INDUCTION OF POLYPLOIDY IN GIANT MISCANTHUS (*MISCANTHUS* × *GIGANTEUS* GREEF ET DEU.)

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Results of obtaining miscanthus polyploid lines using both classic and new dinitroanilines to improve productivity of biomass as well as quality for biofuel production are represented. All of compounds applied in the study appeared to be able to induce polyploidy in Miscanthus × giganteus. It was found that new antimitotics have a lower phytotoxicity level compared to classic dinitroanilines. Ploidy level of obtained lines was confirmed by cytological studies using light microscopy. These lines are acclimatised for growing in open soil conditions. Currently, morphometric parameters of selected lines are being studied and analysed.

Key words: antimitotic agents, cell culture, dinitroanilines, tubulin binding, polyploidisation.

INTRODUCTION

Popularity of alternative energy sources and, in particular, the second generation biofuel production technologies, is conditioned by requirement of modern progress in sustainable supply of energy resources (Clifton-Brown *et al.*, 2008). Utilisation of renewable energy sources, such as wind, solar radiation and biomass, is one of the main components of sustainable economic prosperity that has significant environmental and energy results. In addition, cultivation of technical crops to produce biofuels as well as biomass production solve the problem of contaminated lands utilisation as well as lands unsuitable for growing food crops (Tytko and Kalynychnenko, 2010).

Giant miscanthus (*Miscanthus* × *giganteus*), belonging to the family Poaceae, is one of the most promising species for biofuel production (Weijde *et al.*, 2017). Representatives of this perennial grass use the C₄ photosynthesis pathway, which gives them an advantage of higher utilisation of solar energy and water use efficiency (Weijde *et al.*, 2013). *M. × giganteus* has high productivity while having low requirements for soil conditions, as it has high drought tolerance and a low level of nitrogen uptake from soil. These features make it one of the best candidates for biofuel production (Somerville *et al.*, 2008). According to published data, dry

yield of *M. × giganteus* can reach 39.89 t/ha (Bilandzija *et al.*, 2018). However, as an interspecific hybrid between diploid *M. sinensis* (2n = 2x = 38) and tetraploid *M. sacchariflorus* (2n = 4x = 76), *M. × giganteus* (2n = 3x = 57) has 57 chromosomes and is a sterile allotriploid (Rayburn *et al.*, 2009; Chramiec-Glabik *et al.*, 2012). In the case of sterile *M. × giganteus*, polyploidisation is an indispensable method for obtaining new plant populations and providing genetic diversity. The majority of polyploid plants have an advantage due to larger cell size and plant biomass and, as a result, higher potential yield (Birchler *et al.*, 2003). Polyploidisation methods for obtaining tetraploids, fertile amphidiploids or hexaploids have been successfully applied to miscanthus (Yu *et al.* 2009; Petersen *et al.*, 2002; Petersen *et al.*, 2003; Glowacka *et al.*, 2010b; Dalton, 2013).

A classic antimitotic agent that has been used to double chromosomes and has been known since the 1930s is colchicine (Blakeslee and Avery, 1937; Thomas, 1993; Yemets and Blume, 2008). Plant material treatment with compounds belonging to dinitroanilines similarly as treatment with colchicine also leads to polyploidisation (Yemets and Blume, 2008). However, affinity of colchicine as well as related compounds to plant tubulin is relatively low (Banerjee *et al.*, 2007; Spasevska *et al.*, 2017), so the use of dinitroanilines as substances to block microtubule polymeri-

