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Stable Artificial Autopolyploids of the Zn/Cd Accumulator *Arabidopsis arenosa*—A Promising Genetic Resource for Phytoremediation

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Abstract: *Arabidopsis arenosa* is a good candidate for phytoremediation due to its high tolerance to Zn and Cd as well as its accumulation ability. However, its small size and low biomass are the largest obstacles to applying it on a broad scale. The aim was to obtain polyploid specimens, which tend to have higher biomass to increase the accumulation and translocation capacity of heavy metals in this metal-tolerant plant. Doubled polyploids (octaploids) were obtained via indirect organogenesis on a $\frac{1}{2}$ MS medium supplemented with 1 mg L^{-1} TDZ, followed by rooting on the same medium without growth regulators. Callus tissue of a high endopolyploidy level (the (Σ>2C)/2C ratio over 2.5) obtained on seedling fragments on $\frac{1}{2}$ MS supplemented with 2 mg L^{-1} 2,4-D + 2 mg L^{-1} BAP served as a source material. Among the regenerants successfully obtained (without using antimitotic agents), over half, regardless of the stage of regenerant development, were octaploid (54–78%; 2C DNA = 1.642 pg). Octaploids were not affected by ploidy or in vitro culture conditions; they were fully fertile, produced normal pollen (~97% of viability), and set seeds capable of germinating (78%). Their cell and organ size was affected by genome doubling resulting in longer stomata, bigger pollen grains, and flowers with a larger area and width in comparison with tetraploid regenerants and initial plants. The promising results of measurements of morpho-anatomical, physiological, and reproductive parameters indicate that, in the future, after passing tolerance tests, the obtained polyploids could be used in phytoremediation of metal-contaminated areas.

Keywords: polyploidization; in vitro culture; morpho-anatomical characteristics; sexual reproduction

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1. Introduction

Phytoremediation, including mainly phytoextraction and phytostabilization, consists of recently developed and improved technologies of soil cleaning with the use of innate plant abilities [1,2]. New species, genotypes adapted to local environmental conditions, are being investigated and tested [3,4]. Studies particularly focus on hyperaccumulators, unique plant species defined by two coefficients, the bioaccumulation factor (ratio of the metal content in plants to its content in soils) and the translocation factor (ratio of the metal content in the plant shoots to that in their roots), reaching above one and the high shoot content of heavy metal reaching 100–10,000 mg kg^{-1} (at least), depending on the metal [5]. Direct use of hyperaccumulators is not without its limitations, such as low biomass and slow growth of many tolerant genotypes. Different methods, including

traditional breeding or genetic engineering, are employed to either improve the growth rate and biomass of hyperaccumulators or introduce hyperaccumulation traits [6]. Transgenic plants with enhanced ability to tolerate and hyperaccumulate metals are obtained via somatic hybridization, mutagenesis including gene stacking, epigenetic modifications, or gene editing (for a revision, see [7–9]). Despite the obvious advantages, such as the short time to obtain transgenic plants compared to traditional breeding, such modifications are not without drawbacks. Genetic manipulation of many genes underlying metal tolerance, e.g., encoding heavy metal transporters, chelators, and genes encoding components or even chloroplasts, genomes of antioxidant machinery and accumulation, is a complex procedure [1,10]. Moreover, such plants often arouse public opposition; hence there are difficulties in obtaining approval for their field testing [11]. Some, therefore, believe that the natural potential of plants that have evolved to adapt to high concentrations of heavy metals in the soil should be explored [3]. Thus, it is important to look for the ecotypic variability of metallicolous populations [12].

Arabidopsis arenosa (L.) Lawalrée (formerly *Cardaminopsis arenosa* (L.) Hayek) is one such species with high potential for phytoremediation, occurring as a dominant species (metal tolerant populations) on calamine (Zn/Pb/Cd) soils in Western Europe [13]. This montane and subalpine species of Central and Western Europe, and Scandinavia (lower altitudes), could be either diploid ($2n = 2x = 16$) or tetraploid ($2n = 4x = 32$). Tetraploid cytotypes are commonly distributed across the whole range, while diploid cytotypes were exclusively found on the Balkan Peninsula and in the Carpathians [14]. Though the tetraploid cytotype is autopolyploid, tetraploid individuals of *A. arenosa* run meiosis typical for diploids, where bivalents are selected randomly from four homologs [15,16]. Recent studies have shown that these polyploid populations can rapidly adapt to adverse, extreme soil conditions based on pre-existing and novel variations [17]. Thereby they inhabit heterogeneous habitats, including serpentine outcrops, calamine soils, and railway lines going beyond the sites occupied by diploids [18,19]. The tolerance to heavy metals in this species is induced (metal tolerance is developed only in some populations after contact with heavy metals), and metallicolous populations differ morphologically from non-metallicolous ones [14,20–27]. Metal-tolerant populations from extremely polluted sites could accumulate or even hyperaccumulate Zn and Cd [25–27]. *A. arenosa* does not appear to be a typical metallophyte; however it is a heavy metal tolerant species, possibly also related to drought tolerance, similar to *Viola rupestris* [14,28,29].

Extreme environmental adaptation could be favored by gene duplication that have evolved new functions or increased the protein content [30]. A good example is the split of *Arabidopsis halleri* and *A. lyrata,* which is likely joined with the tandem duplication of Heavy Metal ATPase4 (HMA4), that was an ecological speciation leading to improvement of heavy metal tolerance in *A. halleri* [31]. The whole genome duplication manifested in natural autopolyploids is often overlooked due to the resemblance to their initial monoploids [32]. However, recent studies have shown that autoploid populations of hyperaccumulators of Ni *Odontarrhena bertolonii* (formerly *Alyssum bertolonii*) and the Cu-tolerant plant *Mimulus guttatus* represent higher fitness than initial plants [33–35]. It is known that agricultural and horticultural crops with increased ploidy exhibit morpho-anatomical modifications and enhanced different parameters e.g., stress tolerance, biomass and yield, which make them more valuable for the commercial purposes [36]. Colchicine and other antimitotic agent treatments which disturb microtubule formation during cell division and lead to endomitosis are common chemicals used to generate synthetic polyploids [37].

