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Original Paper

ESTABLISHING OF AN AXENIC *LEMNA MINOR* LINE AS A MODEL ORGANISM FOR A WIDE SPECTRE OF BIOLOGICAL AND ENVIRONMENTAL INVESTIGATIONS

Kārlis Žagata[1,#], Dalius Butkauskas[1,2], Regina Vyšniauskienė[2], Vida Rančelienė[2], Ieva Ignatavičienė[2], Tūrs Selga[1], Andra Miķelsone[1], Isaak Rashal[1], and Dace Grauda[1]

[1] Institute of Biology, University of Latvia, 1 Jelgavas Str., Rīga, LV-1004, LATVIA

[2] Nature Research Centre, 2 Akademijos Str., Vilnius, 08412, LITHUANIA

[#] Corresponding author, karlis.zagata@lu.lv

Contributed by Isaak Rashal

*Common duckweed (*Lemna minor *L.) is a widely used plant for phytoremediation and environment monitoring of wastewater* in vivo*, as this plant can be efficiently grown in Petri dishes and quickly multiplied in laboratory conditions. It is also a valuable test object to study the impact of various environmental factors by measuring growth and biochemical parameters. Our goal was to establish an axenic diploid line of* L. minor *free from symbionts, for use as a model plant to obtain reproducible results in experiments. In this work, we used 29* L. minor *clones collected in natural conditions in Lithuania and Latvia, which were naturally inhabited with bacteria and algae. The* L. minor *clone entitled* Sta2 *was found to be best to fit requirements to establish a laboratory line suitable for testing of the genetic and physiological influence of environmental factors. Application of flow cytometry, confocal laser scanning microscopy, and sequencing of catalase, glutathione peroxidase, and ascorbate peroxidase genes proved that the selected and specifically sterilised line* Sta2 *was diploid and free from symbiotic bacteria or algae. Media for storage and for rapid propagation of* L. minor *biomass were also developed. The axenic line* Sta2 *can be maintained in sterile laboratory conditions and can be used as a model organism in a wide spectrum of biological and environmental investigations.*

Keywords: *common duckweed, flow cytometry, confocal laser scanning microscopy, sequencing.*

INTRODUCTION

Common duckweed (*Lemna minor* L.) is a floating monocotyledonous species, which in natural conditions is widespread in freshwater aquatic environments. *L. minor* plants lack true stems and leaves. Their plant body consists of expanded, flat leaf-like fronds that float on the water surface or are slightly submerged. The roots are attached on the lower surface of the fronds. *L. minor* produces mainly vegetatively by budding; therefore, plants can multiply very rapidly forming genetic clones (Stomp, 2005; Aliferis *et al*., 2009). *L. minor* can be easily cultivated artificially under laboratory conditions. Due to their small size, possibility to use clones with genetically identical plants, and high sensitivity both to organic and inorganic substances, *L. minor* is widely used as a model plant species for studies in genetics, plant physiology, ecology, environmental monitoring etc. (Brain and Solomon, 2007; Scherr *et al*., 2008, Aliferis *et al*., 2009; Zhang *et al*., 2010; Wang *et al*., 2011, Kostopoulou *et al*., 2020). The potential industrial use of *L. minor* for bioenergy production and pharmaceutical applications has also been considered (Yamamoto *et al*., 2001).

In natural conditions plants of *L. minor* are contaminated with a wide range of co-existing microorganisms (bacteria, algae, etc.) (Pala *et al*., 2019; Radulović *et al*., 2019b),

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which have significant effects on plant growth by increasing nutrient acquisition or alleviating biotic and abiotic stresses (Glick, 2012; Ishizawa *et al*., 2017). Therefore, in investigations with samples of *L. minor* collected in natural conditions, the obtained results can be significantly affected by microbiota of co-existing microorganisms. To exclude this influence, in some investigations one- or multistep sterilisation of fronds surface has been carried out (Thomson and Dennis, 2013; Pala *et al*., 2019; Acosta *et al*., 2020; Radulović *et al*., 2020), but the effectiveness of the used sterilisation methods has not been proven.

For use of *L. minor* as a model plant for biological testing of phenotypic, genetic, epigenetic, physiological, and other effects on organism of different factors like electromagnetic fields, UV radiation, and chemical pollution, it is very important to have genetically stable plant clones that are free from contamination. Therefore, the aim of this work was to establish a stable *L. minor* clonal axenic laboratory line suitable for a wide range of investigations.