sation, thus blocking the formation of mitotic spindle by these compounds, has become more and more widespread (Yemets and Blume, 2008; Îzheredov *et al.*, 2009). Oryzalin and trifluralin are the most commonly used representatives of dinitroanilines. Mode of action of these compounds is to block the polymerisation of microtubules (Britsun *et al.*, 2009). Results of several researchers suggest that effectiveness of oryzalin is higher than in the case of colchicine for *in vitro* polyploidisation of *M. × giganteus*. At the same time, oryzalin treatment leads to a decrease in number of regenerated polyploids, in comparison to colchicine treatment, which indicates a high level of phytotoxicity of the former (Yu *et al.*, 2009). Similar results were also obtained in studies of polyploidisation in other cereals (Wan *et al.*, 1991). These studies reveal low efficiency of classic dinitroaniline application for obtaining polyploids, which is associated with the high level of their phytotoxicity. Therefore, a promising aspect was screening and application of new dinitroanilines that are significantly less phytotoxic, compared to commonly used representatives of this class of compounds (oryzalin, trifluralin), but are not significantly inferior in their ability of blocking tubulin polymerisation (Îzheredov *et al.*, 2009; Îzheredov *et al.*, 2010). The accumulated knowledge on plant genomics and applying *in silico* methods allows mass screening of low molecular weight compounds with low phytotoxicity to their affinity to α -tubulin of a particular organism, in order to find the

most promising compounds for further application in *in vitro* induction of polyploidy in *M. × giganteus* (Melnychuk *et al.*, 2016).

The aim of the work was to develop an effective method of polyploidisation *in vitro* for *Miscanthus* genus representatives using promising antimitotic compounds of class dinitroaniline that have low phytotoxicity, to obtain polyploid lines of miscanthus for further application as raw biofuel material for breeding.

MATERIALS AND METHODS

Plant material. Aseptic *M. × giganteus* plants, variety Gulliver, were used in the experiments. Plant material was obtained from the collection of the Department of New Cultures of M. M. Hryshko National Botanical Garden, National Academy of Sciences of Ukraine.

Compounds for polyploidisation. Commercially available dinitroanilines — trifluralin (2,6-dinitro-*N,N*-dipropyl-1-4-(trifluoromethyl)aniline, $C_{13}H_{16}N_3F_3O_4$, *M* -335.5) and oryzalin (4-(Dipropylamino)-3,5-dinitrobenzenesulfonamide, $C_{12}H_{18}N_4O_6S$, *M* -346.36) were from Fluka (Switzerland). Other promising dinitroanilines used in the experiments (Table 1) were provided by the Institute of Organic Chemistry of the National

Table 1. List of tested newly synthesised antimitotic compounds used for polyploidisation of *M. × giganteus*

No	Chemical name	Formula	
1	4-methylsulfonyl-2,6-dinitroaniline	$C_7H_7N_3SO_6$	
2	N'-(N''-(2,6-dinitro-4-trifluoromethylphenyl)propyl)morpholine	$C_{14}H_{17}O_5N_4F_3$	
3	N,N'-bis-(2-nitro-phenyl)-hexylene-1,6-diamine	$C_{18}H_{22}N_4O_4$	
4	N N*1*-(2,6-dinitro-4-trifluoromethyl-phenyl)-ethylene-1,2-diamine hydrochloride	$C_9H_{10}ClF_3N_4O_4$	
5	1-1-{3-(2-(2,6-dinitro-4-trifluoromethyl-phenylamino)-ethyl)-4-methyl-2-phenylamino-2,3-dihydro-thiazole-5-yl}-ethanol hydrochloride	$C_{21}H_{19}ClF_3N_5O_5S$	
6	{2-(4-(2,4-dichlorophenyl)-2-phenylamino-thiazole-3-yl)-ethyl}-(2,6-dinitro-4-trifluoromethyl-phenyl)-amine hydrochloride	$C_{24}H_{17}Cl_3F_3N_5O_4S$	

Academy of Sciences of Ukraine. Dinitroaniline derivatives were dissolved in dimethyl sulfoxide (DMSO). Stock solutions were prepared at concentration 10 µM and stored at –20 °C. For the experiments, fresh drug solutions of various concentrations were prepared by dilution of stock solution with distilled water. Total concentration of DMSO in solutions did not exceed 0.5% (Robinson and Engelborghs, 1982).

