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An Attempt to Restore the Fertility of *Miscanthus* × *giganteus*

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Abstract: *Miscanthus* × *giganteus* is a popular industrial plant with great potential in ecological agriculture. It forms numerous rhizomes that are important in the sequestration of carbon dioxide. The plant can be a source of lignin and cellulose, biomass for renewable energy production, and can be used in small garden architecture, or to strengthen the banks of landslides. Breeding this species is difficult, as it is a sterile allotriploid with 57 chromosomes. The aim of the study was to obtain fertile plants of this species by treating its callus and regenerants with chromosome doubling agents such as colchicine, oryzalin, trifluralin, and caffeine at variable concentrations and durations. Callus cells naturally showed large variations in the number of chromosomes but only euploid cells regenerated plants. Treatment of the regenerants with 1252 µM colchicine for 18 h allowed for obtaining two hexaploid shoots; however, they died before flowering. Colchicine and oryzalin stimulated the formation of mixoploid shoots. The investigated substances, except for caffeine, were highly toxic to plants. *M.* × *giganteus* plants with 114 chromosomes may die because such a high number of chromosomes may be unfavorable for cells of this species.

Keywords: callus; chromosomes; hexaploids; *Miscanthus*; mixoploids; polyploids

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1. Introduction

Miscanthus × *giganteus* (Greef and Deuter ex Hodkinson and Renvoize) is a C4 grass. It is an allotriploid hybrid, resulting from a natural crossing of diploid *M. sinensis* with tetraploid *M. sacchariflorus* [1]. The product of this cross is a grass with 57 chromosomes and a basic chromosome number of 19. The species is sterile, and although it produces inflorescence panicles, it propagates only vegetatively, by dividing the rhizome [2]. Due to its sterility, it has low genetic variability. Attempts to breed cultivars with more favorable economic characteristics have not yet yielded the expected results. Plants of the genus *Miscanthus* inhabit tropical and subtropical areas. Interest in *M.* × *giganteus* was initiated by the Danish botanist and gardener Aksel Olsen, who, in 1935, brought plants to Europe from Jokohama (Kanagawa prefecture, Honshu, Japan) [3]. These plants are not the only natural hybrid resulting from the crossing of *M. sinensis* with *M. sacchariflorus*. Other natural hybrids were also found on Honshu, in the prefecture of Hyogo and Gifu, and on Kyushu, in the Kumamoto and Miyazaki prefectures [4,5]. Around 1980, subsequent plants of *M.* × *giganteus* from natural crossing were brought to England, directly from Japan. They constituted the second genotype of this species brought to Europe [6].

M. × *giganteus* produces high biomass. Due to its low soil requirements, marginal soils and wasteland can be used for its cultivation. Three years after *M.* × *giganteus* is planted, biomass yields can reach as much as 30 tons of dry matter per hectare, with low fertilization. Biomass can be used to obtain cellulose or lignin [7]. The biomass is an interesting raw material for the paper industry, and an alternative to woody materials [8]. *M.* × *giganteus* biomass is a source of renewable energy used to produce heat or electricity [9]. Biomass is treated in thermochemical processing or biochemical conversion. The easiest method

of thermal processing of raw material is its direct combustion. In obtaining electricity or heat, straw can be the only raw material used or co-fired with coal [7]. It is possible to produce fuel in solid, liquid, or gaseous form by gasification, pyrolysis, or liquefaction of biomass. [10]. In the results of biomass alcohol or anaerobic fermentation, gas or bioethanol can be obtained [11,12]. Moreover, *M.* × *giganteus* can be used for soil phytoremediation, especially for heavy metal phytostabilization [13]. In Japan, *M.* × *giganteus* shoots are used as animal feed or to build thatched roofs that cover traditional houses [7]. In some countries, *M.* × *giganteus* is planted on the shores of drying lakes to prevent soil erosion, and to restore ecosystems in degraded environments [14]. Rhizomes of this species efficiently accumulate carbon dioxide, which is a greenhouse gas, thus reducing its concentration in the air. In late autumn, *M.* × *giganteus* plants reallocate assimilates to the rhizomes in order to overwinter in regions of low temperature. *Miscanthus* can be grown at the same site for at least 20 years, which makes them highly efficient at long-term carbon sequestration [9,15].

M. × *giganteus* plants, obtained from the natural environment, still have imperfect agricultural and economic features. Despite the fact that *M.* × *giganteus* has great economic potential, it is still not sufficiently used in some countries. Pellet producers favor rape or wheat straw over *M.* × *giganteus* due to its hard stems. On the other hand, thatch manufacturers require *M.* × *giganteus* shoots to be long and of even length. Modification of the plant using traditional breeding methods is impossible due to a very low genetic variability of this sterile species. This means breeders are required to develop new methods to improve the cultivation and utility features of this grass.

In previous studies, we tried to obtain haploid *M.* × *giganteus* plants by androgenesis to eventually grow fertile double haploids [16]. Our results indicated that the induction of in vitro androgenesis is possible in this species; however, the microspores were produced in low numbers in the inflorescences and they were short-lived. Very low efficiency of the process and lack of regeneration of the androgenic structures renders this technique unworkable. A cytological analysis showed that the cause of the androgenic recalcitrancy is the hybrid origin of *M.* × *giganteus* deriving from interploidy crosses and resulting in irregular meiosis in microsporocytes.