The aim of this work was to obtain and develop a mass production protocol of stable octoploids of Zn/Cd-tolerant *A. arenosa* and conduct an assessment of their vigor, fertility, genetic stability, and preliminary suitability for phytoremediation purposes. Obtaining such more phytoremediation-efficient plants, bypassing socially objectionable genetic modification processes, is crucial for applied science, and so far, few have succeeded in this regard.

2. Materials and Methods

2.1. Plant Material

Seeds of *Arabidopsis arenosa* were randomly collected from two sites in Bukowno (Southern Poland), close to the ZGH Bolesław operating zinc smelter (N 50°16′57″, E 19°28′33″). These sites are known for heavy pollution in soil which could reach 25,000 mg kg^{-1} Zn, 5000 mg kg^{-1} Pb, and 129 mg kg^{-1} Cd [38].

2.2. Obtaining Tetra- and Octaploid Plantlets

2.2.1. In Vitro and Hydroponic Culture

Seeds of *A. arenosa* were sterilized in 70% ethanol for 90 s, next in 25% commercial bleach for 12 min, and rinsed in sterile distilled water for 2, 3, and 5 min. Sterile seeds were sowed and cultured on solidified half-strength Murashige & Skoog medium ($\frac{1}{2}$ MS medium). They were kept in the dark at 4 °C for 4–5 days and afterward in a growth chamber for germination. Sterile fragments of seedlings (leaves, roots, cotyledons + hypocotyls) were collected and placed on the $\frac{1}{2}$ MS medium with different combinations of auxins and cytokinins: 2 mg L^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid) + 2 mg L^{-1} KIN (Kinetin), 0.5 mg L^{-1} 2,4-D + 0.05 mg L^{-1} KIN, 2 mg L^{-1} 2,4-D + 2 mg L^{-1} BAP (6-Benzylaminopurine), and 1 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP to obtain callus tissue. Fragments of the obtained callus tissue were placed on media supplemented with 1 mg L^{-1} TDZ (Thidiazuron) or 2 mg L^{-1} TDZ to induce shoot formation or the $\frac{1}{2}$ MS medium as a control (hormone-free medium). Then, the obtained adventitious shoots were transferred onto $\frac{1}{2}$ MS media containing two auxins in different concentrations (0.5 mg L^{-1} IAA (Indole-3-acetic acid), 1 mg L^{-1} IAA, 0.5 mg L^{-1} IBA (Indole 3-butyric acid), 1 mg L^{-1} IBA) or without hormones for rooting. All media contained 30 g L^{-1} sucrose and were solidified with 8 g L^{-1} agar. The pH was adjusted to 5.7–5.8. The seeds, plants, and culture were maintained in stable growth-chamber conditions at 25 ± 3 °C with a 16-h photoperiod under cool-white, fluorescent lamps (flux 70–100 µmol $m^{-2}s^{-1}$). Regenerated, rooted plantlets with roots a minimum of 15 mm in length were transferred into a hydroponic culture with a modified half-strength Hoagland medium [39]. All chemicals were supplied by Sigma-Aldrich (Saint Louis, MO, USA).

2.2.2. Acclimatization of Regenerated Plants

Rooted plants, after 2–5 weeks of cultivation in a hydroponic culture, were transferred to 0.5–2 L pots filled with garden soil (Biovita, Tenczynek, Poland), kept under a cover in stable growth-chamber conditions (24 °C, 16/8 h photoperiod), and regularly watered. Then, from June to September 2022, plants in pots were introduced to outdoor conditions (temperate climate of Nothern Hemisphere, Świątniki Górne, Poland, N 49°56′04″, E 19°57′21″) and observed till the end of the season. In November 2022, plants were dug into the experimental plot in Świątniki Górne.

2.3. Genome Size and Endopolyploid Estimation by Flow Cytometry

For the estimation of genome size, leaves of plants collected in the field, adventitious shoots, rooted plants on $\frac{1}{2}$ MS, and regenerated plants in hydroponic and soil cultures were used. Samples were prepared as previously described [40], using Galbraith's buffer [41] supplemented with propidium iodide (PI; 50 µg mL^{-1}) and ribonuclease A (50 µg mL^{-1}). *Solanum lycopersicum* cv. Stupické (2C = 1.96 pg; [42]) was used as an internal standard. For each sample, PI fluorescence in 3000–5000 nuclei was measured using a CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer. Analyses were performed using linear amplification. Histograms were evaluated using the FloMax program (Partec GmbH, Münster, Germany). The coefficient of variation (CV) of the G_0/G_1 peak of *A. arenosa* ranged between 2.59 and 6.92%. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of *Arabidopsis*/*Solanum* on a histogram of fluorescence intensities.

For endopolyploid estimation, callus derived from cotyledon + hypocotyl explants cultured on the $\frac{1}{2}$ MS medium supplemented with 2 mg L^{-1} 2,4-D and 2 mg L^{-1} BAP

and callus kept on 1 mg L^{-1} TDZ for 3 weeks and leaves of initial 3–6-week-old seedlings were used. The samples were prepared and analyzed similarly to those of genome size analysis, however, PI fluorescence was measured in 5000–10,000 nuclei, and logarithmic amplification of the signal was used. After histogram evaluation, the percentages of the nuclei with different DNA content (i.e., of different ploidy) and the ($\Sigma > 2C$)/2C ratio [43] were calculated.

