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Original scientific paper

Propagation of miscantus plant by micropropagation procedure

Abstract
Miscanthus is a perennial grass native to East Asia. Although miscanthus primarily spread in Europe and North America for its use as an ornamental grass, growing this plant is financially demanding, resulting in a limited area of cultivation in Europe. The aim of this work is to describe in detail different micropropagation systems applied to the miscanthus plant. Miscanthus propagation is done vegetatively, using underground rhizomes or young plants grown by tissue culture, known as micropropagation. Micropropagation stands out as an alternative and extremely efficient method of reproduction, given the high rate of multiplication and the additional advantage of preventing disease transmission. It represents a method of vegetative reproduction that takes place on artificially created nutrient media, controlled growth conditions and in vitro sterile conditions. Research materials and methods were based on latent buds of three-year Miscanthus × giganteus rhizomes. A 70 percent ethanol solution and a 0.1 percent HgCl2 solution were used for sterilization. Micropropagation of the miscanthus plant is an important technique for breeding purposes in the future as it offers a diverse range of advantages compared to the limitations of common and natural reproductive barriers in conventional breeding techniques. The results of this research are based on the fact that the process ensures a quick process of obtaining a large number of plants that are identical in genetic potential, growth and development of the species. This paper is structured in such a way as to point out the importance of miscanthus production by micropropagation, its characteristics, current knowledge about this plant, the achievements, future and perspective of miscanthus micropropagation using the in vitro technique.
Key words: *in vitro, miscanthus, plant, micropropagation, propagation, reproduction, sterile conditions*

Introduction
In today's contemporary society, we are facing various challenges, including an energy crisis, concerns about food security, and environmental degradation and pollution. All these issues prompt the need to find alternatives to fossil fuels, whose use results in significant carbon dioxide emissions and adverse environmental impacts, leading to increased pollution. Plant-based biofuels can contribute to an overall reduction in greenhouse gas emissions by fixing atmospheric carbon in useful plant biomass (Zheng et al., 2021). Within this perspective, species of the Miscanthus genus stand out as exceptional candidates for sustainable biomass production, given their potential for high dry matter yields and adaptability to diverse climate conditions in Europe.

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The intensive cultivation of energy crops in Croatia does not have a long tradition. Clifton-Brown and colleagues (2017) state that Miscanthus is now considered one of the leading perennial energy grasses in Europe due to its ability to grow in various climatic conditions and high biomass yield potential. Current research is evaluating different hybrid Miscanthus species in various climate regions and soils, including marginal lands, with the aim of producing high-quality biomass for various energy purposes. Due to its C4 photosynthesis and high water use efficiency, Miscanthus shows exceptional potential in biomass production, making it a promising candidate for biofuel and fiber production in Europe.

The plant typically reproduces through rhizome division, but their production is very slow, and the success of establishing new crops is strongly influenced by the age of the parent plant (Lewandowski et al., 2018). Methods of Miscanthus propagation, such as vegetative propagation through underground rhizomes or tissue culture micropropagation, are currently being researched to optimize and enable commercial production. Despite the lack of well-established micropropagation methods for various Miscanthus species, it is believed that this grass will play a key role in sustainable agriculture in the near future. According to recent projections, Miscanthus is expected to meet 5 % of global energy needs by 2090, especially on 11 million square kilometers of marginal lands in the EU, where its cultivation is considered suitable due to high tolerance to abiotic stresses and water use efficiency. Morphological and molecular characterization of Miscanthus indicates a close relationship with other plants such as sugarcane and corn, emphasizing its diversity and adaptability to different climatic conditions (Ivanyshyn et al., 2018).

Currently, various hybrid varieties of Miscanthus are being examined and evaluated in different climatic regions and soils, including marginal lands less suitable for food production, with the ultimate goal of supplying high-quality biomass suitable for various purposes. Due to its C4 photosynthesis and high water use efficiency, Miscanthus tends to exhibit a very high potential in biomass production, making it highly suitable and promising for biofuel and fiber production in Europe. Miscanthus is propagated vegetatively through underground rhizomes or young plants grown by tissue culture (micropropagation).

There are currently no well-established or recommended micropropagation methods for different Miscanthus species suitable for commercial production, but it is believed that in the near future, Miscanthus grass will play an important role in sustainable agriculture. Recent projections anticipate that Miscanthus will meet 5 % of global energy needs by the 2090s. Eleven million square kilometers of marginal land in the EU is considered suitable for Miscanthus cultivation due to its high tolerance to abiotic stresses and the efficiency of water use in the C4 plant cycle. The morphological and molecular characterization of Miscanthus indicates its close relationship with sugarcane and corn (Ivanyshyn et al., 2018). Unlike sugarcane, corn, and other C4 plants, Miscanthus can thrive in cold climate conditions. Various studies report a varying number of species within the Miscanthus genus, ranging from 14 to 23 species (Lewandowski et al., 2018).

Micropropagation

Micropropagation of plants represents an integral process in which cells, tissues, or organs from selected plants are isolated, sterilized, and incubated in an aseptic environment to stimulate growth, with the aim of obtaining numerous cloned seedlings (Gupta et al., 2020). In specific cases, micropropagation stands out as a key technique that not only supports cultivation but also enhances the economic value of certain agricultural species. Plant mi-

cropropagation has proven essential in addressing numerous phytosanitary challenges, providing growers from various countries and economic environments access to high-quality plants. This technique has effectively contributed to the expansion of agriculture during this and the previous century, enabling the expansion and improvement of access to quality plants (Cardoso et al., 2018).

