych do liczby eksplantatów pierwotnych.

** Cadmium was added to the medium in the form of cadmium chloride, while lead as lead nitrate; µM, mM – concentration units: micromolar, millimolar.

** Do pożywek kadm był podawany jako chlorek, a ołów jako azotan; µM, mM – jednostki stężeń: mikromol, milimol.

addition of 5.0 µM $CdCl_2$ and 0.5 mM $Pb(NO_3)_2$, as well as the same propagation medium but without the addition of metal salts (Muszyńska et al. 2017). Interestingly, microplants with a properly developed root system were obtained regardless of the concentrations of cadmium or lead ions applied. Micropropagation on medium supplemented with higher metal concentrations was slightly more efficient than on control medium, although the observed increase in the value of the micropropagation coefficient (MC) was not statistically significant (Table 1). Moreover, the rosettes regenerated on media with heavy metals were viable, and showed no chlorotic or necrotic changes, although their diameter was reduced by about 25% compared to the control ones. Notwithstanding, the fresh and dry mass of rosettes treated with cadmium and lead ions was higher than in control culture, which suggests the formation of more numerous but smaller and/or thicker leaves. Root number, length, and dry matter content declined under the highest concentration, and only root fresh weight remained unchanged (Table 1). The very high doses of tested metals impacted *B. laevigata* biology to some extent, however a high level of tolerance to cadmium and lead ions was still confirmed in the buckler mustard. It has also been shown that lines with enhanced capacity for growth and development on the media highly contaminated with cadmium and lead can be produced under *in vitro* conditions (Muszyńska et al. 2017).

Assessments of the genotoxic action of pollution are among the many types of studies that

can be comfortably conducted in *in vitro* culture systems. In *in vitro* cultures of the buckler mustard calamine ecotype, cadmium genotoxicity as well as the efficiency of its amelioration via application of exogenous (not produced by the plant itself) phytohormones were evaluated. Seedlings obtained from seeds of the calamine ecotype (as described above) were multiplicated on proliferation medium (Hanus-Fajerska et al. 2012), and then obtained adventitious shoots were transferred onto media containing cadmium salt (16 μM $CdCl_2$), and onto media with cadmium and phytohormones: jasmonic acid or gibberellic acid. A comet assay was conducted on nuclei isolated from leaves of shoots that were exposed to cadmium for 2 weeks. During the comet assay, isolated cell nuclei are treated with an electric field, which causes the migration of genetic material in agarose gel. The higher level of DNA degradation, the further can its fragments migrate. This results in the nucleus obtaining a specific shape, which resembles a comet. The head of this comet is composed of undamaged genetic material, while its tail is formed by the shorter DNA fragments, resulting from the action of the toxic agent. In the buckler mustard, this test revealed increased levels of DNA damage in cells of cadmium-treated leaves in comparison with control conditions. It is thought that the DNA damage is linked to considerably high genotoxicity of this metal. However, it was revealed that the application of phytohormones diminished cadmium genotoxicity, leading to lower number of damaged cell nuclei.

Biscutella laevigata callus, suspension, protoplast, and root cultures

Among the plant *in vitro* techniques, cultures of callus, cell suspensions and protoplasts are widely used. Callus tissue (wound contacting) in natural conditions arises from mechanical damage to the plant tissues. In *in vitro* conditions, using an appropriate medium composition, callus can be initiated from different plant organs and tissues (Cardoza 2016, Shahzad et al., 2017). This tissue is composed of cells of various sizes and shapes, and it has the ability to dedifferentiate into meristematic cells (cells that can divide) from which new organs (shoots, roots) and/or somatic embryos can be formed. Due to its loosely arranged and easily separating cells, callus is used to initiate cell suspension culture (Cardoza 2016). Protoplast cultures are established from the cell wall-deprived plant cells, called naked cells. Suspension cultures are mixtures of single cells and cell aggregates, whereas protoplast culture is the only technique by which truly single cells are obtained (Eriksson 2018). Cultures of callus, cell suspensions, or protoplasts are also used in basic research on cell differentiation, primary and secondary cell metabolism, and stress responses at the cellular level. Apart from their application in plant breeding, these types of culture are successfully used in *in vitro* selection (Rai et al. 2011), allowing the selection of resistant (or tolerant) tissues/cells for specific environmental conditions, including increased concentrations of heavy metals in the medium (Ashrafzadeh and Leung 2015).