Some papers reported obtaining hexaploid *M.* × *giganteus* plants [17–19]. A lack of reports on growing *M.* × *giganteus* with a doubled set of chromosomes in breeding and industry encouraged us to undertake further research on polyploidization of this species. We especially wanted to focus on the number of chromosomes in the callus cells treated with chromosome doubling agents. The primary aim of the experiments was to obtain fertile *M.* × *giganteus* plants. In plant breeding research, doubling the number of chromosomes is often performed to restore plant fertility by introducing homologous chromosome pairing into meiosis and regular segregation [20]. Plant polyploidization procedures usually involve the use of antimitotic compounds, among which colchicine is the most common. However, it shows low specificity for plant tubulin, can disturb plant growth, is responsible for the loss of chromosomes or their rearrangement, and also causes gene mutations [21,22]. Some polyploidization procedures also include the use of compounds such as oryzalin, caffeine, or trifluralin [23]. In tissue cultures, plant fragments, callus tissue, or regenerated plants can be subjected to treatment with chromosome doubling agents [24,25]. In our experiments, we used morphogenic callus tissue and regenerants obtained in vitro. Both types of plant material were exposed to colchicine, caffeine, trifluralin, and oryzalin at different times. Changes in the number of chromosomes in the callus cells, leaves, and roots of the resulting plants were observed, and the percentage of survived plants and the number of new shoots produced after the mutation were determined.

2. Materials and Methods

2.1. Reagents

Murashige and Skoog (MS) basal medium, sucrose, agar, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), kinetin (KIN), glacial acetic acid, α-bromonaphthalene, casein hydrolysate, dimethyl sulfoxide (DMSO), Tween 20, colchicine, trifluralin, oryzaline,

caffeine, and Entellan were bought from Merck KGaA (Darmstadt, Germany). Ethanol, Schiff's reagent, hydrochloric acid (HCl), salts for Hoagland medium were purchased from Chempur (Piekary Śląskie, Poland). Honey (multiflorous) was bought at a beekeeping farm Sądecki Bartnik Sp. z o. o. (Stróże, Poland). Reagents for flow cytometry analysis were acquired from Sysmex (Chuo-ku, Kobe, Japan), dedicated to the CyFlow Ploidy Analyzer (Sysmex, Chuo-ku, Kobe, Japan).

2.2. Plant Material and Growth Conditions

The experiments were performed on plants obtained from *M.* × *giganteus* collection of the University of Agriculture in Kraków (Poland). Donor plants for in vitro culture explants were grown in a greenhouse in 15 dm^3 containers filled with commercial soil (pH 6.0), at 24 °C and 65% air humidity. The greenhouse was located at the latitude of 50°04′10″ N, and longitude of 19°50′44″ E. Daylight was supplemented with light at 300 µmol m^{-2} s^{-1} from sodium lamps (AGRO Philips), at a 16-h photoperiod. The plants were fertilized once a month with a multi-component fertilizer Azofoska (GRUPA INCO S.A., Warsaw, Poland).

2.3. Callus Induction and Plant Regeneration

Callus induction and plant regeneration were performed as described earlier (Figure 1) [26]. The callus was induced from fragments of immature inflorescences cultured on a sterile induction medium (MSI) containing MS medium (Murashige and Skoog, 1962), supplemented with 6.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg dm^{-3} 6-benzylaminopurine (BAP), 500 mg dm^{-3} casein hydrolysate, 30 g dm^{-3} commercial multiflorous honey, and solidified with 8 g dm^{-3} agar (pH was set at 5.8) The plants were regenerated by transplantation of the morphogenic calli on a regeneration medium (RM) containing MS medium of the same pH, supplemented with 0.05 mg dm^{-3} kinetin (KIN), 30 g dm^{-3} sucrose, and solidified with 8 g dm^{-3} agar. Both media were autoclaved for 20 min at 121 °C and 0.1 MPa. For plant acclimation to the greenhouse conditions, firstly the regenerants were transferred into small pots (Ø 7 cm) with sterile perlite soaked with Hoagland's medium (Hoagland and Arnon 1950) and grown under phytotronic conditions. Next, the plants were re-potted to larger pots (Ø 13 cm) filled with a soil–peat–sand (2:2:1 $v/v/v$) mixture with pH 6.0 and grown in the greenhouse at 24 °C for three weeks. Daylight was supplemented with light at 300 µmol m^{-2} s^{-1} from sodium lamps (AGRO Philips), at a 16-h photoperiod.

2.4. Polyploidization Using Callus Tissue

Morphogenic callus was divided into 2–5 mm pieces and passaged on MSI medium containing colchicine, oryzalin, trifluralin, or caffeine, and DMSO (10% v/v). Concentrations of chromosome doubling agents were selected after our previous unpublished preliminary research. The chromosome doubling agents dissolved in DMSO were sterilized using syringe filters with 0.22 µm mesh (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and added to the autoclaved media. Two experimental concentrations of the chromosome doubling agents and two callus exposure times were used (Table 1). Then, the calli were transferred into single Petri dishes (Ø 6 cm) containing 10 cm^3 of the medium as an experimental repetition. Each polyploidization procedure was carried out in triplicate. The morphogenic callus cultures grown on the media containing the chromosome doubling agents were incubated in the dark at 26 °C. After two or four days, the calli were washed three times with sterile, redistilled water and passaged to an RM medium. During the passage, a fragment of each callus was collected for the analysis of the chromosome number. In addition, a control group of calli grown on the medium without the chromosome doubling agents was cultured. The regenerated plants were acclimated to greenhouse conditions. Twelve weeks after planting the plants in the greenhouse, the number of shoots was counted and fragments of the youngest leaves were collected for flow cytometry analysis. Based on the shoot number, the plant propagation coefficient (PPC), which is defined as the average number of shoots formed by one plant, was calculated.