Types of miscanthus micropropagation

There are two key modalities of micropropagation: direct and indirect. The direct method, categorized as in vitro cultivation, involves the development of shoots from axillary nodes and apical meristems, with a particular emphasis on preserving genetic uniformity, resulting in individuals of identical genotypes and phenotypes. In contrast, indirect micropropagation methods focus on somatic embryogenesis through immature inflorescence cultures, providing an advantage for facilitating genetic transformation to enhance species characteristics. Micropropagation, a complex process, consists of a series of key steps. It is crucial to perform each step with precision to establish an in vitro culture. Achieving successful micropropagation requires going through five main phases, as highlighted by Gupta and colleagues (2020).

The zero phase involves the selection and preparation of the parent plant, while the first phase relates to establishing an aseptic culture. The second phase, the multiplication phase, focuses on increasing the number of cloned seedlings. The third phase, in vitro rooting, allows for root formation, while the fourth phase, acclimatization, prepares plants for transfer from laboratory conditions to the natural environment (ex vitro conditions). Within micropropagation, it is crucial to ensure high-quality explants, i.e., selected plant cells, tissues, or organs. Their careful manipulation after removal from the original plants is essential to avoid physical damage, contamination, or deterioration before reaching the laboratory. Technical aspects, including asepsis, cleanroom technology, disinfection and sterilization, and environmental conditions such as temperature, relative humidity, and light, play a crucial role in the successful implementation of micropropagation procedures.

Advantages and disadvantages of micropropagation

Micropropagation stands as a significant leap forward in enhancing the quality, quantity, and economic efficiency of plant reproduction compared to traditional vegetative methods. This technique offers numerous benefits for cultivation interests, facilitating the preservation of genetic uniformity and the attainment of desired phenotypic characteristics. However, despite these advantages, micropropagation comes with its specific drawbacks and substantial gaps in understanding the fundamental mechanisms of plant tissues, necessitating a deeper comprehension of how plant tissue culture functions, or not, and why. The incomplete understanding of the basics of micropropagation, encompassing its specificities, efficacy, and limitations, poses a significant challenge in the broader promotion of plant biotechnologies. The growing awareness of these challenges encourages further exploration of improved methods and more favorable outcomes in the context of the commercial application of micropropagation. This research plays a pivotal role in optimizing micropropagation procedures with the aim of enhancing productivity and the broader applicability of this technology in the field of plant biotechnology.

<u>Advantages:</u> The production of a substantial number of clonal propagules in a relatively short time through micropropagation is a pivotal aspect compared to the same plant species propagated through conventional techniques, as emphasized by Gupta and colleagues

(2020). The primary justifications for the application of micropropagation and in vitro techniques lie in their ability to provide essential tools for manipulating explants and reproductive material without the presence of pathogens. In the context of commercial micropropagation, the crucial capability is the precise control and consistent similarity between the characteristics of the original plant and the resulting plant product. This consistency encompasses various aspects such as size, shape, flower color, the presence and concentration of metabolites, and other relevant characteristics. The integration of plant micropropagation techniques into new technologies often serves as an effective means of addressing issues in horticulture and in the fields of agriculture, chemistry, medicine, and pharmacy. One of the key advantages of micropropagation, as emphasized, lies in its ability to provide rapid, efficient, and relatively economical production of large quantities of plant material. This effectively reduces or eliminates the physical distance between research and development centers and laboratories, contributing to the acceleration of material delivery and the optimization of research efforts.

Shortcomings: Micropropagation in the context of commercial application on a large scale encompasses a wide range of issues that significantly impact the economic feasibility of this process. Recognized problems include frequent mutations, limited understanding of organogenesis or embryogenesis, internal infections, vitrification, toxic exudates, increased levels of ethylene and CO2, neglect of the role of physical growth factors (light, temperature, humidity, and gaseous phases), losses during the transition from in vitro to acclimatization phase, labor costs, and limited mechanization. Many newly developed techniques are often not economically viable, while commercial production is frequently inadequately controlled. Significant economic losses, both direct and indirect, in commercial micropropagation often result from endogenous and environmentally induced contamination of plant cultures. Large losses occur during the transfer of plant material from Phase III to Phase IV, i.e., during the acclimatization phase.

Key success and sustainability criteria for commercial micropropagation include not only the multiplication coefficient but also the plant's ability to successfully adapt during the transition from the heterotrophic/mixotrophic in vitro system to the photoautotrophic state in the greenhouse and field. Advanced technology, infrastructure, and operational costs, coupled with the labor-intensive nature of mikropropagation, often make this method economically unfeasible. The costs arising from these challenges are often unsustainable for certain plant species, varieties, products, or markets, necessitating further research and improvement of methods to address these issues and enhance the economic viability of commercial.