In this study, nuclei with at least 8C DNA were considered to be endopolyploid since it was not possible to distinguish by flow cytometry the 4C nuclei originating from the cells that have just entered endoreduplication from those originating from the cells in the G_2 phase of the mitotic cycle.

2.4. Chromosome Counting

Fifty seedlings cultured on the $\frac{1}{2}$ MS medium (see Section 2.2.1.) were incubated in a saturated solution of α-bromonaphtalene for 4 h at 20 °C, washed with distilled water and fixed in a mixture of 96% ethanol and glacial acetic acid (3:1; v:v). Seedlings were stained with 2% orcein for at least 72 h, rinsed several times with 45% acetic acid, and then heated to boiling. Stained root tips were dissected and squashed in a drop of 45% acetic acid with a cover slip. Semi-permanent slides were observed and photographed under a light microscope.

2.5. Morpho-Anatomical and Physiological Parameter Measurements

Plant differentiation between 4× and 8× for all these measurements was based on flow cytometry results.

2.5.1. Photosystem II Efficiency by a Fluorometer

Fast chlorophyll fluorescence kinetics using a Handy PEA portable device from Hansatech Instruments (King's Lynn, UK) was measured on at least 50 leaves from 5–10 randomly selected plants of each group (in vitro derived tetraploids, in vitro derived octaploids, and initial plants from the seeds) growing in hydroponics. Plant leaves were darkened for 20 min to quench the reaction centers. Among all the obtained parameters, only the F_v/F_m parameter defining whether Photosysystem II is negatively affected was chosen for consideration.

2.5.2. Pollen Diameter and Viability by the Alexander Test

Thirty buds of octaploids, tetraploids and initial plants (10 for each group) growing in hydroponics were fixed in the same way as seedlings for chromosome counting (see Section 2.4.). Isolated with needles, pollen grains were stained by Alexander dye [44]. 100 and 5 randomly selected pollen grains per bud were analyzed for viability (stainability) and size (diameter), respectively, using a light microscope combined with NIS-Elements Viewer imaging software.

2.5.3. Length of Stomata

The stomata length (25 per leaf) was measured in the middle part of the abaxial blade of octaploids, tetraploids, and initial plants (4 leaves for each group) growing in hydroponics. The epidermis was pulled out and placed into a drop of water on a microscopic slide, and the stomata length was measured using a light microscope combined with NIS-Elements Viewer imaging software.

2.5.4. Width and Area of the Flowers

Fifty flowers of octaploids, tetraploids, and initial plants grown in hydroponics were collected and photographed, and their narrower width and area were measured using ImageJ 1.8.0.

2.6. Seed Set in Tetra- and Octaploids and Germination of Octaploid Seeds

Due to the self-incompatibility of *A. arenosa*, the obtained tetra- and octaploid regenerants flowering in growth chambers were hand-pollinated reciprocally to obtain progeny. The obtained seeds were sterilized, pre-treated, and germinated according to the procedure described above (see Section 2.2.1.). The percentage of seed germination in octaploids based on 68 seeds was determined 12 days after sowing. Fifty seeds of initial plants and octaploids were photographed, and their length was measured using ImageJ 1.8.0.

2.7. Micro- and Stereoscopy Usage

For pollen, stomata, and chromosome analyses, a Nikon Eclipse 80i (Tokyo, Japan) microscope equipped with a Canon camera was used. Successive stages of in vitro plant regeneration, flowers, and seeds were observed and photographed using an Olympus stereo microscope (Olympus CX31, Tokyo, Japan).

2.8. Statistical Analyses

Statistical analyses were conducted in R (version 2.14.0; [45]). One-way ANOVA, followed by the Tukey HSD test, was used to determine the significance of dependent variables: photosynthesis (F_v/F_m value [46]), stomata and seed length, pollen grain diameter, flower area and width, and frequency of viable pollen grains, for given plant origins (initial plant, tetraploid and octaploid regenerants). Average and standard deviation (given in brackets) were calculated in Microsoft Office 365.

3. Results

3.1. In Vitro Derivation and Ex Vitro Cultivation of Tetra- and Octaploids

Callus tissue was induced on all media used 4–8 weeks after explants (leaves, roots, cotyledons + hypocotyls) were placed, but the fastest growing and the most vigorous, yellow–green callus was obtained from cotyledons with hypocotyl fragments on the $\frac{1}{2}$ MS medium supplemented with 2 mg L^{-1} 2,4-D + 2 mg L^{-1} BAP (Figure 1A,B, Table 1). The largest (multiple, uncountable) numbers of adventitious shoots were obtained from callus tissue derived from cotyledons with hypocotyl fragments on 1 mg L^{-1} TDZ (Figures 1C and 2, Table 1). The shoots obtained on this medium were successfully rooted on all tested media with the highest frequency (above 36%) on a hormone-free $\frac{1}{2}$ MS medium (Figure 1D,E, Table 1).

Table 1. Callus induction, organogenesis, and rooting in *Arabidopsis arenosa* on tested explants and media (1/2 MS medium with or without additives). The numbers in front of the growth regulator abbreviations are values in mg L^{-1}. +, ++, +++—low, moderate, high productivity, respectively. Experiments were performed in duplicate to triplicate; N (number of explants) $\geq$ 30.

