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## PECULIARITIES OF CREATION OF *MISCANTHUS SINENSIS* AND *MISCANTHUS SACCHARIFLORUS* TETRAPLOID LINES

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### ABSTRACT

The work aimed at creating tetraploid lines of *Miscanthus sinensis* and *Miscanthus sacchariflorus* species. To achieve this goal, we used methods of microclonal propagation, fluorescence cytophotometry, and genomic status differentiation using computer software of AP 'Partec' (Germany). It was found that the percentage of cultivated shoots of *Miscanthus sacchariflorus* for an exposure period of 1 day was  $18.86 \pm 5.37\%$ . To compare, it was  $51.78 \pm 6.51\%$  in *Miscanthus sinensis*. The best indicators of tetraploid induction in *Miscanthus sinensis* were observed for the exposure to colchicine for 2 days with polyploidization efficiency of 31.25% and 21.42%, and in *Miscanthus sacchariflorus* for 2 hours and 6 hours with rates of 35.0% and 27.3%, respectively. To stabilize the tetraploid level of genome ploidy, we used Murashige and Skoog liquid media (1962) supplemented with 0.005% colchicine and an exposure period of myxoploids for 6 hours. The flowering of new tetraploid clones in the conditions of Ukraine was observed on the second year of vegetation in late September and the beginning of October with the formation of fertile pollen grains. However, development of a microgametophyte depends on temperature conditions, both for *Miscanthus sinensis* (4x) and *Miscanthus sacchariflorus* (4x). Breeding schemes for the formation of anisoploid populations have also been developed: *M. sinensis* (4x) x *M. sinensis* (2x); *M. sacchariflorus* (4x) x *M. sinensis* (2x); *M. sinensis* (4x) x *M. sacchariflorus* (2x).

**Keywords:** Osmosis, Fruits, Preservation, Mass transfer kinetics, extend shelf life, dehydration

### INTRODUCTION

*Miscanthus* family involves about 12 species, among which the most valuable for biomass production are *M. sacchariflorus*, *M. sinensis*, *M. x giganteus*, and *M. floridulus* (Maksimovic *et al.*, 2014). In Europe, the most common is *M. x giganteus* which is an interspecific hybrid resulted from the natural hybridization of diploid *M. sinensis* ( $2x = 2x = 38$ ) and tetraploid *M. sacchariflorus* ( $2n = 3x = 76$ ) (Linde-Laursen, 1993).

Although *M. giganteus* has a number of advantages as the potential bioenergy crop of Europe and America, there are also some restrictions on its spread. Given that it was formed in a warm humid climate in southern Japan, its yield potential may not be fully revealed in different climatic conditions (Honda, 1939). In addition, *M. giganteus* roots badly in cold climates and northern latitudes (Long, 1983). In addition to the increased risk of disease, there are some hindrances in the breeding process associated with its triploid nature and incompatibility of chromosome conjugation (Yu *et al.*, 2009).

According to the results of molecular genetic research, it was found that the species formation in *Miscanthus* is a complex and dynamic process (Clark *et*

*al.*, 2015). Thus, gene introgression between diploid and tetraploid populations from diploids to tetraploids occurs due to unreduced gametes or chromosomal rearrangements (bridges) and can have major evolutionary consequences (Wang *et al.*, 2014).

Japanese researchers believe that natural hybridization between *Miscanthus* species is rare (Nishiwaki *et al.*, 2011). However, hybridization within sympatric populations is easy, unless there are reproductive barriers (Lee *et al.*, 2012).

New triploid hybrids *Miscanthus giganteus* can be a source of genetic variability for the introduction of disease resistance properties and high adaptive potential (Maksimovic *et al.*, 2014; Zub & Brancourt-Hulmel, 2010; Nishiwaki *et al.*, 2011). Plants of *Miscanthus* form practically unviable seeds; therefore, vegetative propagation by rhizomes, division of rhizomes is recognized as the most promising also in Ukraine (Roik and Kovalchuk, 2017).