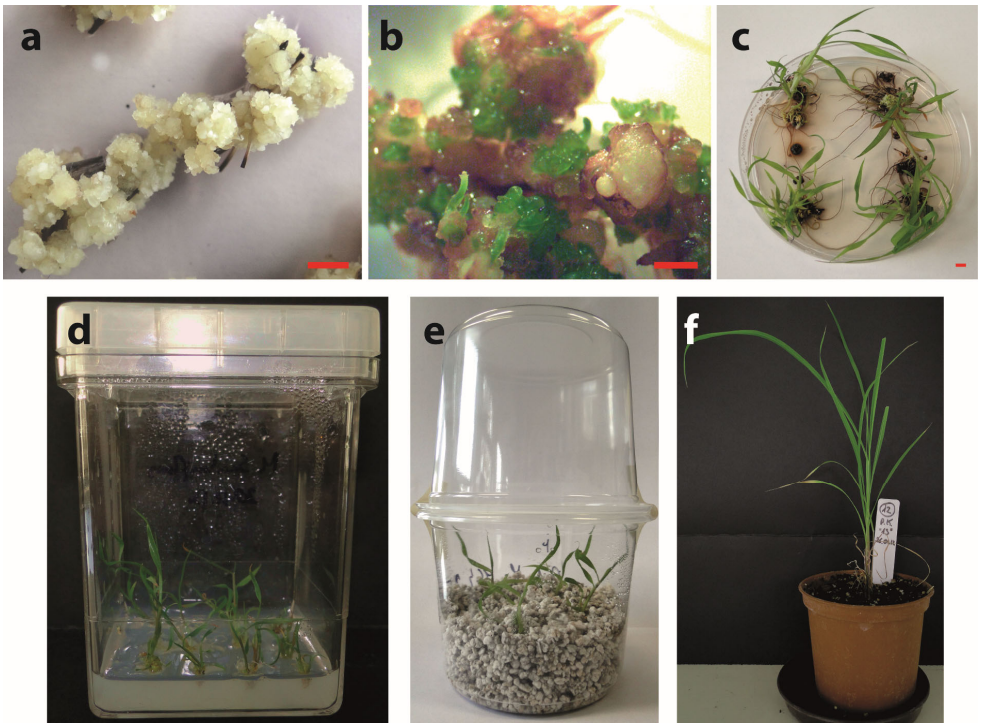

Figure 1. *Miscanthus* × *giganteus* callus induction, plant regeneration, and acclimatization: (**a**) morphogenic callus on an induction medium; (**b**) callus regenerating shoots on a regeneration medium; (**c**) regenerated shoots; (**d**) shoots rooting on a regeneration medium; (**e**) acclimatization of regenerants; (**f**) regenerants acclimated to greenhouse conditions. Bars in (**a**,**b**) = 2.5 mm.

Table 1. Concentrations of reagents used for chromosome doubling in *Miscanthus* × *giganteus* and time of the plant material exposure to their impact in two experiments: (a) polyploidization using callus tissue; (b) polyploidization of regenerants.

Chromosome Doubling Agent	(a) Callus Treatment		(b) Regenerant Treatment	
	[µM]	Time	[µM]	Time
Colchicine	313	2 or 4 days	1252	9 or 18 h
	626		2504	
Oryzalin	10		25	
	20		50	
Trifluralin	10		25	
	20		50	
Caffeine	2575		2575	
	5150		5150	

2.5. Polyploidization of Regenerants

The plants regenerated from the callus with their roots cut up to 2 cm in length were placed into Erlenmeyer flasks (250 cm^3) with colchicine, oryzalin, trifluralin, or caffeine aqueous solution so that the crowns were completely immersed. Two experimental concentrations of the chromosome doubling agents and two regenerant exposure times were used (Table 1). The concentrations of chromosome doubling agents were selected after our previous unpublished preliminary experiments with *M.* × *giganteus* seedlings. All the solutions were supplemented with DMSO and Tween 20. DMSO was used at 10 cm^3 per 1 dm^3 of a solution, while Tween 20 was used at 10 drops per 1 dm^3 of a solution. Oryzalin

and trifluralin were first dissolved in DMSO and then an appropriate amount of water was added. Colchicine and caffeine were dissolved directly in water. The regenerants were incubated in a phytotron chamber under light of 80 µmol m^{-2} s^{-1} and at 23 °C. Each polyploidization procedure was carried out in triplicate with 10 plants per replication. After 9 or 18 h, the plants were removed from the solutions and washed under running tap water. In addition, control plants regenerated on RM medium were also acclimated to the greenhouse conditions. For the acclimation to the greenhouse conditions, the plants were planted into pots (Ø 7 cm) with sterile perlite soaked in Hoagland's medium [27] and grown under phytotronic conditions. After three weeks, the plants were transplanted into pots (Ø 23 cm) with commercial soil (pH 6.0) and cultivated in the greenhouse conditions in daylight and at 24 °C (65% of air humidity). The number of plants that undertook further growth and development, as well as the number of newly formed shoots, was counted 12 weeks after planting them in the soil. Based on the number of newly formed shoots, the PPC was calculated. At the same time, the youngest leaf fragments were collected for flow cytometry analysis.

2.6. Determination of Plant Ploidy Levels

In the callus cells treated with the chromosome doubling agents, the chromosome number was counted using the Feulgen method. The calli were placed in a saturated solution of α-bromonaphthalene for two hours and then fixed in a mixture of ethanol and glacial acetic acid (3:1 v/v). The fixed calli were hydrolyzed in 1 N HCl (60 °C; 11 min) and then treated with Schiff's reagent (60 min). Darkly colored callus fragments were taken for analysis and crushed on a slide. The preparations were frozen in liquid nitrogen and sealed in Entellan. The chromosomes were counted in three cells in metaphase plates in each callus used in the experiment.