In vitro culture establishment. Surface sterilisation of plant material was conducted in aseptic conditions. As explants, root adventitious buds with small fragments of rhizomes were used. Explants were taken from fresh roots that were thoroughly washed from soil. The main criteria for selection of explants were: optimal size of adventitious buds of rhizomes (0.3–1 cm depending on miscanthus type), viability of explants and absence of mechanical damage. The method involved pretreatment of explants with a 3% solution of sodium hypochlorite for 20 min with subsequent removal of dead tissues, rhizomes fragments and washing with sterile water. The pretreated material was sterilised in a 0.08% solution of silver nitrate (AgNO₃) for 20 min. with subsequent washing with sterile distilled water (Melnichuk *et al.*, 2015). After surface sterilisation explants were cultured on a solid MC medium (Murashige and Skoog, 1962) and supplemented with 30 g/l sucrose as a carbon source. The medium was supplemented with 50 mg/l cysteine to reduce phenolic compounds accumulation. All other variants of media used in the experiments also contained cysteine (Gubisova *et al.*, 2013). Cultivation conditions were: 16/8 h photoperiod and temperature 24 °C.

Micropropagation through direct morphogenesis. Solid medium MC was used to obtain a sufficient number of *in vitro* shoots by micropropagation. 6-benzyl aminopurine (BAP) in the amount of 2 mg/l was added to the medium to enhance formation of adventitious buds, shoot formation and shoot growth. Cultivation conditions were: 16/8 h photoperiod and temperature 24 °C.

Micropropagation through indirect morphogenesis. Induction of callusogenesis was conducted by cultivating shoots *in vitro* on solid MC medium, using 30 g/l sucrose as a carbon source. With the aim to determine the most effective ratio of auxins and cytokinins for callusogenesis, BAP (0.05 mg/l, 0.1 mg/l, 0.2 mg/l) and 2,4-dichlorophenoxyacetic acid (2,4 D) (2.5 mg/l, 5 mg/l) were added to nutrition media at various proportions. Adventitious buds from rhizomes and shoots *in vitro* obtained by micropropagation were used as explants. Cultivation was carried out in Petri dishes containing 30 ml medium. Explants 50–100 mm in length were placed without immersion in the medium, 5 per dish. Explants were cultured at 24 °C in the dark. Every 14 days, explants were transferred to fresh medium with an appropriate combination of growth regulators. For plantlet regeneration from callus, the explants were transferred to solid medium MC with addition of 2 mg/l BAP. As a carbon source 30 g/l sucrose was used. Cultivation conditions were: 16/8 h photoperiod and temperature 24 °C.

Polyploidisation *in vitro*. Polyploidisation of *M. × giganteus* was induced in two ways:

- Regeneration of plants from callus culture on media supplemented with antimetabolic agents at various concentrations (3.5 and 10 µM) for 7 and 14 days with subsequent transfer to media free of antimetabolic agents. Cultivation conditions were: 16/8 h photoperiod and temperature 24 °C.
- Cultivation of shoots *in vitro* was on micropropagation media supplemented with antimetabolic agents at various concentrations (3, 5, 10, 25 and 50 µM) for 7 and 14 days, followed by transfer to medium free of antimetabolics. Cultivation conditions were: 16/8 h photoperiod and temperature 24 °C.

Rooting, potting and transplanting to soil of obtaining lines. Rooting of obtained lines was conducted on solid MC medium with addition of 1 mg/l 1-naphthaleneacetic acid (NAA). Cultivation conditions were: 16/8 h photoperiod and temperature 24 °C (Gubisova *et al.*, 2013). After three weeks of cultivation, 3–10 well-developed roots appeared on the basal part of the shoots. Rooted shoots were washed under tap water to remove residues of medium and planted in cassettes for seedlings (77 cells), 45 × 45 × 62 mm in size, filled with a universal substrate for seedlings, which included peat, 1–6 mm in size, sand, perlite and microelements (Fe, Mn, B, Cu, Zn, Mo), pH 5.5–6.5. Planted individuals in soil were cultivated at temperature 18–25 °C with regular watering of the substrate.