### MATERIALS AND METHODS

The material for the creation of new tetraploid forms of *Miscanthus sinensis* and *Miscanthus sacchariflorus* were the following cultivars:

**Table 1.** The efficiency of obtaining tetraploids *M. sinensis* and *M. sacchariflorus* affected by the duration of exposure to colchicine 0.05%

| Starting material / year of exposure                               | Number of studied clones | Exposure time (hours per day) | Viable shoots (%) | Number of regenerated shoots | Ploidy of the obtained regenerants |    |        | Percentage of polyploidization |
|--------------------------------------------------------------------|--------------------------|-------------------------------|-------------------|------------------------------|------------------------------------|----|--------|--------------------------------|
|                                                                    |                          |                               |                   |                              | 2x                                 | 4x | 2x, 4x |                                |
| <i>M. sinensis</i> ecotype 1 'Poland', 2017 control*               | 125                      | 2 hour                        | 100 ± 0           | 39                           | 27                                 | -  | -      |                                |
|                                                                    |                          | 6 hour                        | 100 ± 0           | 25                           | 14                                 | -  | -      |                                |
|                                                                    |                          | 1 day                         | 100 ± 0           | 37                           | 29                                 | -  | -      |                                |
|                                                                    |                          | 2 days                        | 100 ± 0           | 57                           | 19                                 | -  | -      |                                |
| <i>M. sinensis</i> ecotype 1 'Poland', 2017                        | 197                      | 2 hour                        | 71.42 ± 9.85      | 20                           | 5                                  | 7  | 8      | 35.0                           |
|                                                                    |                          | 6 hour                        | 62.85 ± 8.16      | 28                           | 15                                 | 3  | 10     | 10.71                          |
|                                                                    |                          | 1 day                         | 18.86 ± 5.37      | 16                           | 12                                 | -  | 4      | -                              |
|                                                                    |                          | 2 days                        | -                 | -                            | -                                  | -  | -      | -                              |
|                                                                    |                          | 3 days                        | -                 | -                            | -                                  | -  | -      | -                              |
| <i>Miscanthus sinensis</i> new 'Germany' ecotype 2, 'Jelitto' 2018 | 175                      | 2 hour                        | 65.52 ± 8.82      | 22                           | 4                                  | 6  | 12     | 27.3                           |
|                                                                    |                          | 6 hour                        | 37.84 ± 7.9       | 13                           | 5                                  | 1  | 7      | 7.69                           |
|                                                                    |                          | 1 day                         | 14.71 ± 6.07      | 10                           | 7                                  | -  | 3      | -                              |
|                                                                    |                          | 2 days                        | -                 | -                            | -                                  | -  | -      | -                              |
|                                                                    |                          | 3 days                        | -                 | -                            | -                                  | -  | -      | -                              |
| <i>Miscanthus sacchariflorus</i> ecotype 1 'Poland' No.1 (2017)    | 184                      | 2 hour                        | 87.09 ± 6.02      | 26                           | 21                                 | -  | 5      | -                              |
|                                                                    |                          | 6 hour                        | 70.37 ± 8.73      | 21                           | 9                                  | 1  | 11     | 4.76                           |
|                                                                    |                          | 1 day                         | 51.78 ± 6.51      | 16                           | -                                  | 2  | 14     | 14 (28%)                       |
|                                                                    |                          | 2 days                        | 18.36 ± 5.57      | 14                           | 7                                  | 3  | 4      | 21:42                          |
|                                                                    |                          | 3 days                        | -                 | -                            | -                                  | -  | -      | -                              |
| <i>Miscanthus sacchariflorus</i> ecotype 1 'Poland' No. 2, (2018)  | 145                      | 2 hour                        | 88.0 ± 6.49       | 20                           | 17                                 | -  | 3      | -                              |
|                                                                    |                          | 6 hour                        | 82.17 ± 7.9       | 17                           | 6                                  | 1  | 10     | 8.8                            |
|                                                                    |                          | 1 day                         | 48.57 ± 8.4       | 22                           | 7                                  | 2  | 13     | 9.09                           |
|                                                                    |                          | 2 days                        | 39.13 ± 10.17     | 16                           | 1                                  | 5  | 10     | 31.25                          |
|                                                                    |                          | 3 days                        | 2.56 ± 5.26       | 2                            | 2                                  | -  | -      | -                              |

- *Miscanthus sinensis* ecotype 1 'Poland' 2x = 38;
- *Miscanthus sacchariflorus* ecotype 1 'Poland' 2x = 38;
- *Miscanthus sinensis* new 'Germany' ecotype 2 firm 'Jelitto' 2x = 38;
- *Miscanthus giganteus* (ecotype 1 'Poland', ecotype 2 'Austria', ecotype 3 'Great Britain' 3x = 57.