The flow cytometry analysis was performed for leaf fragments. Nuclei from the youngest leaves were isolated, stained with 4′,6′-diamidino-2-phenylindole (DAPI), and analyzed with a CyFlow Ploidy Analyzer (Sysmex, Chuo-ku, Kobe, Japan) by the Cytogenetics Laboratory of White Beet Breeding in Kutno (Poland). Plant ploidy composition was estimated by comparing the peaks in the nuclear DNA histogram with the plants after the polyploidization procedure with known ploidy.

2.7. Statistical Analysis

The experiments were based on a randomized block design. Two-way analysis of variance (ANOVA) and the Tukey multiple range test ($p < 0.05$) were performed using the statistical package STATISTICA 13.0 (Stat-Soft Inc., Tulsa, OK, USA). In the analysis of variance, one factor was the polyploidization procedure (type of chromosome doubling agent with its concentration), and the other factor was the treatment time. Compliance of the distribution of features in the groups with the normal distribution was verified by the Shapiro–Wilk test. However, in the case of data deviating from the normal distribution, the $y = \log(x + 1)$ transformation was used.

3. Results

3.1. Polyploidization in Callus Tissue

The morphogenic callus induced from fragments of immature inflorescences was subjected to polyploidization. After each polyploidization procedure, the number of chromosomes in the callus cells was counted before the calli transplantation on the regeneration medium. In each analyzed callus, cells with a different number of chromosomes were observed. This phenomenon is called mosaicism. The frequency of cells with different numbers of chromosomes is shown in Figure 2. Most of the cells had 57 chromosomes, which is characteristic of the species. The remaining cells demonstrated between 23 and 87 chromosomes. The mosaicism was not influenced by the chromosome doubling agents (Figure 3). The callus tissue cultured on the medium without additional substances also

showed different numbers of chromosomes in individual cells. However, no cells with doubled chromosome number (114) were found in the studied material.

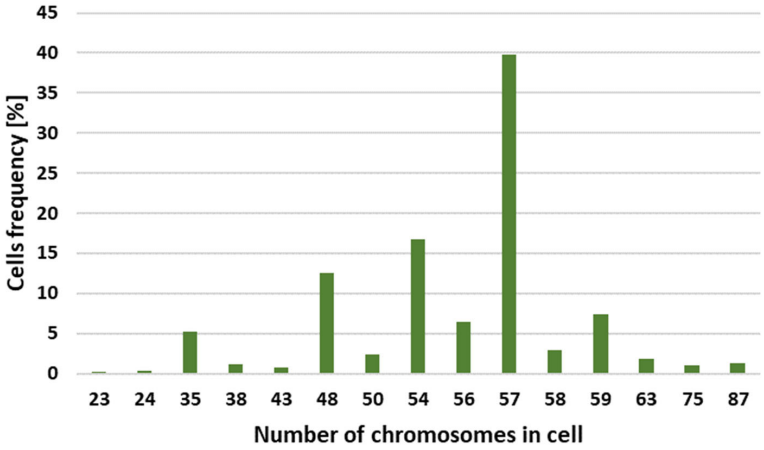

Figure 2. Chromosome variation in callus of *Miscanthus* × *giganteus*, regardless of the treatment with chromosome doubling agents.

The callus ability to regenerate plants was drastically limited by the antimitotic agents. Their use at the lowest concentrations, i.e., 313 µM colchicine, 10 µM oryzalin, and 10 µM trifluralin, regardless of the treatment time, still enabled the plants to regenerate. However, the higher concentrations (626 µM colchicine, 20 µM oryzalin, and 20 µM trifluralin) completely inhibited this process. Four hundred and eighty pieces of callus were treated with the chromosome doubling agents. Among them, only 108 calli (22.5%) survived and regenerated rooted green plantlets (Table 2). Of the 30 untreated control calli, 29 regenerated plants (96.7%). Caffeine had the least toxic effect on the callus. After its use, the percentage of regenerating calli ranged from 53.3 to 76.7%, depending on the applied concentration and time of exposure.

Table 2. Effect of treating the callus of *M.* × *giganteus* with chromosome doubling agents on the plant regeneration ability and effectiveness of shoot propagation. Values marked with the same letter do not differ statistically (Tukey test; $p < 0.05$; $n = 30$).

Chromosome Doubling Agent	Concentration [µM]	Time of Exposure [Days]	No. of Regenerating Calli (Percentage of Regenerating Calli)		No. of Green Plantlets	PPC *
Colchicine	313	2	4 ^{d}	(13.3%)	9 ^{de}	2.18 ^{b}
		4	4 ^{d}	(13.3%)	10 ^{d}	2.18 ^{b}
	626	2	0 ^{e}	(0%)	0 ^{f}	0 ^{e}
		4	0 ^{e}	(0%)	0 ^{f}	0 ^{e}
Oryzalin	10	2	5 ^{d}	(16.7%)	5 ^{e}	1.00 ^{d}
		4	6 ^{d}	(20.0%)	6 ^{e}	1.19 ^{d}
	20	2	0 ^{e}	(0%)	0 ^{f}	0 ^{e}
		4	0 ^{e}	(0%)	0 ^{f}	0 ^{e}
Trifluralin	10	2	7 ^{d}	(0%)	8 ^{e}	1.10 ^{d}
		4	4 ^{d}	(23.3%)	5 ^{e}	1.38 ^{d}
	20	2	0 ^{e}	(0%)	0 ^{f}	0 ^{e}
		4	0 ^{e}	(0%)	0 ^{f}	0 ^{e}
Caffeine	2575	2	16 ^{c}	(53.3%)	34 ^{c}	2.15 ^{b}
		4	23 ^{ab}	(76.7%)	42 ^{b}	1.82 ^{c}
	5150	2	19 ^{b}	(63.3%)	35 ^{c}	1.84 ^{c}
		4	20 ^{ab}	(66.7%)	41 ^{b}	2.16 ^{bc}
Control			29 ^{a}	(96.7%)	71 ^{a}	2.42 ^{a}

* Plant propagation coefficient–the average number of shoots formed by one plant.