Callus Induction on the $\frac{1}{2}$ MS Medium Supplemented with 2 mg L^{-1} 2,4-D and 2 mg L^{-1} BAP (after 6–8 Weeks)		**Organogenesis (after 8 Weeks)**		**Rooting (after 4–12 Weeks)**	
Explant Type	**Productivity**	**Medium**	**Productivity**	**Medium**	**Frequency (%)**
Leaves	++	$\frac{1}{2}$ MS	+	0.5 IAA	4.88
Cotyledons + hypocotyl	+++	1 TDZ	+++	1 IAA	16.25
Roots	++	2 TDZ	++	0.5 IBA	22.50
				1 IBA	11.11
				$\frac{1}{2}$ MS	36.36

2,4-D—2,4-Dichlorophenoxyacetic acid; BAP—6-Benzylaminopurine; IAA—Indole-3-acetic acid; IBA—Indole-3-butyric acid; MS—Murashige and Skoog medium; TDZ—Thidiazuron.

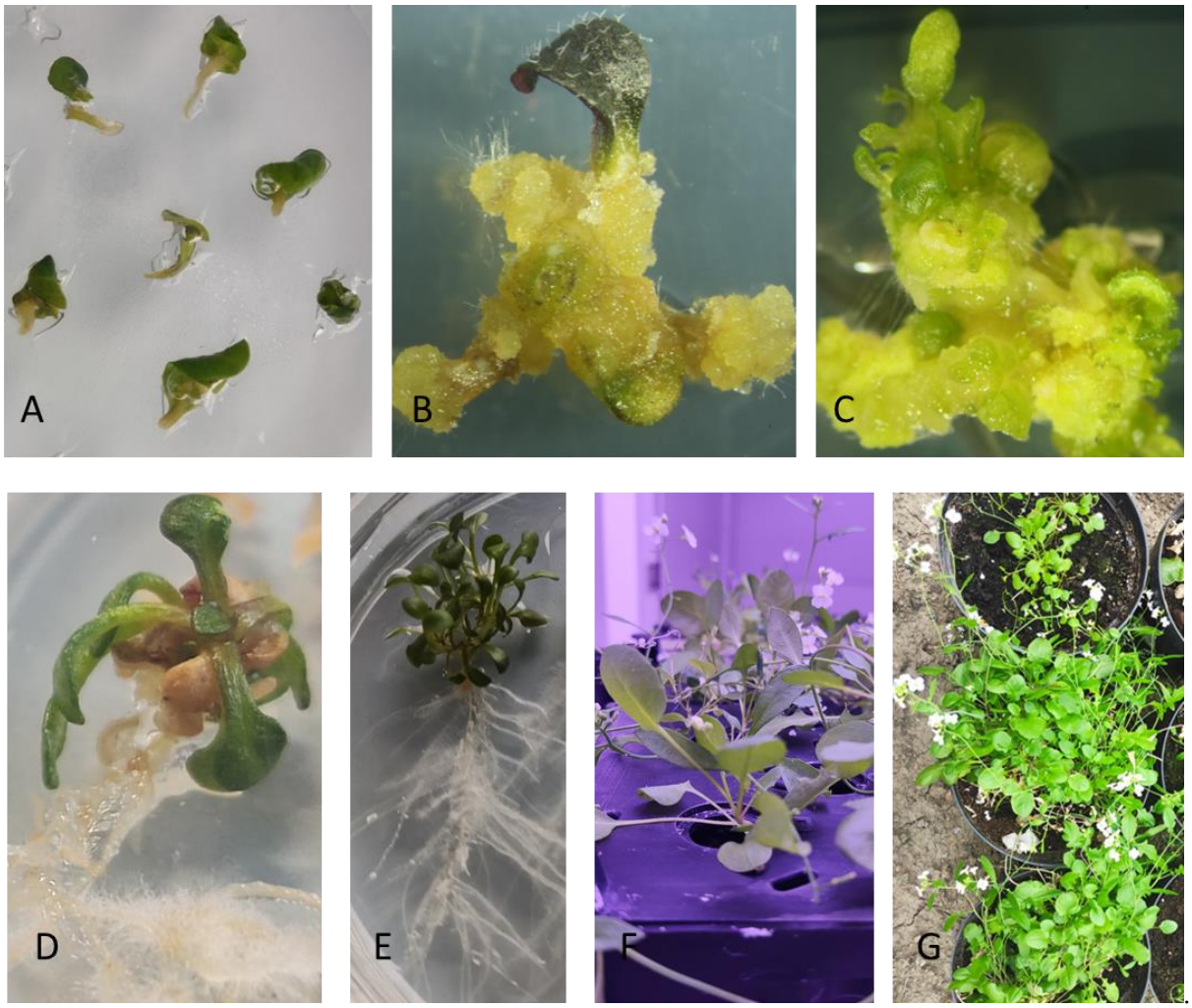

Figure 1. Successive stages of obtaining *Arabidopsis arenosa* regenerants. Fragments of seedlings laid out on the callus induction medium (**A**), callus tissue derived from the hypocotyl with cotyledons on the $\frac{1}{2}$ MS medium supplemented with 2 mg L^{-1} 2,4-D and 2 mg L^{-1} BAP (**B**), adventitious shoots on the $\frac{1}{2}$ MS medium supplemented with 1 mg L^{-1} TDZ (**C**), rooted plant on the $\frac{1}{2}$ MS after 10 weeks on a rooting medium (**D**), regenerated plant on $\frac{1}{2}$ MS ready for transfer to hydroponics (**E**), flowering plants maintained in a hydroponic culture (**F**), and acclimatized to outdoor conditions (**G**). 2,4-D—2,4-Dichlorophenoxyacetic acid; BAP—6-Benzylaminopurine; MS—Murashige and Skoog medium; TDZ—Thidiazuron.

Rooted plantlets acclimatized to the ex vitro conditions developed normally, reach generative phases in 1–4 weeks (Figure 1F,G).