Ploidy level determination. Determination of the ploidy level of obtained lines was carried out by counting chromosome number in root tips apical meristem. Material for microscopy was fixed for 18 h at 18–20 °C in Clark solution (96% ethanol and acetic acid, 3 : 1) and washed in 70% ethanol (Kundelchuk *et al.*, 2002). Specimens were macerated in 1N HCl and stained with 1% carmine in 45% acetic acid. Temporary specimens were prepared by squashing in a drop of 45% lactic acid (Pausheva, 1988). Chromosome counting was performed using a microscope Zeiss, Axioscop 40. Results were documented using a digital camera Zeiss, AxioCam MRC 5 and software Axivision Rel. 4.7.

RESULTS

In our experiments different methods of plant material treatments with dinitroanilines were studied to determine the most efficient method for polyploidisation. Cultivation of callus was conducted on regeneration media supplemented with antimetabolic compounds of various concentrations (3–10 µM) and of aseptic shoots on media for micropropagation with addition of antimetabolic compounds in different concentrations (3–50 µM) with durations 7 and 14 days, followed by transfer to free of antimetabolic compounds media.

To determine optimal combination of growth regulators for induction of callusogenesis in *M. × giganteus*, six different combinations of auxins and cytokinins were tested (Ta-

Table 2. Influence of growth regulators combinations on callusogenesis of *M. × giganteus* (the 30th day)

	Growth regulator	Concentration, mg/l	Survived, %	Explants with embryogenic and shoot forming callus, %	Explants with dark, unviable callus, %
1	BAP	0.05	82.1	12.5	29.7
	2,4-D	2.5			
2	BAP	0.1	86.7	6.0	32.0
	2,4-D	2.5			
3	BAP	0.2	85.9	8.1	31.2
	2,4-D	2.5			
4	BAP	0.05	92.2	34.6	27.2
	2,4-D	5.0			
5	BAP	0.1	87.7	61.6	34.8
	2,4-D	5.0			
6	BAP	0.2	92.7	31.4	48.3
	2,4-D	5.0			

ble 2). The resulting callus culture was assessed on the 30th day of cultivation. Number of explants with induced callus culture was determined. Obtained calli were estimated by colour and structure. Callus was evaluated visually distinguishing four types: white embryogenic callus; shoot-forming callus, in good condition; dark or yellow callus; dark brown, the last two are not viable callus types. According to the results of the study, the highest number of embryogenic callus and shoot-forming callus were induced on medium supplemented with 2,4D — 5 mg/l and BAP — 0.1 mg/l (Table 2). Similar results were obtained for both types of explants: adventitious buds from rhizomes and shoots *in vitro* obtained by micropropagation (Fig. 1).

After 16–18 days of cultivation, the first dedifferentiated tissue started to appear on explants, and after two months of cultivation, explants were completely covered with callus with a large number of embryo-like structures. The obtained callus was used for further regeneration and polyploidisation *in vitro* on solid MS medium with addition of dinitroanilines. Table 3 shows the results of plant regenera-

tion from callus on media with oryzalin and trifluralin. Concentrations of antimitotic drugs in the range of 3–10 µM resulted in significant reduction in survival of callus and, especially, in number of regenerated plants. Thus, after callus cultivation on medium with oryzalin, survival rates of explants were 6.7–20%, respectively. Callus cultivation on medium with trifluralin resulted in a 9.3–33.3 % survival rate of explants (Table 3).