Materials and methods

Morphogenesis of miscanthus x giganteus in vitro/klyachenko

This study aimed to compare various micropropagation methods to determine the most favorable technique for propagating Miscanthus. Numerous authors have studied and developed new biotechnological methods for the reproduction of Miscanthus and the development of novel starting forms to enhance genetic variability. The first detailed information on in vitro cultures based on callus production in Miscanthus x giganteus was published by Lewandowski (1992.) and Lewandowski and Kahnt (1993. a, b, c). The initial results of in vitro micropropagation were presented by Moller-Nielsen et al. (1993). In this work, a comparison was made with studies on the micropropagation of morphogenesis in Miscanthus x giganteus, including the

research conducted by Klyachenko et al. (2018.) on the regeneration of Miscanthus giganteus plants, emphasizing the effects of callus types, age, and cultivation methods on regeneration capacity. Additionally, the effective and simple in vitro regeneration system for Miscanthus sinensis, M. × giganteus, and M. sacchariflorus for planting and biotechnological purposes, as investigated by Ślusarkiewicz-Jarzina et al. (2017.), was analyzed.

This research was conducted to compare various micropropagation methods to determine the most favorable technique for propagating Miscanthus. The authors explore different biotechnological approaches to Miscanthus reproduction and develop new starting forms to enhance genetic variability. The first detailed information on in vitro cultures based on callus production in Miscanthus x giganteus was published by Lewandowski (1992.) and Lewandowski and Kahnt (1993. a, b, c). Moller-Nielsen et al. (1993.) were the first to present results of micropropagation through in vitro cultivation. Within this study, a comparison is made of the results of the micropropagation of morphogenesis in Miscanthus x giganteus, analyzing the studies by Klychenko et al. (2018.) on the regeneration of Miscanthus giganteus plants with a focus on the influence of callus types, age, and cultivation methods on regeneration capacity. Additionally, the effective and straightforward in vitro regeneration system for Miscanthus sinensis, M. × giganteus, and M. sacchariflorus for planting and biotechnological purposes, as investigated by Ślusarkiewicz-Jarzina et al. (2017.), is analyzed. This approach allows for a comprehensive overview and critical analysis of various studies investigating Miscanthus micropropagation from different perspectives, including genetic variability, plant regeneration, and the application of in vitro systems for biotechnological purposes.

This study utilized latent buds from three-year-old rhizomes of Miscanthus × giganteus as the research material. The explant sterilization procedure involved the application of a 70 % ethanol solution and a 0.1 % HgCl2 solution. The explants underwent sterilization for 1.5 minutes in a 70 % ethanol solution, followed by 22 minutes in a 0.1 % mercuric chloride (HgCl2) solution, with subsequent rinsing in three changes of water lasting 7 to 10 minutes. Sterile explants were then transferred to test tubes containing 10 ml of Murashige and Skoog (MS) hormone-free medium. Subsequently, they were transferred to a modified MS culture medium containing BAP8 (0.2 mg/l) + sucrose (30 mg/l) or a combination of BAP (0.75 mg/l) and kinetin (1.2 mg/l) + sucrose (30 mg/l). Miscanthus shoots were rooted in MS medium with half the concentration of macro- and microelements and without the addition of growth regulators.

The explants were cultivated in a controlled culture room at a temperature of 23–25 °C, relative humidity of 60–70 %, and illumination of 3000 lx, with a photoperiod of 16 hours. Subsequently, the plants were acclimated to in vivo conditions in a growth chamber using various soil mixtures, including peat:sand:perlite (2:1:1), peat:sand (2:1), and peat:perlite:-soil (2:2:1), at a temperature of 24–25 °C, relative humidity of 70–80 %, light intensity of 1500 lx, and a photoperiod of 16 hours. The aim of the research was to obtain a significant quantity of planting material of Miscanthus × giganteus to study the peculiarities of morphogenesis in isolated meristem cultures. This methodological approach allows for systematic investigation into plant development and creates conditions that stimulate the specificities of morphogenesis in isolated meristem cultures within the context of research goals.

Plant regeneration in miscanthus x giganteus: effect of callus types, age, and cultivation methods/kim

In this study, tissues from immature inflorescences approximately 5-20 mm in length were collected from greenhouse-grown Miscanthus giganteus plants and sterilized by immersion

in a 0.5 % NaOCl solution for 3 minutes. The sterilized explants, cut into 5 to 7 mm segments, were placed on callus induction medium (M1BA). This medium consisted of MS basal salts and MS vitamins according to Murashige & Skoog (1962), supplemented with 2,4-D (13.6 µM), BA (0.44 µM), 2.88 g L^-1 proline, 30 g L^-1 sucrose, and 750 mg L^-1 MgCl2·6H2O, following Petersen (1997). All media contained 2 g L^-1 Phytagel (Sigma-Aldrich, St. Louis, MO, USA) and were adjusted to pH 5.5 before autoclaving. Each callus induction medium (M1BA) was set with 15 explants, totaling 15 repetitions. Cultures were incubated in the dark at a temperature of 27 ± 2 °C and subcultured weekly during the first 2 weeks, followed by bi-weekly intervals for the next 4 weeks.

After six weeks from the start of the culture, the total number of induced calli was recorded to calculate the percentage of callus induction. Different types of calli were classified based on visual appearance, and the count for each type was documented. Callus classification followed the guidelines of Lewandowski (1997), Holme and Petersen (1996), and Petersen (1997) for assessing Miscanthus giganteus calli. A callus unit was defined as a callus with a diameter of 2 mm. The percentage of each type of callus was calculated as follows: percentage of callus type = (number of each callus type/total number of calli) x 100. This approach allows for a systematic analysis of callus induction and the classification of different callus types, contributing to an overall understanding of morphogenesis under experimental conditions.