3.2. Genome Size, Endopolyploidy and Chromosome Number at Different Stages of Plant Culture

Plants used for in vitro culture were tetraploids (4*x*) and possessed 2C = 0.829 [±0.028] pg DNA. Similarly, the calli obtained from these plants contained 0.823 [±0.029] pg/2C DNA, which confirmed that the nuclei of the lowest ploidy present in the calli were tetraploid. Nevertheless, at the stage of adventitious shoots after 4–6 weeks of cultivation of the calli on 1 mg L^{-1} TDZ, besides tetraploids, plant material with double amount of DNA (about 1.6–1.7 pg/2C; representative for octaploids, 8*x*) was also detected; octaploids also occurred among regenerated acclimatized (both originating from hydroponic and from soil cultures) plants. The frequency of octaploids was high in all examined stages of regeneration (54.2–78%) (Table 2). The mean genome size of 90 octaploid regenerants was

2C = 1.642 [±0.042] pg. Moreover, among the rooted plants after 5–12 weeks of culturing, seven plants possessed DNA content characteristic for 16*x* (2C = 3.265 [±0.064] pg) (Table 2).

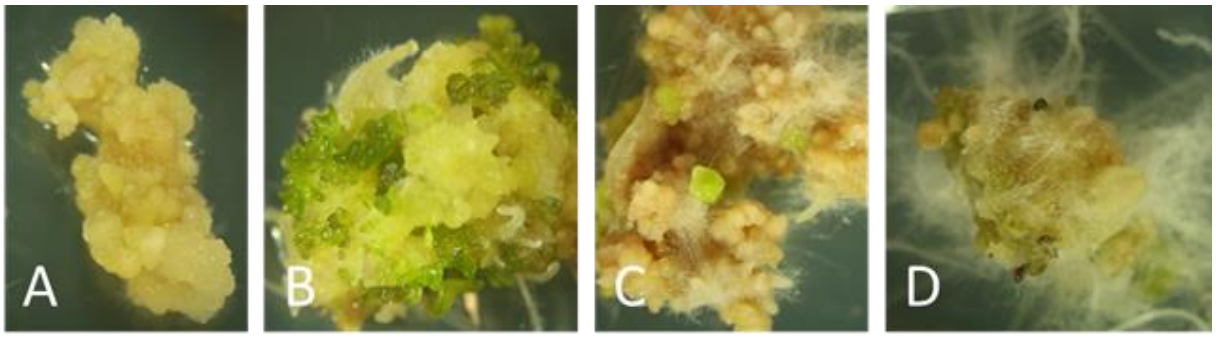

Figure 2. Varied response (organogenesis) of 4-5-week-old callus (**A**) of *Arabidopsis arenosa* initiated on the $\frac{1}{2}$ MS medium supplemented with 2 mg L^{-1} 2,4-D and 2 mg L^{-1} BAP and then transferred onto media with or without different plant growth regulators. Organogenesis; on $\frac{1}{2}$ MS supplemented with 1 mg L^{-1} TDZ (**B**), on $\frac{1}{2}$ MS supplemented with 2 mg L^{-1} TDZ (**C**), and on $\frac{1}{2}$ MS (**D**). Note the green adventitious shoots (multiple in **B**, single in **C**) and white roots (unwanted in **D**). 2,4-D—2,4-Dichlorophenoxyacetic acid; BAP—6-Benzylaminopurine; MS—Murashige and Skoog medium; TDZ—Thidiazuron.

Table 2. Number and frequency (%) of 4*x*, 8*x*, and 16*x* at different stages of *Arabidopsis arenosa* culture.

Plant Material	4*x* (%)	8*x* (%)	16*x* (%)
Initial plants	13 (100)	0 (0)	0 (0)
Callus obtained on $\frac{1}{2}$ MS supplemented with 2 mg L^{-1} 2,4-D + 2 mg L^{-1} BAP (8–16 weeks after induction) *	15 (100)	0 (0)	0 (0)
Adventitious shoots after 4–6 weeks on $\frac{1}{2}$ MS supplemented with 1 mg L^{-1} TDZ	11 (45.8)	13 (54.2)	0 (0)
Rooted plants after 5–12 weeks on rooting $\frac{1}{2}$ MS medium	14 (23.7)	38 (64.4)	7 (11.9)
Regenerated plants (from hydroponic culture and soil)	11 (22)	39 (78)	0 (0)

* The DNA-ploidy of a callus was estimated according to the ploidy of the first peak present in the flow cytometric histogram. MS—Murashige and Skoog medium; 2,4-D—2,4-Dichlorophenoxyacetic acid; BAP—6-Benzylaminopurine; TDZ—Thidiazuron.

In all analyzed plant materials, nuclei with 2C, 4C, 8C, 16C, and 32C were detected; however, they differed in endopolyploidy level; the highest (Σ > 2C)/2C ratio was observed in 10–15-week-old callus on $\frac{1}{2}$ MS supplemented with 2 mg L^{-1} 2,4-D + 2 mg L^{-1} BAP (over 2.5; Figure 3B). The lowest endopolyploidy occurred in young leaves of the seedlings (the (Σ > 2C)/2C ratio almost 0.3); most nuclei (almost 80%) contained 2C DNA, and endopolyploid nuclei (> 4C) constituted only slightly over 4% (Figure 3A). In calli, regardless of the medium/treatment, the frequency of 2C nuclei essentially decreased, and this of endopolyploid nuclei increased to about 16% and 13%, in 10–15-week-old callus and callus treated for 3 weeks with 1 mg L^{-1} TDZ, respectively (Figure 3B,C). It must be noted, however, that the calli differed greatly in endopolyploidy level (see high standard deviation).

The chromosome number of the initial 4*x* plants was 2*n* = 32 [20], and 8*x* progeny was 2*n* = 64 (Figure 4A).