Solid medium MS was used to inducing polyploidy by cultivation *in vitro* shoots. Shoots were cultured on media with dinitroanilines for 7 and 14 days, and then the shoots were transferred to test tubes with fresh medium free of antimitotic drugs (Fig. 2). Number of survived shoots was counted on the 30th day after beginning of cultivation. The obtained new shoots were counted, divided into separate lines and micropropagated. Subsequently, ploidy level of these lines was determined. Survival rates of *in vitro* shoots on media with dinitroanilines are presented in Table 4. Oryzalin and trifluralin had higher level of phytotoxicity compared to newly tested dinitroanilines. After *in vitro* shoot cultivation for 7 days on medium with oryzalin and trifluralin in concentration 10 µM, the survival rate of explants was 44.9 and 74.5%, respectively, and after increase of concentration up to 50 µM the survival rate was only 16.6 and 8.3%. At the same time, when new dinitroanilines were used in concentration 10 µM, survival rate of explants was 96.0–98.8%, and when the concentration increased up to 50 µM the rate was 83.3–91.6% (Table 4). Similar results were obtained after 14 days of cultivation on media with classic dinitroanilines. After *in vitro* shoot cultivation for 14 days on media supplemented with 10 µM of oryzalin and trifluralin, survival rates of explants were 21 and 54%, respectively, and after increase of concentration up to 50 µM, survival rate of explants was 8.3% for both. Newly tested dinitroanilines in concentration 10 µM showed a survival rate of explants within the range of 95.3–98.0% and at 50 µM — 83.3–91.6% (Table 5). Therefore, application of new dinitroanilines is more effective to produce polyploid plants of *M. × giganteus*.

Fig. 1. Callusogenesis induced from *in vitro* shoots (A) and buds (B, C) of *M. × giganteus*.

Table 3. Regeneration of *M. × giganteus* from callus on media with antimitotic compounds (first 7 days) on the 30th day of cultivation

Agent	Concentration, µM					
	3.0		5.0		10.0	
	regenerated, %	survived, %	regenerated, %	survived, %	survived, %	survived, %
Trifluralin	16.0	33.3	16.3	20.0	18.3	9.3
Oryzalin	22.6	20.0	22.7	11.0	23.3	6.7

Fig. 2. In vitro cultivation of *M. x giganteus* shoots on media with anti-mitotics agents.

Table 4. Survival of *M. x giganteus* explants after 7 days of cultivation on media with dinitroanilines (30 days)

Agent	Concentration, μM				
	3	5	10	25	50
Trifluralin	90.4	88.0	74.5	27.3	8.3
Oryzalin	89.0	85.7	44.9	25.0	16.6
#1 from Tab. 1	97.0	98.4	96.0	91.6	91.6
#2 from Tab. 1	98.8	98.6	98.0	91.6	83.3
#3 from Tab. 1	98.8	98.8	98.8	91.6	91.6
#4 from Tab. 1	98.5	96.8	96.4	91.6	85.7
#5 from Tab. 1	98.8	96.6	97.5	90.9	90.0
#6 from Tab. 1	98.8	98.5	97.0	91.6	90.9
Control	100.0	100.0	100.0	100.0	100.0

Table 5. Survival of *M. x giganteus* explants after 14 days of cultivation on media with dinitroanilines (30 days)

Agent	Concentration, μM				
	3	5	10	25	50
Trifluralin	88.0	68.5	54.0	16.6	8.3
Oryzalin	84.0	76.0	21.0	16.6	8.3
#1 from Tab. 1	97.1	96.7	95.3	91.6	91.6
#2 from Tab. 1	98.8	98.0	97.6	90.9	83.3
#3 from Tab. 1	98.8	98.8	98.0	84.6	85.7
#4 from Tab. 1	97.0	97.0	96.4	91.6	85.3
#5 from Tab. 1	98.0	96.8	97.4	90.0	90.0
#6 from Tab. 1	98.0	97.6	97.2	90.9	83.3
Control	100.0	100.0	100.0	100.0	100.0

Full plant regeneration obtained lines were transferred onto rooting medium and cultivated for 30 days. Plants with well developed root systems were transplanted under aseptic conditions for further acclimatisation and adaptation to open soil conditions (Figs. 3, 4) In order to determine ploidy level of the resulting lines separate roots were used for microscopic studies. Determination of ploidy level of obtained lines was carried out by counting of chromosome number in the apical meristem from root tips. As a result, lines with changed ploidy were obtained (Fig. 5). All substances used in the experiments proved to be able to induce polyploidy in *M. x giganteus*.