Efficient and simple in vitro regeneration system for miscanthus sinensis, m. × giganteus, and m. sacchariflorus for planting and biotechnological purposes by ślusarkiewicz

In this study, the material for investigation originated from three Miscanthus species, encompassing a total of six forms selected from a well-established field collection of the Institute of Plant Genetics of the Polish Academy of Sciences. In vitro cultures were initiated from explants collected during June and August in the early stage of inflorescence development of field-grown plants. Explants, composed of distal segments of shoots measuring 10-20 cm, including inflorescences in the developmental stage within leaf sheaths, were carefully cleaned and sterilized in a 5 % calcium hypochlorite solution for 10 minutes, followed by thorough rinsing with sterile water. Three different types of explants were isolated after removing the leaf sheaths.

In the first experiment, all types of explants were cultivated on MS basal medium supplemented with 30 gL−1 sucrose, 5.0 mg L−1 2,4-D + 0.5 mg L−1 BAP, while plants from induced calli were regenerated on MS medium with sucrose and 2.0 mg L−1 BAP. In the second experiment, the explants selected in the first trial were grown on MS medium with 30 gL−1 sucrose or modified C17 medium with sucrose replaced by 90 gL−1 maltose. The media were supplemented with different combinations of growth regulators. In the third experiment, calli were induced from explants selected in the first trial on the medium established in the second experiment, while seedlings were regenerated on various media with the addition of 30 gL−1 sucrose and different combinations of growth regulators. All used media were adjusted to pH 5.7 and gelled with 0.7 % agar. Callus induction was conducted at 24 °C in the dark for 2 months, after which calli were excised from the original explants and transferred to the regeneration medium. Continuous cultivation, including seedling development, growth, and rooting, took place at 22 °C in a 16-hour photoperiod. This approach enables a systematic analysis of different culture phases, contributing to an overall understanding of morphogenesis under experimental conditions.

Results and discussion

In vitro morphogenesis of miscanthus × giganteus/klyachenko

The study involved the analysis of the influence of different culture media on the development of meristematic cultures of Miscanthus x giganteus. In the process of sterilizing latent buds, the highest sterility percentage (70 %) was achieved by consistent treatment in a 70 % ethanol solution (1.5 min) followed by transfer to a 0.1 % HgCl2 solution (22 min) and triple rinsing in sterile distilled water. After extracting latent buds, an increase in their size was observed from the 5th to the 6th day after planting in the culture medium. From the 8th to the 10th day, they were transferred to the MSR1 and MSR2 culture media to evaluate the efficiency of the selected media. Systematic measurements of morphometric indices, including shoot length, number of shoots, and frequency of multiple shoots, were conducted over a period of 30 days to obtain quantitative data on the development of meristematic cultures of Miscanthus x giganteus. Details regarding the impact of substrate composition on the mentioned parameters are presented in Table 1. These results provide insights into the optimal culture conditions supporting the development of meristematic cultures of Miscanthus x giganteus (Klyachenko et al., 2018).

Table 1. Influence of Substrate Composition on the Development of Meristematic Cultures of Miscanthus × giganteus
Tablica 1. Utjecaj sastava podloge na razvoj meristemskih kultura Miscanthus x giganteus

Broj medija kulture/ Culture Media	Sastav medija/ Composition	Dužina klice, mm / Shoot Length (mm)	Broj klica/ Number of Shoots	Koeficijent reprodukcije/ Replication Coefficient
MSR1	MS + BAP (0.2 mg/l) + saharoza (30 mg/l) MS + BAP (0.75 mg/l) and kinetin (1.2 mg/l) + saharoza (30 mg/l)	4.6 ± 0.9	2.8 ± 0.5	4.6 ± 1.1
MSR2	MS + BAP (0.75 mg/l) and kinetin (1.2 mg/l) + saharoza (30 mg/l)	13.3 ± 1.0	5.2 ± 0.6	11.4 ± 1.2

Izvor/Source: Autor

In the first three weeks of cultivation, the regeneration processes in isolated miscanthus plants exhibited the highest activity in the presence of an increased concentration of BAP and kinetin. Kinetin (1.2 mg/l) particularly stimulated the formation of buds and additional shoots. This phenomenon aligns with scientific findings indicating the ability of specific cytokinins, such as kinetin, to suppress apical dominance and promote shoot development from axillary buds. In this study, the addition of BAP (0.75 mg/l) resulted in the active formation of 3-7 additional shoots in the 4th-5th week of cultivation. It is noteworthy that sucrose (30 mg/l) in this growth stage of isolated explants demonstrated favorable effects as a carbohydrate source. The research identified MSR2 as the optimal culture medium for microshoot regeneration, supplemented with BAP (0.75 mg/l) and kinetin (1.2 mg/l). In this context, the frequency of shoot regeneration reached high values of 90.0–100.0 %, encompassing the development of the main shoot and multiple branching with a frequency of 85.0–100.0 % (Figure 1). These results underscore the importance of precise selection of culture medium components to achieve an optimal microshoot regeneration process in miscanthus.