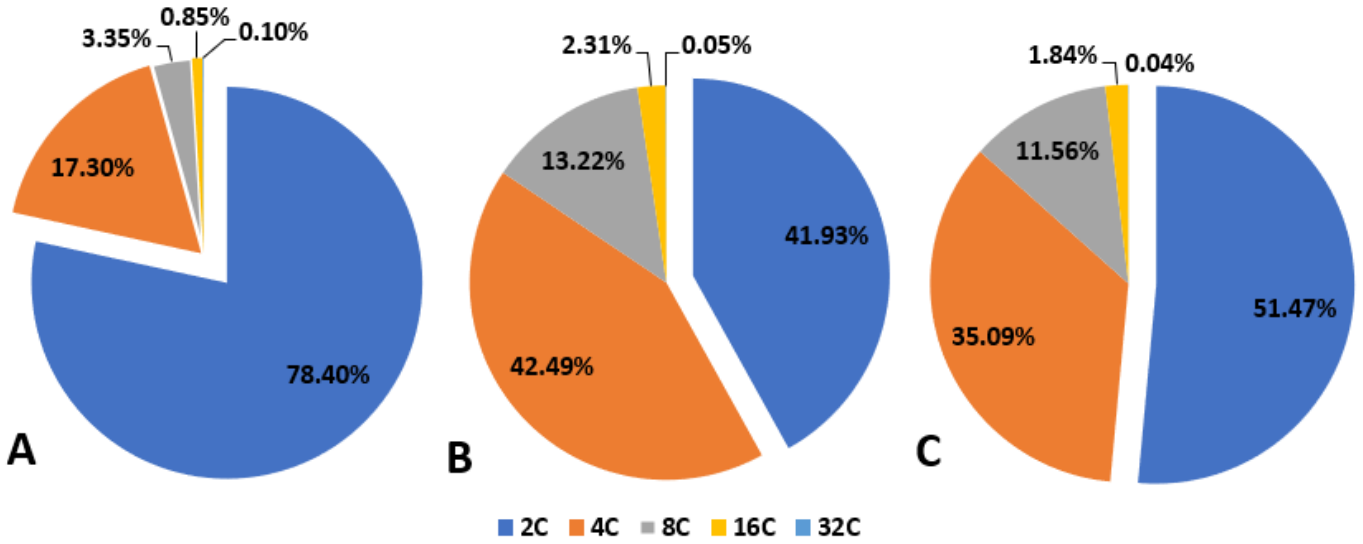

Figure 3. Frequency of nuclei with different DNA contents in various plant materials of *Arabidopsis arenosa*. Leaf (blade with petiole) of initial plants (**A**), 10–15-week-old callus on $\frac{1}{2}$ MS supplemented with 2 mg L^{-1} 2,4-D + 2 mg L^{-1} BAP (**B**), callus treated with 1 mg L^{-1} TDZ for 3 weeks (**C**). The (Σ > 2C)/2C ratio was 0.292 [±0.161], 2.559 [±3.012], and 1.334 [±1.452] for **A**, **B**, and **C**, respectively. 2,4-D—2,4-Dichlorophenoxyacetic acid; BAP—6-Benzylaminopurine; MS—Murashige and Skoog medium; TDZ—Thidiazuron.

3.3. Morpho-Anatomical Characteristics of Regenerated Octaploids

The obtained octaploids produced normal and viable pollen (96.8% vs. 96.0% in regenerated tetraploids and 96.5% in initial plants) of higher diameter (than tetraploids, both, initials and regenerants) and longer (than initial plants) seeds able to germinate. The frequency of seed germination in octaploids was 77.9%. The nucleotypic effect (cell size increases resulting from the increases in the 2C value) was also visible in the stomata length of octaploids. The latter ones produced the longest stomata among the analyzed groups. Similarly, flower diameter and area in octaploids were greater than in tetraploid regenerants and tetraploids obtained from seeds (initial plants) (Table 3, Figure 4).

Table 3. Morpho-anatomical characteristics of initial tetraploids, tetraploid and octaploid regenerants of *Arabidopsis arenosa* grown in hydroponics. Different letters mean statistical differences ($p \leq 0.05$) with one-way ANOVA, followed by the Tukey HSD test between each dependent variable.

	Initial Plant (Tetraploid)	Tetraploid Regenerant	Octaploid Regenerant
F_v/F_m value	0.822 [±0.059] a	0.806 [±0.101] a	0.815 [±0.074] a
Stomata length [µm]	21.4 [±1.74] b	20.5 [±2.28] a	27.2 [±3.85] c
Pollen diameter [µm]	19.3 [±1.70] a	20.0 [±1.47] a	24.7 [±1.47] b
Flower diameter [mm]	3.4 [±0.362] a	3.39 [±0.329] a	4.6 [±0.555] b
Flower area [mm^2]	37.0 [±8.11] a	35.6 [±4.50] a	45.9 [±8.99] b
Pollen viability [%]	96.5 [±3.67] a	96.0 [±8.75] a	96.8 [±5.58] a
Seed length [mm]	0.892 [±0.086] a	n.m.	1.170 [±0.109] b

[±st. dev.]; n.m.—non-measured.

F_v/F_m, indicating the maximum potential quantum efficiency of Photosystem II when all capable reaction centers are open, showed no significant differences between the three groups of plants, indicating that Photosystem II was not affected by the ploidy level or in vitro culture. Furthermore, the average values of F_v/F_m were in the range of the optimal values of most plant species (0.79–0.84).