Plant material for callus induction and protoplast isolation were stable shoot cultures of the buckler mustard calamine ecotype (Fig. 4A). Callus was initiated from fully developed leaves (Fig. 4B). Leaf fragments were explanted on ten different MS media enriched with various growth regulators and additives (Table 2). After 4 weeks of culture, callus tissue appeared on explants cultivated on several medium types (Fig. 4C, E, F). The resulting callus had a green or yellow-green color, initially a compact and grainy structure, which in the next passage (a transfer to fresh medium)

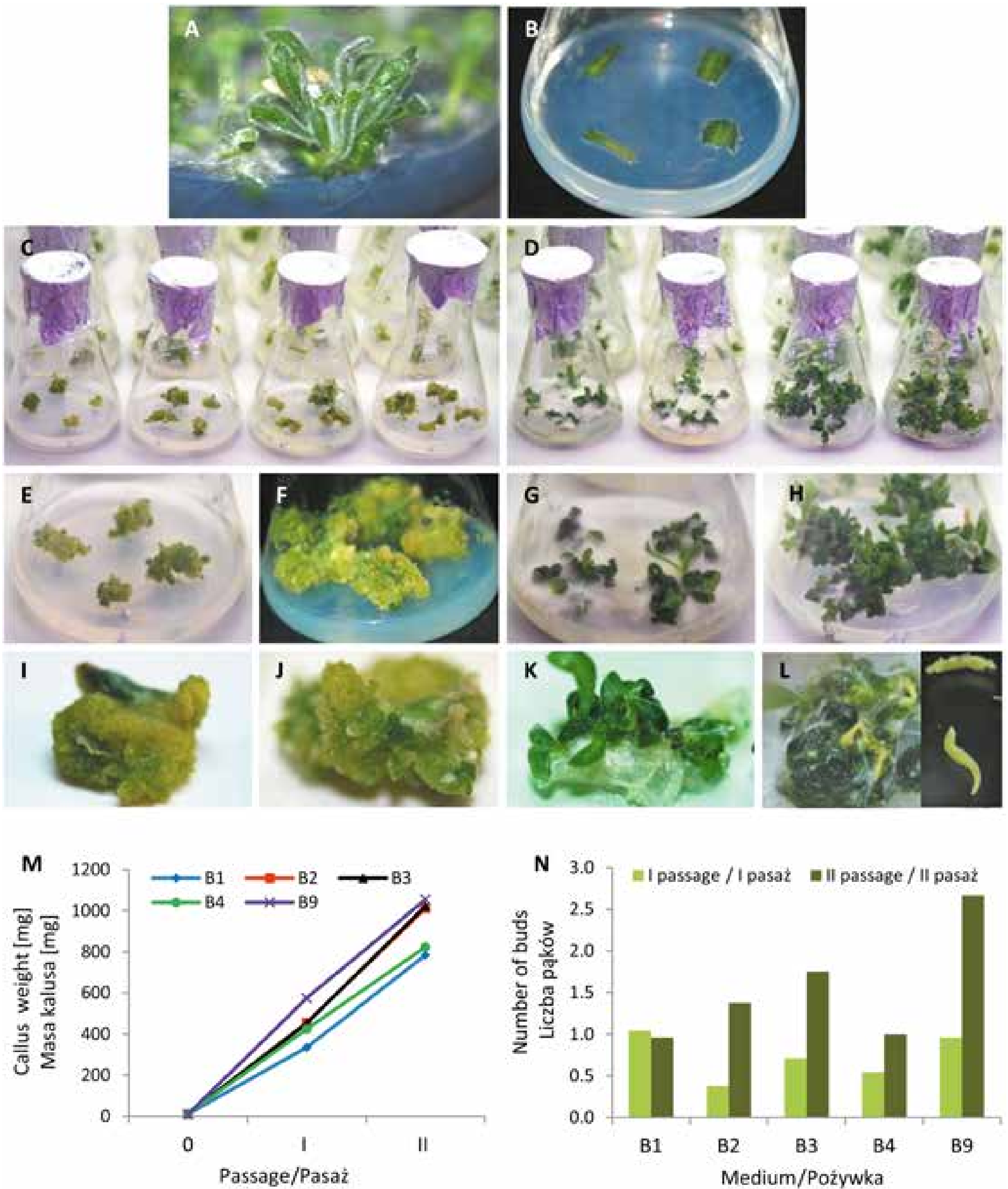


Fig. 4. Callus induction and proliferation of *Biscutella laevigata* calamine ecotype. A – donor plant cultivated in *in vitro* conditions. B – fragments of leaves explanted on one of ten tested media. C, D – 4-week cultures on various media: (C) B1–B4 media and (D) B5–B9 media (cf. Table 2). E, F, I, J – callus tissue on the explant: (E, I) after four weeks of culture (I passage) and (F, J) after eight weeks of culture (II passage). G, H, K, L – organogenesis and somatic embryogenesis: (G, K) explants with numerous roots, (H) explants with numerous shoots, (L) explants with early stages of somatic embryogenesis. M – average gain of callus weight after I and II passage (n = 24, explant no.). N – average number of adventitious buds after I and II passage (n = 24, explant no.), (cf. Table 2 for medium compositions)

Table 2. Composition of the media for callus induction and proliferation of *Biscutella laevigata* calamine ecotype
Tabela 2. Skład pożywek zastosowanych do indukcji i namnażania kalusa ekotypu galmanowego *Biscutella laevigata*