Figure 1: Cultivation of miscanthus shoots on substrates of different compositions (a- MSR1, b- MSR2)
Slika 1: Uzgoj izdanaka miskantusa na supstratima različitog sastava (a- MSR1, b- MSR2)
(Source/Izvor: Klyachenko et al., 2018)

It is emphasized that the process of rhizogenesis is of significant importance. In in vitro conditions, the rooting of Miscanthus × giganteus shoots depended on their size and the number of passages conducted. Shoots measuring 5 – 6 cm (aged 8 – 9 weeks) were transferred to MS culture medium with half the dose of macro- and micronutrients, without added growth regulators. Miscanthus × giganteus plants demonstrated their ability for normal development in this culture medium, with the formation of the root system observed between the 8th and 11th day of cultivation (Figure 2). These results highlight the importance of a proper rooting procedure for the successful micropropagation of Miscanthus × giganteus under in vitro conditions.

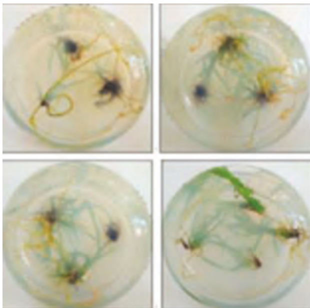

Figure 2: Rhizogenesis in Miscanthus x giganteus
Slika 2: Rizogeneza Miscanthus x giganteus. *(Source/Izvor: Klyachenko et al., 2018)*

Table 2. Rooting of Miscanthus x giganteus in vitro
Tablica 2. Ukorjenjivanje Miscanthus x giganteus in vitro

Dan Day	Prosječan broj korijena po biljci/ Average Number of Roots per Plant	Dužina klice, mm/ Average Root Length (cm)
10	6.3 ± 0.3	1.5 ± 0.07
20	10.5 ± 0.5	4.3 ± 0.2
30	14.1 ± 0.7	9.6 ± 0.5
10	6.3 ± 0.3	1.5 ± 0.07

Laboratory-grown Miscanthus × giganteus plants, aged 12 - 14 weeks (depending on the rooting substrate cultivation period), demonstrated the ability to continue successful growth in greenhouse conditions. Regenerated plants, measuring 5 to 7 cm in height with well-developed root systems, were carefully removed with tweezers. After thorough rinsing of the roots to prevent rotting and decay, the plants were transplanted into different substrates. It is evident that substrate number 1 proved to be the most effective for Miscanthus × giganteus, as it promoted rapid rooting, shoot growth, and development, unlike substrates number 2 and 3. Four weeks after transplantation, 3 - 4 leaves and a milky root system had

formed on the plants. In these conditions, the survival rate of microplants ranged between 91 % and 95 %. These results indicate a successful Miscanthus × giganteus micropropagation process in laboratory conditions, with a promising survival rate and development after transplantation in the greenhouse.

Regeneration of miscanthus giganteus plants: the effect of callus types, age and cultivation methods on the ability to regenerate / kim

The explants of immature inflorescences showed the initiation of callus formation approximately 2 weeks after cultivation on M1BA medium containing 13.6 mM 2,4-D and 0.44 mM BA, with a frequency of 78 %. After 6 weeks of culture initiation, three distinct types of callus were identified (depicted in Figure 3a-c). This result indicates heterogeneity in the callus formation process, where different morphological types of callus developed during the culture period.

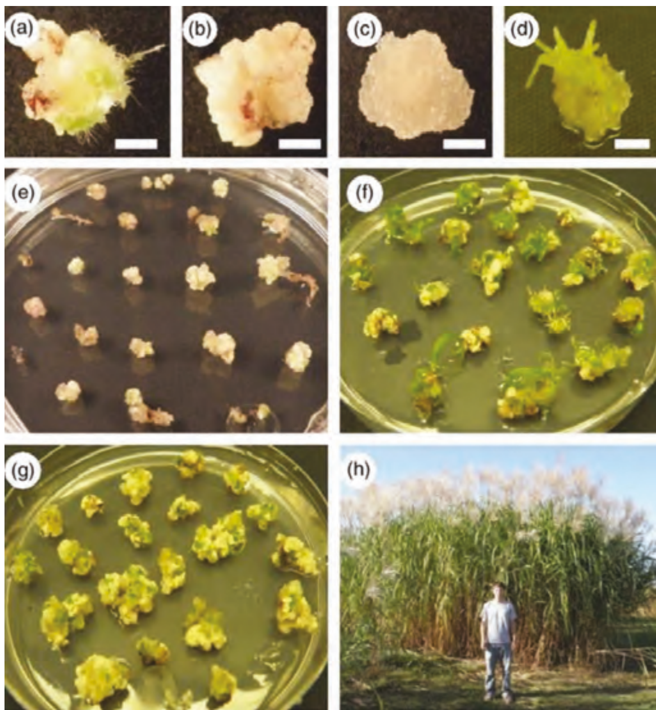

Figure 3: Plant regeneration from miscanthus callus
Slika 3: Regeneracija biljke iz kalusa miskantusa
(Source/Izvor: Kim et al., 2010.)

The dominant type of callus, constituting 41 ± 4 % of the total, was a yellow or white compact callus with pronounced light green structures, indicating callus formation with shoot development (shown in Figure 3a). Other types of callus included a compact, white callus similar to the embryogenic type (22 ± 2.1 %, shown in Figure 3b) and a soft, friable callus (37 ± 3.7 %, shown in Figure 3c). After continuous subculture of the shoot-forming callus on a solid induction medium, most calli changed color to brown.