To determine the number of chromosomes we used the method of acetoorsein staining of seedlings obtained from seeds and underground rhizomes with a reduction of chromosomes of 0.03% 8-orthoxyquinoline and cold processing for 12 hours at a temperature of 4°C, described by Pausheva Z.P. (Pausheva, 1980) and modified for *Miscanthus*.

Liquid selective media supplemented with the composition of Murashige and Skoogmacro- and micro

salts were used for clonal micropropagation with the addition of sucrose 30,000 mg/l, BAP 0.2–0.5 mg/l, kinetin 0.2–0.5 mg/l, and gibberellin 0.1 mg/l.

For polyploidization *in vitro*, we used liquid selective media with colchicine, the percentage of which is 0.05%, and then incubated at 26 ± 1°C for 2 hours, 6 hours, 1 day, 2 days, and 3 days. The colchicine-free medium was used as a control.

After treatment, the shoots were washed with sterile distilled water and cultured on medium to restore growth processes according to the following recipe: ½ weight fraction of Murashige and Skoogmacro salts, sucrose 30,000 mg, mesoinoside 100 mg/l. After 14 days, the sprouts were planted in medium to regenerate clones and stimulate growth processes.

The experimental material after the action of colchicine was propagated for 3 passages in agar media with

**Table 2.** Rhizogenesis of tetraploid clones *Miscanthus sacchariflorus* and *Miscanthus sinensis*

| Source material                                              | Ploidy   | Experimental numbers           | Number of planted shoots | Of these rooted (%) |
|--------------------------------------------------------------|----------|--------------------------------|--------------------------|---------------------|
| <i>Miscanthus sinensis</i> ecotype 1 'Poland'                | (4x)     | 2/3-10-5, 2/3-10-3, 2 / 3-10-1 | 200                      | 40.7 ± 3.47         |
| <i>Miscanthus sinensis</i> new «Germany» ecotype 2 'Jelitto' | (4x, 2x) | 2/3-10, 2/1                    | 100                      | 31.5 ± 4.66         |
| <i>Miscanthus sacchariflorus</i> ecotype 1 'Poland'          | (4x)     | 3/8-4, 3 / 8-2, 3/3, 3/4       | 200                      | 91.5 ± 1.97         |
| <i>Miscanthus sinensis</i> ecotype 1 'Poland'                | (2x)     | M son 2                        | 50                       | 56.7 ± 7.01         |
| <i>Miscanthus sacchariflorus</i> ecotype 1 'Poland'          | (2x)     | Msac 4                         | 50                       | 33.4 ± 6.66         |

**Table 3.** Features of development of a male gametophyte of tetraploid forms *M. sinensis* (4x) and *M. sacchariflorus* (4x)

| The origin of the source material                   | Ploidy | Total pollen grain number | Of them (%) |             |              |
|-----------------------------------------------------|--------|---------------------------|-------------|-------------|--------------|
|                                                     |        |                           | sterile     | undeveloped | fertile      |
| <i>Miscanthus sinensis</i> ecotype 1 'Poland'       | 2x     | 2104                      | 8.61 ± 0.61 | -           | 79.99 ± 0.87 |
| <i>Miscanthus sinensis</i> ecotype 2 'Poland'       | 4X     | 1505                      | 56.5 ± 1.27 | 20.0 ± 1.03 | 13.5 ± 0.44  |
| <i>Miscanthus sacchariflorus</i> ecotype 1 'Poland' | 2x     | 2851                      | 9.54 ± 0.17 | -           | 90.46 ± 0.55 |
| <i>Miscanthus sacchariflorus</i> ecotype 'Poland'   | 4X     | 1015                      | 35.8 ± 1.48 | 30.7 ± 1.45 | 35.5 ± 1.5   |

the addition of hormones, biologically active substances, and sucrose to restore growth processes and selected for ploidy using AP 'Partec'. Replication of isolated tetraploid shoots was performed on nutrient media with the addition of 0.5 ml/l BAP, 30,000 mg/l sucrose, 0.1 mg/l gibberellin, and 0.15 mg/l kinetin.