Fig. 3. Rooting of obtained lines *M. x giganteus*.

Fig. 4. Chromosome number in of *M. x giganteus* root apical meristematic cells:

A, control, 57 chromosomes; B, C, lines with 76 chromosomes; D, E, F, lines with 114 chromosomes.

Fig. 5. Polyploid *M. x giganteus* lines transplanted in soil: A, B, in pots; C, in open soil conditions.

Currently, analysis of selected *M. x giganteus* lines with changed ploidy level and study of their morphometric parameters is ongoing.

DISCUSSION

Today questions on induction of callusogenesis and plant regeneration from callus *M. x giganteus* are well studied (Petersen *et al.*, 2003; Plazek and Dubert, 2010; Wang *et al.*, 2011; Zhang *et al.*, 2012). There are many published results of callus induction from various types of explants, like shoot apices, immature inflorescences, primordia and frag-

ments of leaves. Despite seasonality, immature inflorescences are most often used for callus induction. The main advantage of this type of explants is that it is possible to obtain more than 200 explants from one immature inflorescence. In addition, there is a low percentage of explant contamination during *in vitro* culture establishment compared to other types of explants (Lewandowski, 1997). For induction of callusogenesis in maize, as well as in most cereals, immature embryo proved to be the best explant (Armstrong *et al.*, 1985; Rhodes *et al.*, 1986). Explant type plays an important role in the induction of morphogenic callus for different types of callus. Apex shoots for callusogenesis induction is considered to be less productive compared to use of immature inflorescences, but better in comparison with leaf fragments (Dalton, 2013). Moreover, several researchers indicated that smaller inflorescences produce more embryogenetic and shoot forming types of callus than other types (Holme and Petersen, 1996; Glowacka *et al.*, 2010a; Gubisova *et al.*, 2013). Mature seed is good explant for *in vitro* culture establishment and allows to obtain a sufficient amount of callus. Wang *et al.* (2011) successfully induced callus from seeds on MS medium supplemented with relatively high concentration (5 mg/l) of 2,4-D and a relatively low (0.01 mg/l) BA. This ratio of growth regulators allowed to obtain the highest percentage of compact callus (22.9%) compared with other variants (Wang *et al.*, 2011). However, this method can not be used to induce callusogenesis in *M. × giganteus* as a result of sterility of its seeds.

According to published data, in the case of callusogenesis induction from *M. sacchariflorus* shoot apices, 90% of induced callus were of embryogenic type, whereas when lateral buds were used — only 31%. However, when shoot apices of *M. × giganteus* were used as explants, only 17% of explants induced callus, and in the case of lateral buds — only 10% (Dalton *et al.*, 1999). Inducing of callusogenesis from large shoot tips of *M. × giganteus* (6–9 cm in length) resulted in appearance of morphogenic callus on 100% explants (Christian and Haase, 2001). It is well known that when rhizome fragments from soil and large shoot tips are used for *in vitro* culture establishment, a significant proportion of explants remains contaminated, which is the main obstacle for use of underground parts of miscanthus as explants (Lewandowski, 1997). Earlier we have reported about development of *in vitro* culture methods for *M. × giganteus*, which allowed use of rhizomes and large shoot tips for induction of callusogenesis (Melnychuk *et al.*, 2015).