Maintenance of shoot-forming callus on a solid medium with only 2,4-D mostly resulted in a yellowish and semi-soft callus with a small portion of embryogenic callus. Shoot-forming callus maintained in a liquid medium also turned into a yellowish and semi-soft callus, and most then formed roots on the regeneration medium (shown in Figure 3d). The best results were achieved

with one-month-old shoots forming shoots. After one month of subculture on a solid callus maintenance medium, the estimated frequency of shoot-forming callus regeneration dropped to 0.3 and 0.1 plantlets per callus on the MR1-1 and MR1-2 regeneration media. Experimental data indicate that the types and age of calli induced from immature flower tissues are crucial factors influencing the efficiency of plantlet regeneration in Miscanthus giganteus. Calli forming shoots, induced from immature flower tissues, showed the highest regeneration frequency in this experiment. Plant regeneration was recorded to a greater extent using calli forming shoots induced from leaves and shoot apices of Miscanthus giganteus. The combination of 2,4-D and BA in the callus induction medium proved effective in improving plant regeneration, as well as the induction frequency of shoot-forming callus in M. giganteus. Preliminary experiments on the regeneration of callus induced from immature flowers with or without BA also showed higher frequencies of regeneration from calli induced by 2,4-D and BA compared to callus induced by 2,4-D alone. The loss of regeneration ability with increasing callus age poses a critical problem for the maintenance and regeneration of callus in M. giganteus, as observed in other grass species as well. Previous experiments by Holme et al. (1997) established a suspension culture system for maintaining embryogenic callus cultures in M. giganteus, achieving high regeneration efficiency from suspension aggregates aged 18 months. These experiments confirm that the regenerative capacity of callus cultures with embryogenic characteristics can be maintained over an extended culture period using a suspension culture. Furthermore, it was estimated that two auxin-like growth regulators, NAA or 2,4-D, influence the efficiency of shoot-forming callus regeneration. The combination of NAA and BA in the regeneration medium (MR1-1 medium) stimulated faster shoot regeneration and growth from shoot-forming callus compared to the combination of 2,4-D and BA (MR1-2 medium) (visible in Table 3). The frequency of shoot-forming callus regeneration on the MR-1 medium was significantly higher than the frequency with the combination of 2,4-D and BA.

Table 3: Effect of two different media for regeneration (MR1-1 and MR2-2) on rapid regeneration from 6-week-old callus T / **Tablica 3:** Učinak dva različita medija za regeneraciju (MR1-1 i MR2-2) na brzu regeneraciju iz kalusa starog 6 tjedana

After 1 month of incubation*,§	**After 2 months of incubationw,§**	**Total frequency z,§**	**After 1 month of incubation*,§**
MR1-1 (1.3 mM NAA i 22 mM BA)	0.84 ± 0.8a	0.84 ± 0.8a	0.84 ± 0.8a
MR1-2 (4.5 mM 2,4-D i 22 mM BA)	0.29 ± 0.05b	0.29 ± 0.05b	0.29 ± 0.05b

The frequency of regeneration obtained from the MR1-2 medium during the first month of incubation was significantly lower compared to the MR1-1 medium, while the total number of regenerated plants after 2 months of incubation on the regeneration medium was significantly higher on the MR1-1 medium than on the MR1-2 medium. Most regenerated seedlings (69 % of the total number of regenerated seedlings) appeared within the first month of incubation on the MR1-1 medium, while on the MR1-2 medium during the same period, only 30 % of the total number of regenerated seedlings were produced. Calli forming shoots on the MR1-2 medium developed many green spots, indicating the initiation of callus differentiation with several small multiple shoots on the callus surface over 3 weeks (Figure 3g),

but the growth of differentiated shoots was relatively slow compared to regenerated shoots on the MR1-1 medium (Figure 3f). Fine selection and maintenance of compact, white nodular callus type seem to be an important factor influencing the efficiency of regeneration in cultures with embryogenic characteristics. Regenerated plants grown in the field showed normal phenotypic development, with plant heights and stem diameters comparable to rhizome-propagated plants in our experiments. Planting mature rhizomes grown for approximately 3 months in a greenhouse or outdoors without division can accelerate the growth and development of regenerated Miscanthus giganteus plants.

Efficient and simple in vitro regeneration system of miscanthus sinensis, m.× giganteus and m. sacchariflorus for planting and biotechnological purposes / ślusarkiewicz
Embryogenic callus was successfully induced from all types of explants of six different genotypes of Miscanthus, as tested in Experiment I. The somatic embryogenesis process was initiated in the very early stages of callus growth, where globular and polarized embryos began to form around 3-4 weeks after transferring the explants to the callus induction medium (Figure 4a). The development of somatic embryos was grouped, forming various bipolar structures with a single layer of epidermal cells loosely connected to the surface of the callus tissue (Figure 4b).