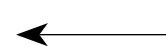
Medium code* Symbol pożywki*	Concentration of growth regulator** Stężenie regulatora wzrostu**
B1	1mg/l 2,4-D
B2	1 mg/l 2,4-D + 100 mg/l casein hydrolyzate / hydrolizat kazeiny
B3	1 mg/l 2,4-D + 0.5 mg/l KIN
B4	1 mg/l 2,4-D + 0.5 mg/l KIN + 100 mg/l casein hydrolyzate / hydrolizat kazeiny
B5	1 mg/l NAA
B6	1 mg/l NAA + 100 mg/l casein hydrolyzate / hydrolizat kazeiny
B7	1 mg/l NAA + 0.5 mg/l BAP
B8	1 mg/l NAA + 0.5 mg/l BAP + 100 mg/l casein hydrolyzate / hydrolizat kazeiny
B9	1 mg/l 2,4-D + 0.5 mg/l BAP
B10	1 mg/l NAA + 0.5 mg/l KIN

* Media based on the composition of macro and microelements and vitamins of MS medium (Murashige and Skoog 1962).
* Pożywki oparte na składzie makro i mikroelementów oraz witamin pożywki MS (Murashige i Skoog 1962).

** Auxins: 2,4-D – dichlorophenoxyacetic acid, NAA – naphtaleneacetic acid; cytokinins: KIN – kinetin, BAP – benzylaminopurine.
** Auksyny: 2,4-D – kwas dichlorofenoksyoctowy, NAA – kwas naftalenooctowy; cytokininy: KIN – kinetyna, BAP – benzyloaminopuryna.

became looser and crumbly (Fig. 2I, J). The composition of the other media was unsuitable for initiating the callussing of explants, but led to intensive organogenesis (shoots and roots formation) and somatic embryogenesis (embryo formation from somatic tissues) (Fig. 4D, G, H, K, L). Growth of callus tissue in all media was very intensive. The callus weight doubled after the second passage (Fig. 4M). In addition, adventitious buds were formed in callus tissue, and on the majority of media their number doubled after the second passage (Fig. 4N).