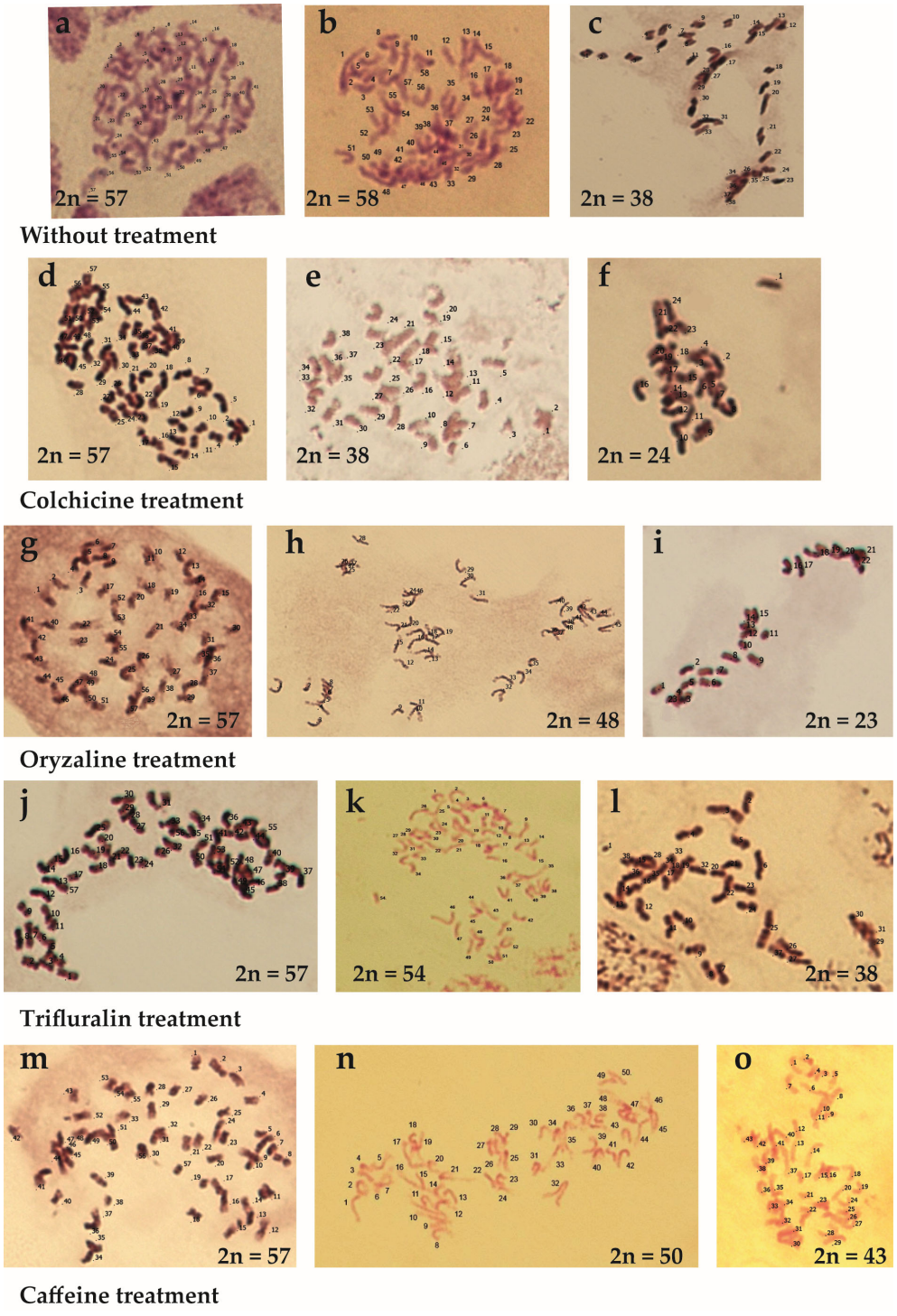

Figure 3. Chromosome numbers in metaphase cells of callus of *Miscanthus* × *giganteus* dyed by the Feulgen method: (**a**–**c**) callus without treatment; (**d**–**f**) callus treated with colchicine; (**g**–**i**) callus treated with oryzalin; (**j**–**l**) callus treated with trifluralin; (**m**–**o**) callus treated with caffeine.

In the group of callus treatment with the chromosome doubling agents, 12 weeks after the callus tissue passage to the regeneration medium, 195 rooted green plantlets were acclimated to the greenhouse conditions (Table 2). In the control group, 71 plantlets regenerated. In both groups, there were no albino shoots. After 12 weeks from the passage to the RM medium, some, though not all, of the morphogenic callus started to form green shoots and regenerated green, rooted plantlets. The largest number of plantlets was obtained after treatment of the callus tissues with caffeine. After cultivation with 2575 and

5150 µM of caffeine for two days, 34 and 35 plants were formed. A prolonged exposure of tissues to this substance (four days) resulted in a greater number of regenerated plants. After cultivation with 2575 µM and 5150 µM of caffeine, 42 and 41 plants regenerated. Contrary to caffeine, the antimitotic substances, especially oryzalin and trifluralin, strongly limited the formation of plantlets. The callus treated with colchicine at 313 µM and 626 µM regenerated nine and ten plants, respectively, while that treated with oryzalin at 10 µM and 20 µM regenerated five and six plants, respectively. The calli treated with trifluralin at 10 µM and 20 µM regenerated eight and five plants, respectively.