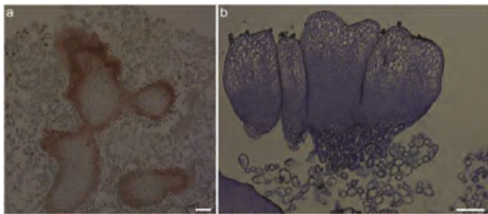

Figure 4a and b.: Histological development of a somatic embryo in a callus from a young flower of M. sinensis genotype 17 after 1 month of culture / **Slika 4a i b.:** Histološki razvoj somatskog embrija u kalusu iz mladog cvijeta M. sinensis genotipa 17 nakon 1 mjeseca kulture *(Source/Izvor: Ślusarkiewicz-Jarzina et al., 2017.)*

The structure and appearance of the callus varied depending on the type of explant, with some variations also related to the genotype. Callus derived from entire immature inflorescences, regardless of the Miscanthus genotype, developed as yellowish and firm nodules, with the presence of soft parts and small white compact clusters (see Figure 5a – d). Explants obtained from the axis of fluorescence, especially genotype MsacR, also resulted in the formation of yellowish and nodular callus with white clusters, but the callus was more brittle and partially rhizogenic. After transfer to light conditions, embryogenic structures turned green and continued to develop into plantlets (see Figure 5e – f).

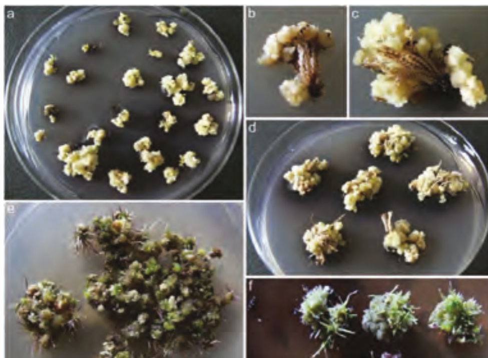

Figure 5: Callus induction of different miscanthus genotypes / **Slika 5:** Indukcija kalusa različitih genotipova miskantusa *(Source/Izvor: Ślusarkiewicz-Jarzina et al., 2017.)*

Regeneration of plantlets from the remaining callus was not observed, and it was found that genotype and type of explant significantly influenced callus induction and subsequent plant regeneration. The height of entire inflorescences varied, with nodes being the lowest. It was determined that plant regeneration depends on the origin of the callus, with the highest regeneration percentages recorded for callus formed from entire immature inflorescences followed by axis explants (insignificant difference). Nodes generally resulted in unproductive explants, except in genotypes Ms16 and Mxg4. An exception was the genotype MsacR, where the results indicated a similar regeneration percentage, but different genotypes behaved differently in callus formation. Experiment II focused on optimizing callus induction media and its impact on later plant development. Twelve variants were compared, containing two basal media, MS and C17, with six different combinations of growth regulators. Despite a decline in both processes compared to the analogous variant of Experiment I, similar patterns were observed in both processes. Miscanthus genotype, along with the substrate and growth regulators, significantly influenced callus induction and plant regeneration. Among the growth regulators, a medium concentration of 2,4-D (5.0 mg/l) proved to be the most effective in promoting callus growth in both media, while BAP showed higher activity than KIN (Table 4).

Table 4: Effect of medium and combination of growth regulators on callus induction rate (CIR) of selected miscanthus genotypes and explants / **Tablica 4:** Učinak medija i kombinacije regulatora rasta na stopu indukcije kalusa (CIR) odabranih genotipova miskantusa i eksplantata

Type and genotype	**M. sinensis**		**M. × giganteus**		**M. sacchariflorus**		
Growth regulators [mg l−1]	**Medium**						
	MS	C17	MS	C17	MS	dC17	
4.0 2,4-D + 0.5 BAP	112.2 cd	237.4 b	123.8 d	246.7 b	135.6 c	178.9 bc	172.4 c
5.0 2,4-D + 0.5 BAP	230.0 a	390.0 a	259.0 a	317.9 a	192.0 a	200.0 a	264.8 a
6.0 2,4-D + 0.5 BAP	201.7 b	371.2 a	145.0 c	288.0 a	154.8 b	187.5 ab	224.7 b
4.0 2,4-D + 0.5 KIN	101.6 d	156.9 d	112.7 d	164.3 d	117.3 d	156.3 d	134.9 d
5.0 2,4-D + 0.5 KIN	123.7 c	190.2 c	198.1 b	197.2 c	127.4 cd	176.6 bc	168.9 c
6.0 2,4-D + 0.5 KIN	109.8 cd	178.0 cd	180.2 b	179.1 cd	119.8 d	170.2 c	156.2 cd
	146.5 c	254.0 a	169.8 bc	232.2 a	141.2 c	178.3 b	

The optimal combination of 2,4-D and BAP for callus induction showed a positive impact on subsequent plant regeneration, although not significant. However, using the C17 medium instead of MS had a synergistic and significantly positive effect on callus induction, which also indirectly influenced plant regeneration. This study highlighted that the efficiency of embryogenic callus induction is crucial for the entire process of Miscanthus regeneration. Diversity in callus induction efficiency was observed among Miscanthus species and genotypes, where M. sinensis and M. × giganteus demonstrated a higher morphogenetic capacity than M. sacchariflorus. Overall, the study results reflect similar patterns to other reports that have mainly investigated M. × giganteus or M. sinensis, with variations in callus formation capacity among species and genotypes. In this experiment, M. sinensis, M. × giganteus, and M. sacchariflorus, along with their respective genotypes, exhibited diverse rates of callus induction and plant regeneration. The observed differences in efficiency among genotypes of

M. sinensis and M. × giganteus, especially compared to their related genotypes, confirm the importance of genotype in determining regeneration capacity. In Experiment III, plantlet regeneration was successful, and further development of these plantlets, including the spontaneous formation of clumps that can be divided, indicates a successful regeneration process. This regeneration phase lasted approximately 4-5 months, and the entire process from callus induction to plantlet rooting represents a crucial step in Miscanthus regeneration (Figure 6).