For the protoplasts culture, we have developed a protoplast isolation protocol from the leaves of the buckler mustard calamine ecotype grown *in vitro* (Fig. 5A). Leaf blade fragments with their lower epidermis removed were incubated for 5 hours in the mixtures of cell wall-degrading enzymes (Table 3) (Fig. 5B, C). The evaluation of the protoplast isolation efficiency (number of protoplasts released from 1 g of tissue) and viability (percentage of viable protoplasts) allowed the selection of the optimal composition of the enzyme mixture (En3) (Fig. 5D–G). Isolated and purified



Ryc. 4. Indukcja (wzbudzenie) i namnażanie kalusa ekotypu galmanowego *Biscutella laevigata*. A – roślina donorowa (macierzysta) hodowana w warunkach *in vitro*. B – fragmenty liści wyłożone na jedną z dziesięciu zastosowanych pożywek. C, D – cztero-tygodniowe kultury na różnych pożywkach: (C) pożywki B1-B4 i (D) pożywki B5-B8 (por. Tabela 2). E, F, I, J – tkanka kalusowa na eksplantacie: (E, I) po czterech tygodniach kultury (I pasaż) i (F, J) po ośmiu tygodniach kultury (II pasaż). G, H, K, L – organogeneza i somatyczna embriogeneza: (G, K) eksplantaty z licznymi korzeniami, (H) eksplantaty z licznymi pędami przybyszowymi, (L) eksplantaty z pierwszymi stadiami somatycznej embriogenezy. M – średni przyrost masy kalusa po I i II pasażu (n = 24, liczba eksplantatów). N – średnia liczba pąków przybyszowych uzyskana po I i II pasażu (n = 24, liczba eksplantatów). Skład pożywek przedstawiono w Tabeli 2

Table 3. Enzyme mixtures composition used for mesophyll protoplasts isolation of *Biscutella laevigata* calamine ecotype

Tabela 3. Skład mieszanin enzymatycznych wykorzystywanych do izolacji protoplastów mezofilowych ekotypu galmanowego *Biscutella laevigata*

Mixture code* Kod mieszaniny*	Enzyme concentration [%] Stężenie enzymu [%]	
	Cellulase Celulaza	Macerozyme Enzym macerujący
En1	0.75	0.3
En2	1.00	0.4
En3	1.25	0.5
En4	1.50	0.6

* Osmotic stabilization of the solution was provided by sorbitol; each mixture was prepared in a variant with 13% and 14% sorbitol.

* Stabilizację ciśnienia osmotycznego roztworu zapewniał sorbitol; każdą mieszaninę przygotowywano w wariancie z 13% i 14% sorbitolem.

protoplasts were cultured in liquid media: B5A (Wiszniewska and Piwowarczyk 2014), K8p (Kao and Michayluk 1975) and modified MS (Hanus-Fajerska et al. 2012). Protoplasts have much higher requirements for growth conditions than cells. This sensitivity is due to their lack of cell walls. Moreover, as a result of experimental treatments, plasma membranes lose their selectivity and become leaky, which may also cause a loss of metabolites (Eriksson 2018). Therefore, the proper choice of medium for protoplast culture is a difficult step in the development of regeneration protocols for new plant species. Observations of viability and changes in morphology of buckler mustard protoplasts carried out in subsequent days of culture allowed for the selection of modified MS medium (Hanus-Fajerska et al. 2012) as the basic medium for further experiments on cell wall regeneration and cell differentiation (Table 4).

The observations also indicated that in *in vitro* culture *B. laevigata* efficiently forms adventitious roots, which cells easily differentiate and regenerate into buds and shoots. It offers the opportunity of root culture establishment, providing a valuable model to study root biochemistry and physiology under heavy metal stress. Our preliminary study revealed that the presence of cadmium ions in the medium stimulates rooting of *B. laevigata* calamine ecotype. Root cultures of this species can be used for obtaining and analyzing the composition of organic compounds exerted by the roots under metallic stress. Such exudates, either facilitating or inhibiting heavy metal uptake from the rhizosphere, and interacting with soil microorganisms, are considered to be crucial elements of plant responses to heavy metal contamination (Malik et al. 2016).