PPC is presented in Table 2. The plantlets from the control group had the highest PPC, amounting to 2.42. The plantlets obtained from the calli treated with caffeine at 2575 µM for two days, 5150 µM for four days, and with colchicine at 313 µL for two and four days were also distinguished by a PPC above 2. In the remaining cases, most plantlets grew only a single shoot.

The level of ploidy was measured in the plantlets regenerated from the callus tissue by flow cytometry. All plants had unchanged ploidy in relation to their donor plants, i.e., they were still triploids.

3.2. Polyploidization of Regenerants

Four hundred and eighty plants regenerated from immature inflorescences were treated with colchicine, oryzalin, trifluralin, and caffeine at two concentrations and two exposure times.

Following the treatment of the plants with the chromosome doubling agents, only 57.5% of the plants continued to grow and develop (Table 3). After 12 weeks, the plants produced 496 new shoots (Figure 4a). The average value of PPC amounted to 1.82 (Table 3).

Table 3. Effects of treating the plantlets of *M.* × *giganteus* regenerated in vitro from immature inflorescences with chromosome doubling agents on the plant survival ability and effectiveness of shoot propagation. Values marked with the same letter do not differ statistically (Tukey test; $p < 0.05$; $n = 30$).

Chromosome Doubling Agent	Concentration [µM]	Time of Exposure [h]	No. of Survived Plants (Percentage of Survived Plants)		No. of All Shoots	PPC *	Ploidy of Shoots		
							3x	3x/6x	6x
Colchicine	1252	9	30 ^{a}	(100%)	58 ^{a}	1.93 ^{ab}	46	12	
		18	20 ^{b}	(66.7%)	46 ^{c}	2.30 ^{a}	33	11	2
	2504	9	28 ^{a}	(93.3%)	50 ^{bc}	1.79 ^{b}	32	18	
		18	8 ^{d}	(26.7%)	11 ^{fg}	1.38 ^{c}	0	11	
Oryzalin	25	9	11 ^{c}	(36.7%)	16 ^{ef}	2.00 ^{a}	16	4	
		18	13 ^{c}	(43.3%)	22 ^{d}	1.69 ^{b}	22		
	50	9	4 ^{e}	(13.3%)	8 ^{g}	2.00 ^{a}	8		
		18	0 ^{f}	(0%)	0 ^{h}	0 ^{d}			
Trifluralin	25	9	13 ^{c}	(43.3%)	22 ^{d}	1.69 ^{b}	22		
		18	13 ^{c}	(43.3%)	19 ^{d}	1.46 ^{bc}	19		
	50	9	11 ^{c}	(36.7%)	18 ^{de}	1.64 ^{b}	18		
		18	5 ^{de}	(16.7%)	11 ^{fg}	2.20 ^{a}	11		
Caffeine	2575	9	30 ^{a}	(100%)	50 ^{bc}	1.67 ^{b}	50		
		18	30 ^{a}	(100%)	60 ^{a}	2.00 ^{a}	60		
	5150	9	30 ^{a}	(100%)	47 ^{c}	1.57 ^{b}	47		
		18	30 ^{a}	(100%)	58 ^{a}	1.93 ^{ab}	58		
Control			30 ^{a}	(100%)	62 ^{a}	2.06 ^{a}	62		

* Plant propagation coefficient—the average number of shoots formed by one plant.