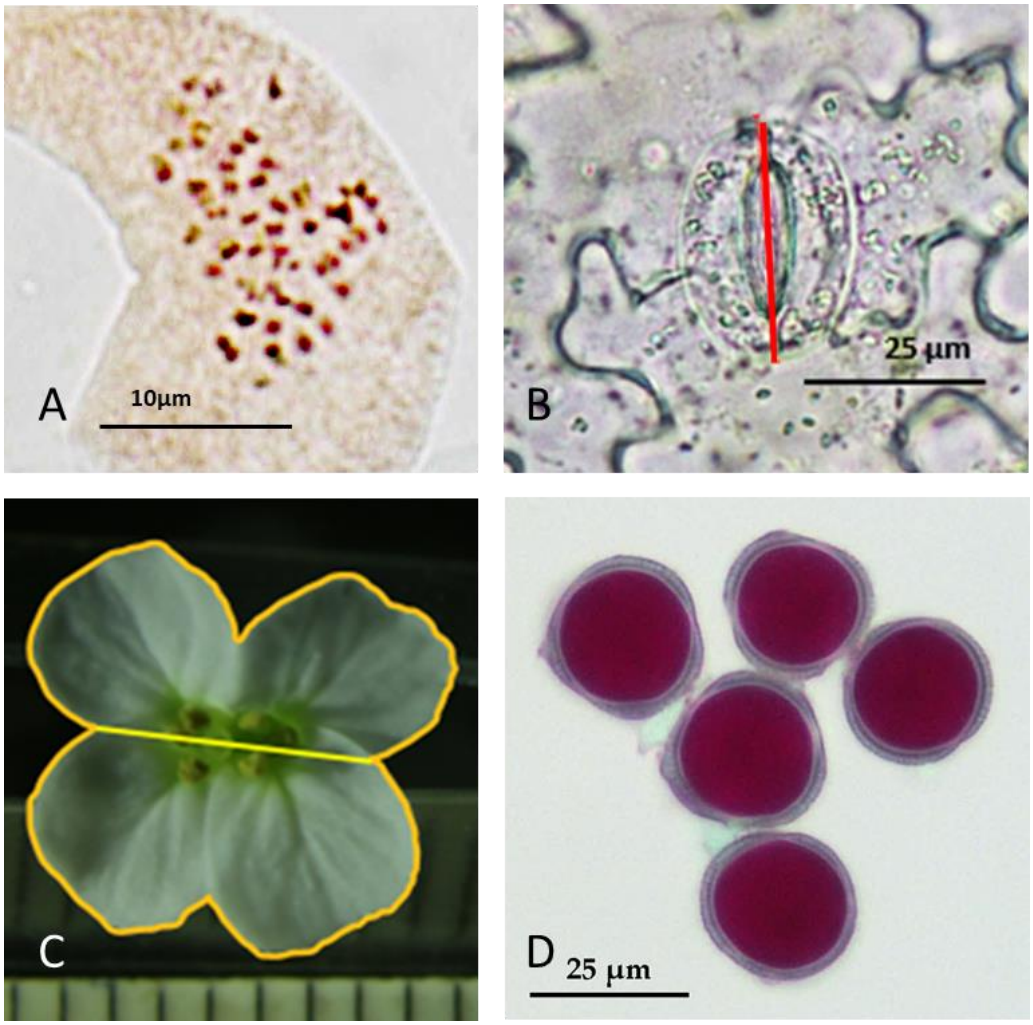

Figure 4. Morpho-anatomical characters of octaploid regenerants of *Arabidopsis arenosa* and chromosome number of their progeny. Metaphase plate $2n \approx 64$ (**A**), stomata length (marked with a red line) (**B**), flower width (marked with a yellow line) and area (marked with an orange line) (**C**), viable pollen grains stained with Alexander dye (**D**). The added lines illustrate how stomata and flowers were measured.

4. Discussion

In vitro culture of many species provides effective production of plant material (e.g., *Dianthus carthusianorum*) and selection to obtain more tolerant genotypes (e.g., *Gypsophila fastigiata*, *Daphne jasminea*, *D. tangutica*, *Viola arvensis*) [47–50]. Successful obtaining of chromosome-doubled breeding lines within metal tolerant plants, to the best of our knowledge, has been described only in the *Sedum alfredii*—Zn/Cd hyperaccumulator. It possesses, similarly to the obtained herein, *Arabidopsis arenosa* octaploid, promising phytoremediation parameters [51].

4.1. Octaploids Derived from Endopolyploid Callus Treated with TDZ

In the present paper, we have developed an effective protocol for the regeneration of octaploid specimens of metal-tolerant *A. arenosa*. The obtained plants turned out to be fertile and produced viable seeds, which provides the opportunity to introduce them to polluted sites also (except of plantlets) in the form of propagules. Furthermore, the obtained octaploid regenerants could also be well propagated vegetatively in artificial conditions (not shown). Both pathways of propagation likely could be complementary in the field. Referring to the fertility of polyploids in *Arabidopsis*, Yu et al. (2010) based on

the analysis of diploid and artificial tetraploid of *A. thaliana*, proposed that this species manifested an inherent and heritable ability to respond to increased chromosome numbers. It was associated with alterations in gene expression (DNA methylation), which are stable, nonstochastic, and developmentally specific [52]. Similarly, the autotetraploid cytotype Warschau of *A. thaliana* was fully fertile. It set fewer seeds per silique; however, it produced more of the latter [53]. Taking all of this into account, including our results, it seems that the genus *Arabidopsis* is a good object for any kind of genetic modification.