Other biotechnological tools applied in studies on *B. laevigata* biology

Molecular biology and genetic techniques, including the use of molecular markers, could facilitate investigations of buckler mustard biology. A genetic marker is a biochemical compound which allows two different physiological conditions or two individuals to be distinguished. Nowadays, molecular markers are considered to be polymorphic DNA sequences: fragments of genetic material (genes or non-coding sequences) that exist in two or more distinguishable versions. Biochemical indicators, such as different variants of enzymatic proteins (so-called alloenzymes), were also used to study variation among plant populations over a decade ago (Tremetsberger et al. 2002). Today, various types of molecular markers facilitate studies of genetic structure and diversity among metallicolous populations or ecotypes of various plant species, giving an insight into their ecological genetics (Wójcik et al. 2013, Bothe and Słomka 2017). In

Table 4. Changes in protoplast viability and morphology of *Biscutella laevigata* calamine ecotype during the first days of culture. The percentage of protoplasts belonging to particular groups in the total number of cultivated protoplasts is shown

Tabela 4. Zmiany żywotności i morfologii protoplastów ekotypu galmanowego *Biscutella laevigata* w ciągu pierwszych pięciu dni kultury. Pokazano procentowy udział protoplastów należących do poszczególnych grup w całkowitej liczbie protoplastów w kulturze

Medium* Pożywka*	After 1 day of culture Po 1 dniu kultury					After 5 days of culture Po 5 dniach kultury				
	Viable Żywe	Spherical Okrągłe	Oval Owalne	Budding Pączkujące	Degenerating Degenerujące	Viable Żywe	Spherical Okrągłe	Oval Owalne	Budding Pączkujące	Degenerating Degenerujące
B5A	50.9	50.4	0.5	0	49.1	5.8	5.4	0.4	0	94.2
K8p	27.1	26.1	1.0	0	72.9	8.7	2.9	4.7	1.1	91.3
MS modified MS zmod.	84.8	79.5	5.3	0	15.2	19.0	18.4	0	0.6	81.0

* B5A (Wiszniewska and Piwowarczyk 2014), K8p (Kao and Michayluk 1975) and MS modified according to Hanus-Fajerska et al. (2012).

* B5A (Wiszniewska i Piwowarczyk 2014), K8p (Kao i Michayluk 1975) i MS z modyfikacjami według Hanus-Fajerskiej i in. (2012).

B. laevigata, polymorphisms of microsatellite sequences (short tandem repeats, 1–6 nucleotides long) were exploited to discover the adaptation strategies of numerous populations from different habitats (Babst-Kostecka et al. 2014, Wąsowicz et al. 2014). Mechanisms of adaptation that allow facultative metallophytes to grow on metalliferous soils are of particular interest (Bothe and Słomka 2017).

Analyses based on molecular markers, complemented with biochemical and physiological studies, were used to assess the differences in heavy metal tolerance among *B. laevigata* populations (Pošćić et al. 2015). Another application of molecular markers is the ability to distinguish stress responses between metallicolous populations. With this view in *B. laevigata*, Pošćić et al. (2015) performed AFLP-based (amplified fragment length polymorphism-based) cluster analysis, allowing the identification of point mutations and changes in the length of repeated sequences in DNA. Furthermore, Geiser et al. (2016) compared the expression level of several genes, and the synthesis of respective proteins. It was postulated that genome duplication increased the number and expression of stress-responding genes, enabling *B. laevigata* to survive periods of limited water supply.

Conclusions

The calamine ecotype of *B. laevigata* is a convenient subject for *in vitro* culture experiments. Effective proliferation and the high capacity for *in vitro* regeneration in this species allows both fundamental and applied studies to be conducted relatively easily by means of *in vitro* culture. In particular, culture methods aimed at propagation of the calamine ecotype were presented, complemented with studies on tolerance levels to cadmium and lead. As briefly shown, biotechnological methods may also be applied in studies on species adaptation strategies and its responses to abiotic stresses. Newly obtained knowledge can be applied in

future breeding programmes to obtain heavy metal tolerant crop species. New cultivars could be applied in the phytoremediation of heavy metal polluted soils.

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