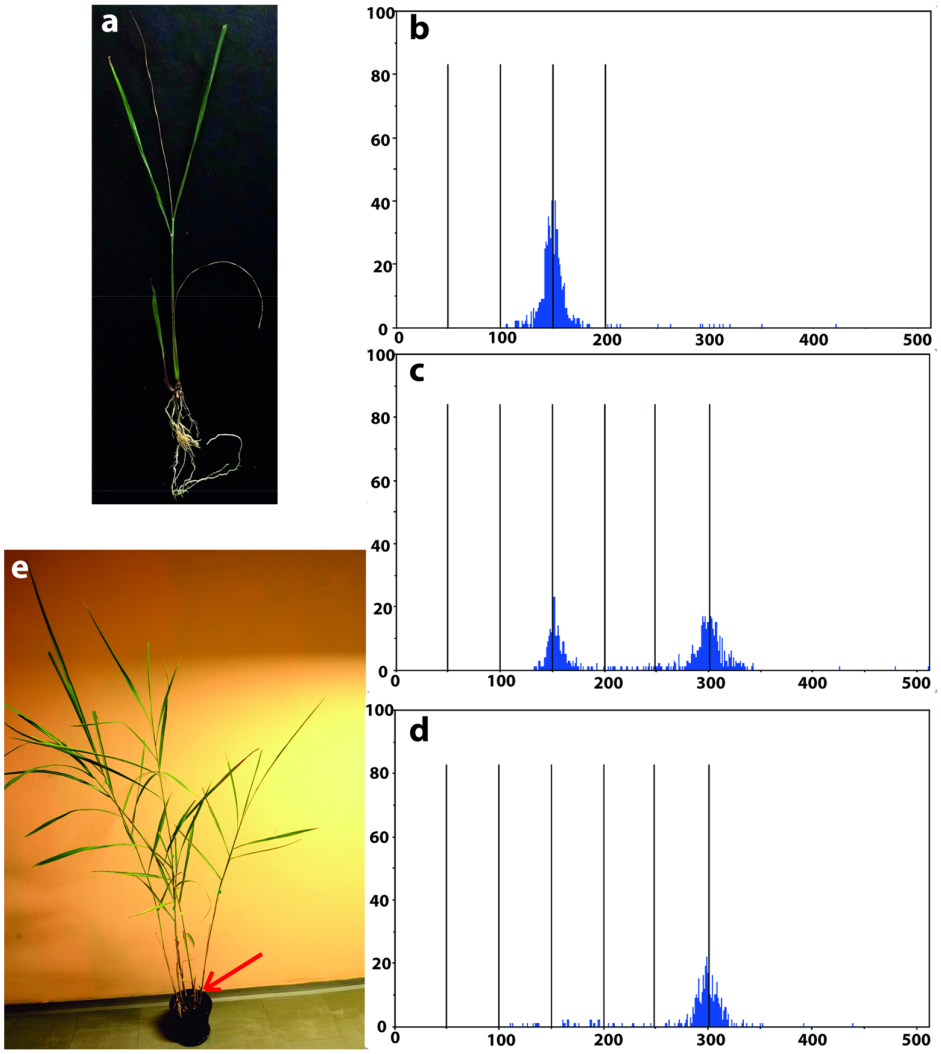

Figure 4. Polyploidization of *Miscanthus* × *giganteus* regenerants: (**a**) plantlets forming a lateral shoot; (**b**) flow cytometry histogram of a triploid shoot; (**c**) flow cytometry histogram of a mixoploid shoot; (**d**) flow cytometry histogram of hexaploid shoots; (**e**) plant with a hexaploidy shoot.

All caffeine-treated plants survived and were successfully acclimated to the greenhouse conditions (Table 3). The same lack of toxicity was shown for colchicine at 1252 µM and 2504 µM and a 9 h treatment. Plant survival decreased when the treatment time with these agents was extended to 18 h. After this time, 66.7% of the plants survived colchicine treatment with 1252 µM, and 26.7% with 2504 µM. Oryzalin and trifluralin were highly toxic to plants. Among the plants treated with 25 µM of these compounds for both 9 and 18 h, 36.7% to 43.3% plants survived, respectively. A similar result was obtained following the plant exposure to 50 µM trifluralin for 9 h. At the same concentration of oryzalin, the plant survival decreased drastically, and after 9 h exposure only 13.3% of plants survived, while after 18 h all the plants died.

The plants treated with colchicine at 1252 µM for 9 h and caffeine at 2575 µM and 5150 µM for 18 h produced the largest number of shoots, while the plants treated with oryzalin and trifluralin produced the lowest number (Table 3). The PPC was the highest for the plants treated with 50 µM of trifluralin for 18 h (2.20); however, it should be noted that only 16% of plants survived this treatment. The plants propagated similarly after the treatment with colchicine at 1252 µM for 9 h (1.93) and 18 h (2.30), oryzalin at 25 µM (2.00) and 50 µM (2.00) for 9 h, and caffeine at 2575 µM (2.00) and 5150 µM (1.93) for 18 h. Control plants obtained in vitro produced 62 shoots, and their PPC ratio was 2.06.

The ploidy of all newly formed shoots was analyzed by flow cytometry. The histograms showed a dominant peak corresponding to cells in G1 phase. Out of 558 shoots tested, 504 were still triploid (2n = 3x); they contained 3C DNA (Table 3; Figure 4b). On the histograms obtained for the remaining 56 shoots, there were two peaks, corresponding to the population of nuclei with 3C DNA and 6C DNA (Figure 4c). This means that the examined shoots were mixoploids (2n = 3x/6x). Their tissues had both cells with 57 chromosomes and cells with a doubled number of chromosomes–114. Mixoploid shoots were obtained after the application of colchicine and 25 µM oryzalin for 9 h. Two plants treated with 1252 µM colchicine for 18 h produced one hexaploid (6C DNA) shoot (2n = 6x) (Figure 4d,e). Due to the low efficiency of polyploidization, the results of the ploidy determination were not statistically analyzed. Hexaploid and mixoploid shoots died approximately 16 weeks after the flow cytometry analysis without producing panicles, while the triploid plants bloomed in the greenhouse conditions.

4. Discussion

Restoring *M.* × *giganteus* fertility by doubling its genome is one way to improve the industrial properties of the crop. The genetic variability of the species can be extended by a controlled crossing of the diploid *M. sinensis* with the tetraploid *M. sacchariflorus*, but the resulting new triploids would also require further crosses that would not be possible. The other ways to improve *M.* × *giganteus* involve mutational breeding, which is inefficient, or the use of new technologies such as genome editing. The latter technique requires knowledge of *M.* × *giganteus* genetics, and moreover, plants with their genome changed in this way are classified as genetically modified organisms and their cultivation is legally prohibited in many countries.

In the presented experiments, four chromosome doubling agents were applied. One of them, colchicine, is a commonly used antimitotic substance. It disrupts mitosis by the inhibition of microtubule formation and polar migration of the chromosomes. However, it has a low affinity for plant microtubules and therefore must be used at relatively high concentrations. A huge disadvantage of colchicine is its toxicity to both plants and animals [28]. Therefore, three other substances were used: oryzalin, trifluralin, and caffeine. Oryzalin and trifluralin are also antimitotic agents. Their advantage is a much higher affinity for plant microtubules than that of colchicine, and they do not bind to animal microtubules, reducing the toxicity risk to humans [29]. The fourth substance used was caffeine, which is not toxic to plants or animals. It inhibits cell plate formation during cytokinesis [30]. In *Miscanthus* callus cultures, we observed the most toxic effect when the antimitotic substances were used at their highest concentrations, i.e., 626 µM for colchicine and 20 µM for oryzalin and trifluralin. Similar results were obtained by Yu et al. [31] during polyploidization of *M.* × *giganteus* callus cultures. These authors examined higher concentrations of the antimitogens in the media, namely 939 µM of colchicine and 30 µM of oryzalin, and demonstrated higher survivability of callus tissue than in our research. This was probably because the DMSO we used in the polyploidization procedure increased tissue penetration by the chemical agents. Głowacka et al. [17] showed that the addition of DMSO to colchicine solution for a treatment of *M.* × *giganteus* and *M. sinensis* regenerants reduced the plant survival and their tillering, and it had no effect on genome doubling. In our experiment on polyploidization of regenerants, a significant decrease in plant survival after soaking in solutions of antimitotic substances with DMSO was noted. Such a toxic effect on the regenerants was not observed after treatment with caffeine solution with DMSO, as all the plants survived, even though no effects on chromosome doubling were seen. In a study by Hamill et al. [32] on polyploidization of other plant species with colchicine, DMSO improved the efficiency of genome doubling.