Thidiazuron (1-Phenyl-3-(1,2,3-thidiazol-5-yl)urea) is a very effective synthetic plant growth regulator commonly used in in vitro culture for inducing organogenesis and somatic embryogenesis [54,55]. It is normal that polyploids are obtained when administered with antimitotic agents (e.g., [56–60]), but it was a positive surprise that without the use of such agents, just in the presence of TDZ, endopolyploid callus of *A. arenosa* initiated polyploids whose frequency was very high, reaching even almost 80%. The varied frequency of polyploids at different stages of their development could have resulted from non-random selection of the specimens. If, for example, octaploids had emerged earlier than tetraploids and the former ones were larger than the latter, which cannot be ruled out, then the collection of samples for measurement could not have been devoid of randomness. Overall, such a high frequency of polyploids could be explained by the immanent properties of the polyploid cells of the specific tissue (here specifically callus), which tended to develop into plants, similar to the studies of Fritsche et al. (2022), who also obtained polyploid plants from endopolyploid protocorm and leaves of orchids [61]. Callus used for the regeneration of *A. arenosa*, both treated and non-treated with TDZ, manifested a high frequency of endopolyploidy (see Figure 3). This phenomenon could also be a result of 2,4-D action, which induced somaclonal variation (genetic or epigenetic changes that arise in vitro between clonal regenerants and their corresponding donor plants) during in vitro culture of other species [62,63]. It was not possible to examine whether obtaining such a high frequency of polyploids was influenced by TDZ, by stimulating the proliferation of endopolyploid cells, or by just the presence of cells with increased ploidy within callus tissue which would have developed into polyploid plants anyway (even without the presence of TDZ). Simply, without TDZ, cells did not undertake regeneration into adventitious shoots; therefore, the role of TDZ here remains unresolved. The mode of action of this cytokinin and auxin-like growth regulator is still unknown [54], though it has been proved that it prolonged the cell division phase [64] and doubled cell diameter [65,66]. Zhai et al. (2022) suggested that in *Brassica napus*, bigger cells may result from TDZ activity, which regulates the transcriptional levels of key genes involved in different processes [66]. However, TDZ ability to increase the DNA amount has never been confirmed. It was observed in our experiments (not shown) that excessively high endopolyploidy resulting from the age of the callus tissue led to a decrease in its regenerative capacity, which is a known phenomenon in plant tissue culture [63]. Despite applying similar conditions, including TDZ applying and running the regeneration in a similar way (indirect organogenesis—via callus), polyploids were obtained in only some violets (in *Viola uliginosa* but not in *V. stagnina* and *V. arvensis*), which suggests that it may be a species-specific process [50,67,68].

4.2. Genetic Stability of Octaploids

The octaploid regenerants obtained here seem to show genetic stability, which could be concluded based on the stable genome size (2C = 1.642 pg), high pollen viability seed setting and germination. The polyploidy seems to be passed to the next generation without significant losses in the genome as the progeny of octaploids manifest a doubled chromosome number ($2n = 8x = 64$). This is likely because they were obtained from a single callus cell (or cell group), not from chimeras which are usually obtained after treatment of the seedlings/seeds with colchicine [69]. In the latter case, the high frequency of mixoploidy and genetic instability of progeny is often detected, which is undesirable in breeding procedures [70,71]. Maintenance of polyploid stability requires the development of mechanisms to control pairing and segregation of more than two homologous chromosomes during

meiosis [15,16,72]. Meiosis in octaploid regenerants of *A. arenosa* must be regular since pollen viability is high, reaching almost 97%, similar to tetraploid regenerants and initial plants. Furthermore, pollen grains size in octaploids did not vary significantly, which means that they were cytologically balanced (likely possessing constant chromosome number, $1n = 32$). It seems that pollen of *A. arenosa* is not very sensitive to both internal and external factors in this species, as in plants growing at heavy-metal polluted sites, male meiosis was almost not impaired by pollution, and pollen was also highly viable [73]. Such highly probable diploid-like chromosome pairings during octaploid meiosis, whose bivalents are selected randomly from eight homologs already in the first generation of regenerants, are amazing. In the synthetic autopolyploid *Physalis ixocarpa*, pollen viability was reduced almost by half (from above 90% in the diploid to 50–60% in the tetraploid), similar to somatic autotetraploid *Hylocereus*, although to a significantly lower extent (97% in the diploid vs. 86% in the tetraploid) [74,75]. Hollister et al. (2012), based on the functional annotations of the *A. thaliana* homologs, indicated candidate genes and processes (regulation of core transcription, epigenetic regulation, DNA repair, cell division and morphogenesis, chromosome synapsis and cohesion, homologous recombination, and chromosome segregation) that may have been important for compensatory adaptation of *A. arenosa* to its genome-doubled state [15]. Similar or other unknown mechanisms may be responsible for such high genetic stability of obtained octaploids of *A. arenosa*.

To sum up, we believe that the higher-level autopolyploids (now diploid, tetraploid, and octaploid cytotypes of *A. arenosa* are available) are an excellent model for studying the increase in ploidy as a mechanism that does not restrict the persistence of neopolyploids in this species. Given the close relationship of the sequenced genome *A. thaliana*, it will be possible in the future to edit their genes, e.g., by PCR in these polyploid lines. This provides a route to generate polyploid mutants for improving the understanding of genome dosage effects in polyploid plants [76].

4.3. Conclusions and Future Perspectives

The obtained herein, without using antimitotic agents polyploids, are genetically stable, fertile and vigor. Their bigger cells, flowers, and seeds resulting from doubled genomes are good predictors that other morphological traits such as plant biomass and size and translocation coefficient will also be greater in octaploids than in the initial tetraploids. Prompt studies on heavy metal tolerance and the accumulation capacity of octaploids are urgently needed to estimate their phytoremediation potential. The developed protocol for octaploids production is reproducible. Next generations of octaploid could be obtained vegetatively or from seeds. Octaploids grow fast, thus processing more than one sowing/planting (generation) in the field per year seems to be feasible. For this reason, expected amount of heavy metal being extracted could also be high.

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