Shoots regenerated and microclonally propagated after the action of colchicine were selected by morphological development of the leaves and transferred to the medium for rooting with the addition of phytohormones NAA 1 mg/l, IAA 0.2 mg/l, sucrose 20000 mg/l, agar 7.5 g/l, and activated carbon 0.3 mg/l.

The plants were grown in a culture room at a temperature of 27°C and a photoperiod of 16/8 hours. The roots were formed within 10–14 days. After that, the plants were acclimatized for 7 days in greenhouse conditions for transplanting. The plants were transplanted into a soil mixture with the following composition (per 100 kg of soil: ammonium nitrate (34%) 40–50 g, superphosphate (19%) 100–110 g, potassium salt (40%) 30–40 g.

Observation of the flowering period of diploid and new tetraploid clones of *M. sinensis* and *M. sacchariflorus* were performed using cytological methods, analysis of fertility and sterility of pollen grains.

## RESULTS AND DISCUSSION

Indicators of viability and efficiency of polyploidization for the two studied diploid species *M. sacchariflorus* and *M. sinensis* in comparison with *M. sinensis* ecotype 1 'Poland' (2x = 38), without the use of colchicine are presented in Table. 1

Among the propagated shoots, *M. sacchariflorus* ecotype 1 'Poland' showed greater viability after the action of colchicine. The percentage of regeneratively capable cultivated plants with an exposure period of 1 day was 51.78 ± 6.51 in 2017 and 48.57 ± 8.4 in 2018. In *M. sinensis*, under the exposure for 1 day, viable shoots were observed in different years 18.86 ± 5.37 and 14.71 ± 6.07. After selection by ploidy for 3 passages and identification of experimental material by the quantitative content of DNA of interphase nuclei, tetraploid and myxoploid forms were isolated and propagated *in vitro*.

After propagation and replication of shoots and re-testing them for ploidy, the best morphologically developed plants were transferred to the hormone-free Murashige and Skoog medium with the addition of mesoinoside 100 mg/l, sucrose 30,000 mg/l, and cultured for 7–10 days.

Indicators of efficiency of rhizogenesis of tetraploid and diploid clones *M. sacchariflorus* and *M. sinensis* are shown in Table 2.

Based on myxoploid shoots stabilized as tetraploids, genome ploidy in the conditions of liquid nutrient media in 2018, tetraploid plants of *M. sinensis* were isolated and rooted.

Analysis of quality indicators of the pollen-forming ability of tetraploid forms *M. sinensis* and *M. sacchariflorus* in comparison with diploid initial forms for the second year of vegetation are given in Table 3

According to the results of cytological studies, it was found that the morphological features of pollen grains in diploid plants *M. sacchariflorus* and *M. sinensis* did not

differ during the first and second years of growing. The percentage of fertile pollen in *M. sacchariflorus* (2x) was  $90.46 \pm 0.55\%$  and in *M. sinensis* (2x)  $79.99 \pm 0.87\%$  that was significantly higher compared to tetraploid forms with  $35.5 \pm 1.5\%$  and  $13.5 \pm 0.44\%$ , respectively.

## CONCLUSIONS

Selective media with colchicine 0.05% and sucrose content of 30,000 mg/l, BAP 0.2–0.5 mg/l, kinetin 0.2–0.5 mg/l, gibberellin 0.1 mg/l for induction of tetraploid biotechnological lines *Miscanthus sinensis* ( $2n = 4x = 76$ ) and *Miscanthus sacchariflorus* ( $2n = 4x = 76$ ) have been developed.

The best indicators of tetraploid induction in *Miscanthus sinensis* were observed for the exposure to colchicine of 2 days with the efficiency of polyploidization 31.25% and 21.42%. In *Miscanthus sacchariflorus*, these indices were 2 hours and 6 hours, 35.0% and 27.3%, respectively.

For the formation of anisoploid populations and hybridization of *Miscanthus* of different ploidy, it is necessary to carry out selection for the fertility and sterility of pollen grains.

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