It has been reported that 2–3 mg/l 2,4-D is sufficient for effective induction of embryogenetic callus from miscanthus explants (Christian and Haase, 2001; Zili *et al.*, 2004; Dalton, 2013). Moreover, a low concentration of auxins allows minimisation of somaclonal variations and does not affect further plant regeneration (Plazek *et al.*, 2007). According to the results of our study, the highest percent of white embryogenic and shoot forming callus types was observed on medium supplemented with 5 mg/l 2,4-D. Reducing 2,4-D concentration in nutrition medium caused a decrease in efficiency of callusogenesis and appearance of white embryo-

genic and shoot forming callus types were observed only on 8.1% of explants (Table 2). Similar results of induction of callusogenesis from *M. × giganteus* explants with addition to nutrition medium 5 mg/l of 2,4-D have been published by other authors (Holme and Petersen, 1996; Petersen, 1997). To induce callusogenesis in *M. × giganteus*, 0.1–2 mg/l BAP can be added to the medium. However, some miscanthus genotypes do not seem prone to dedifferentiate if BAP concentrations are higher than 0.01 mg/l (Dalton, 2013). According to our results, 0.1 mg/l BAP concentration appeared to be the most effective for obtaining white embryogenetic and shoot forming callus. Increase in concentration of BAP up to 0.2 mg/l caused formation of wilted, dark-yellow, nonviable callus at high per cent (48.3%), which was significantly higher than in the case of variant with 0.1 mg/l BAP, for which this parameter was 34.8% (Table 2).

For plant regeneration from callus, media supplemented with different combinations of growth regulators can be used. Most often for this purpose nutrition media based on MS with NAA or 2,4-D in combination with BAP or kinetin are applied (Holme and Petersen, 1996; Holme *et al.*, 1997; Zili *et al.*, 2004; Glowacka and Jezowski, 2009; Yu *et al.*, 2009; Zhang *et al.*, 2012). In our work, for plant regeneration from callus, as well as for micropropagation, media with 2 mg/l BAP were used. This amount of cytokinins provided best results. According to results of other researchers, effective shoot growth takes place on medium that contains 2–2.5 mg/l BAP (Petersen *et al.*, 2003; Glowacka *et al.*, 2010a). *In vitro* shoot cultivation on medium with 1–2 mg/l BAP leads to poor growth and reduction of plant viability in subsequent subcultures (Dalton, 2013).

Antimitotic compounds that block microtubule polymerisation and, consequently, formation of mitotic spindle, are able to induce polyploidy. The most commonly utilised are colchicine, oryzalin and trifluralin. At present, there is a significant number of publications on use of colchicine and oryzalin for *in vitro* polyploidisation of various miscanthus species (Petersen *et al.*, 2002; Petersen *et al.*, 2003; Yu *et al.*, 2009; Glowacka and Jezowski, 2009; Glowacka *et al.*, 2010b). After cultivation on liquid medium using both colchicine (939 µM) and oryzalin (10 µM), 67% of callus resulted in polyploids. The best results were obtained on liquid medium with 3.4 µM oryzalin and 4-day of cultivation of calli. Oryzalin appeared to be more effective than colchicine for polyploidy induction, but percentage of regenerated plants was lower than in the latter, 8.9% and 6%, respectively (Yu *et al.*, 2009).

Derivatives of dinitroanilines, like oryzalin and trifluralin, have previously been applied as pre-germination herbicides to suppress root system development by blocking microtubule polymerisation (Britsun *et al.*, 2009). Their advantage is that they are less toxic than colchicine. In spite of a high level of conservativity of tubulins of different origins, dinitroanilines have high affinity to plant tubulin (Morejohn and Fosket, 1991; Yemets and Blume, 1999) and weakly interact with fungal and animal tubulins (Banerjee *et al.*, 2007; Spasevska *et al.*, 2017). However, there is a problem