In our experiment, where the morphogenic callus was treated with chromosome doubling agents, the number of chromosomes in the callus cells was counted. Regardless of the polyploidization procedure, each callus had cells with a chromosome number deviating from the somatic number of chromosomes (57). No cell demonstrated a duplicate number

of chromosomes (114). Callus mosaicism is a common phenomenon observed in in vitro cultures, but most often it is associated with cytological diversity of plants regenerated from it [33,34]. The regeneration of *M.* × *giganteus* plants occurred exclusively from callus euploid cells. In our experiment, no agent affected regeneration of the hexaploid plants. Głowacka et al. [35] induced callus of both triploid *M.* × *giganteus* and *M. sinensis* cv. 'Goliath' from immature inflorescence culture on the media containing 313 µM or 626 µM of colchicine. They obtained a total of 125 hexaploid *M.* × *giganteus* plants and 111 hexaploid *M. sinensis* cv. 'Goliath' plants, without mixaploid individuals. Similar to our research, Yu et al. [31] treated callus tissue of *M.* × *giganteus* with colchicine or oryzalin. They studied various concentrations of these compounds added to solid or liquid medium and different exposure times. They regenerated a few hexaploid plants, only in the liquid media with 626 µM or 939 µM of colchicine and 5 µM, 10 µM, or 15 µM of oryzalin. They also obtained mixoploid plants. Chae et al. [36] subjected *M.* × *giganteus*, *M. sinensis*, and *M. saccharoflorus* callus to 10 µM oryzalin in a liquid medium. In all cases, the plants doubled their number of chromosomes.

After an 18 h treatment with colchicine at 1252 µM, only two of our plants produced hexaploid shoots. The treatment with colchicine and oryzalin induced the formation of mixoploid shoots, but the shoots that were not triploid died before flowering. The formation of mixoploid shoots of *M. sinensis* in in vitro cultures after treatment with colchicine was also observed by Petersen et al. [37]. These authors also received tetraploid shoots; however, they concluded that in vitro plantlets are not a suitable target for antimitotic treatment, due to a relatively low frequency of doubling of the chromosome number and a high frequency of ploidy chimeras. During polyploidization of *M. sinensis* and *M.* × *giganteus* regenerants, Głowacka et al. [17] also obtained mixoploid shoots. The plantlets were treated with colchicine (626 µM or 1252 µM) for 6, 18, or 24 h, and, additionally, supplementation with DMSO and Tween 20 was tested. Głowacka et al. [17] stated that in *Miscanthus* genus the induction of polyploids with colchicine is highly dependent on genotype, and using DMSO improved the polyploidization rate only with a higher concentration of the antimitotic agent. Hexaploid plants of *M.* × *giganteus* were obtained by Głowacka et al. [18] through polyploidization of in vitro propagated shoots. The possibility of obtaining *M.* × *giganteus* hexaploid plants using commercially available and newly synthesized antimitotic compounds from the dinitroanilines group was confirmed by Melnychuk et al. [19].

In 2012, Touchell and Ranney [38] reported on the receipt of hexaploid *M.* × *giganteus* by oryzalin treatment in in vitro conditions at the Annual Conference of the American Society for Horticultural Science in Miami (FL, USA). The fertility of hexaploid plants was assessed by crossing them with a diploid *M. sinensis*. Many seeds had been aborted and those that remained contained little or no endosperm. By using the in vitro technique, tetraploid plants were obtained from embryos.

Fully duplicated *Miscanthus* genome has 114 chromosomes, which is a large number, although modern cultivated sugarcane typically has 100 to 130 chromosomes [39], while *Ophioglossum reticulatum* has naturally more than 1440 chromosomes [40]. Whole genome duplication often fails to improve the economic value of crops. Doubling the entire chromosome set increases the gene dosage. Duplicated alleles are responsible for additional synthesis of gene products, which can lead to genomic instability, epigenetic remodeling, and mitotic and meiotic abnormalities [41,42].

5. Conclusions

Our study shows that it is possible to obtain hexaploid *M.* × *giganteus* plants. However, the plants with doubled number of chromosomes are highly likely to die before flowering. Antimitotic substances used for polyploidization are highly toxic to plants. Oryzalin and trifluralin exhibit their toxicity at much lower concentrations than colchicine. Caffeine, which inhibits cell plate formation during cytokinesis, does not have a toxic effect on plants, but also does not allow for obtaining hexaploid plants. Antimitotic agents enable the formation of mixoploid plants which, similarly to hexaploids, die out. Callus cells

naturally show large variations in the number of chromosomes, but only euploid cells (with 57 chromosomes) regenerate plants. Only two hexaploid plants were obtained after treating the in vitro regenerated plantlets with colchicine. For the examined *M.* × *giganteus* plants, having 114 chromosomes may be such a disadvantage that it results either in the lack of plant development from cells with a doubled number of chromosomes or death of the hexaploid plants. Some researchers reported receiving hexaploidy plants of *M.* × *giganteus*, but there is no further information on their development and reproduction. It seems that the restoration of *M.* × *giganteus* fertility is not an appropriate way to extend intra-species genetic variability. Alternative methods should be found that combine classical mutagenesis and genomic analytical tools.

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