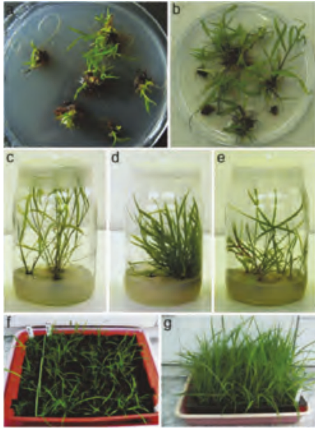

Figure 6: Regeneration of plants of different miscanthus genotypes on medium 190-2 supplemented with 0.5 mg/L NAA and 0.5 mg/L KIN and adaptation to ex vitro conditions
Slika 6: Regeneracija biljaka različitih genotipova miskantusa na mediju 190-2 s dodatkom 0,5 mg/L NAA i 0,5 mg/L KIN-a i prilagodba na ex vitro uvjete
(Izvor/Source: Ślusarkiewicz-Jarzina et al., 2017.)

This experiment confirms similar patterns to other reports, focusing on Miscanthus × giganteus and M. sinensis but also contributing to understanding the regeneration capacity of M. sacchariflorus. The results indicate that M. × giganteus and M. sinensis had a comparable and significantly higher ability to form callus compared to M. sacchariflorus. These findings underscore the importance of genotype in determining the regeneration capacity of different Miscanthus species. Considering the ecological and economic significance of these species, the development of efficient regeneration systems is essential for further research and applications, including biomass production, phytoremediation, genetic, and breeding studies.

Conclusion

This paper explores the significance of micropropagation in Miscanthus cultivation, emphasizing the need for the development of suitable in vitro methods for the commercial production of micropropagated plants. The authors highlight that micropropagation allows a significantly higher number of plants to be produced from a single explant compared to traditional rhizome propagation, which has the potential to reduce costs and increase the efficiency of Miscanthus cultivation. It is also emphasized that different Miscanthus species produced by in vitro culture currently require high establishment costs, limiting the widespread application of this technique.

The authors stress the need to address the complex challenges associated with micropropagation, including reducing overall costs to increase practical applications in agriculture, horticulture, and forestry. This text also recognizes the growing interest in Miscanthus cultivation due to the need for alternative energy sources and bioenergy, highlighting the crucial role of micropropagation in achieving this goal. Overall, the text points to the need for further research and improvement of micropropagation techniques to encourage the broader application of this important technology in Miscanthus cultivation.

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Izvorni znanstveni rad

Razmnožavanje biljke miskantus postupkom mikropropagacije

Sažetak

Miskantus je višegodišnja trava podrijetlom iz istočne Azije. Unatoč tome što se miskantus prvenstveno proširio u Europi i Sjevernoj Americi zbog svoje upotrebe kao ukrasne trave, uzgoj ove biljke je financijski zahtjevan, što rezultira ograničenim područjem uzgoja u Europi. Cilj ovog rada jest detaljno opisati različite sustave mikropropagacije primijenjene na biljku miskantus. Razmnožavanje miskantusa provodi se vegetativno, koristeći podzemne rizome ili mlade biljke uzgojene kulturom tkiva, poznatom kao mikropropagacija. Mikropropagacija se ističe kao alternativni i izuzetno učinkovit način razmnožavanja, obzirom na visoku stopu umnožavanja i dodatnu prednost sprječavanja prijenosa bolesti. Predstavlja metodu vegetativnog razmnožavanja koja se odvija na umjetno stvorenim hranjivim podlogama, kontroliranim uvjetima rasta te in vitro sterilnim uvjetima. Materijali i metode istraživanja temeljili su se na latentni pupovi trogodišnjih rizoma Miscanthus × giganteus. Za sterilizaciju je korištena 70-postotna otopina etanola i 0,1-postotna otopina HgCl2. U ovom radu napravljena je usporedba studija mikropropagacije morfogeneza Miscanthus x giganteus koju su objavili Klychenko i dr. (2018.), regeneracija biljaka Miscanthus giganteus - učinak tipova kalusa, starosti i načina uzgoja na sposobnost regeneracije koju su objavili Kim i dr. (2010.) te učinkovit i jednostavan in vitro sustav regeneracije Miscanthus sinensis, M. × giganteus i M. sacchariflorus za sadnju i biotehnološke svrhe, koju su objavili Ślusarkiewicz-Jarzina i dr. (2017.). Ovaj rad strukturiran je na način da ukaže na važnost proizvodnje miskantusa mikropropagacijom, na njegove karakteristike, na dosadašnje spoznaje o toj biljci, na dostignuća, budućnost i perspektivu mikropropagacije miskantusa in vitro tehnikom.

Ključne riječi: *in vitro, miskantus, biljka, mikropropagacija, razmnožavanje, reprodukcija, sterilni uvjeti*