in application of most known dinitroanilines for inducing polyploidy, due to a high level of phytotoxicity. Numerous studies pointed out high efficiency as well as significant phytotoxicity of trifluralin and oryzalin in polyploidisation of both monocotyledonous and dicotyledonous plants. The high toxicity of both oryzalin and trifluralin was observed in polyploidisation of catnip (*Nepeta sp. L.*), where shoots were cultured on nutrient medium with dinitroanilines. Phytotoxic effect of both dinitroanilines on growth and development of explants was equally high (Zilbervarg *et al.*, 1997). The results of our studies on polyploidisation with oryzalin are in accordance with previously reported results, highlighting phytotoxicity of this compound. After callus cultivation on solid medium containing 10 μM oryzalin, the survival rate of explants did not exceed 6.7% and only 20.0% of them were able to regenerate as green plants (Table 3). Similar results were also obtained after cultivation of *in vitro* shoots for 14 days on media with 50 μM oryzalin and trifluralin. Survival rate of explants for both compounds was only 8.3% (Table 5).

Previously, in order to determine the phytotoxicity level and affinity to plant tubulins of more than 100 newly synthesised derivatives of dinitroaniline, a series of experiments were conducted by our group (Ozheredov *et al.*, 2009; Ozheredov *et al.*, 2010). Testing with *Allium* showed that phytotoxic effect of typical dinitroaniline compounds (trifluralin) appeared even at concentration 1 μM . Increase in concentration up to 10 μM was accompanied by significant increase in phytotoxicity and at concentration 100–1000 μM , partial or complete loss of experimental material was recorded. In our study on polyploidy induction of *M. $\times$ giganteus*, we used a concentration range of 3 to 50 μM , as the most potentially effective. Among the previously selected dinitroanilines, we conducted several studies using *in silico* methods for screening of compounds with high affinity to miscanthus α -tubulin and low phytotoxicity (Melnichuk *et al.*, 2016). The list of selected antimitotic substances used in this research is presented in Table 1.

Compounds selected *in silico* search showed significant reduction in phytotoxicity level compared to variants with trifluralin and oryzalin, which had a positive effect on the survival rate of treated explants. It should be noted that the survival rate of explants in treatments with newly synthesised dinitroanilines varied from 83.3% to 91.6%, which is significantly higher than in the case of such compounds like oryzalin and trifluralin (Table 5). Lines obtained after treatment with antimitotic compounds were cultured on rooting medium and after development of a root system the plants were potted and adapted to *in vivo* conditions with following planting in open soil. *In vitro* rooting of *M. $\times$ giganteus* effectively occurs on solid MC medium with 1 mg/l NAA (Gubisova *et al.*, 2013). Obtained roots with active growth were used to prepare squashed specimens in order to determine ploidy level of the lines by light microscopy methods. Currently, about 40 lines of *M. $\times$ giganteus* of changed ploidy level have been selected. The analysis of obtained material is still ongoing. Selected promising lines are being

acclimatised and planted in open soil conditions, where they are being analysed.

CONCLUSIONS

Effectiveness of new promising antimitotic compounds belonging to dinitroanilines with low phytotoxicity for polyploidisation of genus *Miscanthus* were studied. All compounds used in the work proved to be able to induce polyploidy in *M. $\times$ giganteus*. The most effective was their utilisation in concentrations ranging 10–50 μM . As a result of culturing *in vitro* shoots on media supplemented with new dinitroanilines, 27 hexaploid lines of *M. $\times$ giganteus* with 114 chromosomes were obtained. Selected lines were adapted to growth in open soil conditions. Currently, their biological and economic analysis is underway. Analysis of selected lines and study of their morphometric parameters are ongoing.

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POLIPLOĪDIJAS INDUCĒŠANA MILZU MISKANTEI (*MISCANTHUS* × *GIGANTEUS* GREEF ET DEU.)

Parādīta vairāku jaunu dinitroanilīnu antimitotiskā aktivitāte, kas ļauj efektīvi inducēt augu poliploidiju. Tiem ir zemāks fitotoksicitātes līmenis, salīdzinot ar savienojumiem, kuri tiek tradicionāli izmantoti šim nolūkam. Eksperimentā gaitā iegūtas poliploīdās milzu miskantes līnijas, kuras ir piemērotas audzēšanai atvērtās grunts apstākļos.