MATERIALS AND METHODS

Plant material. In total, 29 *L. minor* individual plants were collected in geographically distant locations from the Nemunas, Neris and Venta River basins in Lithuania and from the Daugava river and Blīdene water reservoir in Latvia. The collected plants were sterilised from surface contamination as follows: plant roots were catted, and the remained fronds were rinsed with deionised water for 10 min and then rinsed with commercial bleach ACE for 60 seconds. Finally, the sterilised fronds were rinsed four times with deionised water.

Sterilised plants were cultivated in sterile conditions on 60 mm Petri dishes with 10 ml of Steinberg medium (according to ISO 20079, see OECD guidelines for the testing of chemicals, 2006) in a light/dark cycle of 18/6 hours at 24 °C. Progenies of each sampled plant were cultivated separately forming a genetically uniform clone.

Examination of cell ploidy of *L. minor* clones. Flow cytometry was applied to evaluate distribution of cells regarding amount of DNA in the cell nucleus (cell ploidity). For this purpose, a BD FACSJazz® flow cytometer with cell sorter function (BD Biosciences) was used to determine the relative fluorescence of DNA stained with a fluorescent dye, which was proportional to the DNA content in the cells. Diploid cells of pea leaf were used as a positive control of cell nucleus fluorescence (Bourge *et al*., 2018). Samples for the analysis were prepared using CyStain propidium iodide (PI) absolute P reagent kits (Sysmex Partec GmbH.) according to the manufacturer protocol. Frond leaves were chopped with a blade, and then added to 500 µl of extraction buffer solution, after 90 s the suspension was filtered with a Partec 40 µm CellTrics disposable filter. Prepared samples were stained by propidium iodide with RNase A treatment and incubated in darkness for 24 hours at 4 °C. The flow cytometry device was equipped with a 100 µm nozzle and calibrated by Sphero^{TM} 8 peaks Rainbow calibration particles (Spherotech, Inc.). The coefficient of variation of the relative fluorescence of calibration particles did not exceed 3%. Cell counting events were triggered by a forward-scattered signal, and the mean fluorescence intensity in relative fluorescence units was detected. The excitation of cell fluorescence was induced by a 488 nm Coherent Sapphire Solid State (blue) laser. The fluorescence emission was measured at 585 nm (bandwidth 29 nm). The intensity of fluorescence as expressed in arbitrary logarithmic units used through the whole research. For each sample 10×10^3 gated nucleuses were analysed. Statistical calculations with significance level of $p = 0.05$ were made using MS Excel software.

Molecular screening. For selection of diploid *L. minor* clones with the most uniform DNA sequences, comparison of DNA sequences of catalase (*CAT*), glutathione peroxidase (*GPx*) and ascorbate peroxidase (*APx*) genes was carried out for some most promising clones that were chosen based on growing parameters and flow cytometry analysis.

DNA extraction**.** Whole plants with fronds and roots were ground in liquid nitrogen and total DNA was extracted using DNeasy Plant Mini Kits (QIAGEN) according to the manufacturer's protocol. DNA extracts were diluted to the final concentration 10 ng/µl in distilled water and stored at –20 °C until use.

PCR and DNA sequencing**.** *L. minor* sequences of target genes used for designation of primers were obtained from the CoGe database (https://genomevolution.org/coge/) (Van Hoeck *et al*., 2015). Specific primers used both for amplification and sequencing of different parts of gene including promoter, introns and exons were designed using Primer3Plus software. For amplification and sequencing of the *CAT* gene fragments, the primers CAT-7L: CGCGGTTTGGTTCAATTCGT and CAT-7R: TGGACTTGATCAGCGGTGAC were used. For amplification and sequencing of the *GPx* gene fragment primers GPx6F: TGTGCAAACACATAATCCCAAT and GPx6R: TGATCATGACCAATAGATCGTT were used.

PCR was performed in 10 µl of solution containing 2 µl DNA solution, 1 µl of each primer (10 µM), 5 µl DreamTaq PCR Master Mix, and 1 µl nuclease-free water. The PCR performing programme was 5 min at 94 °C, followed by 35 cycles at 94 °C for 45 s, specific primer's annealing temperature for 45 s, elongation step at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR amplicons were purified with exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) and then sequenced by 3500 Genetic Analyser (Applied Biosystems). The sequenced DNA fragments were aligned using the MUSCLE (Edgar, 2004) option in MEGA-X (Kumar *et al*., 2018) and the purity of DNA sequences (obtained from different *L. minor* samples) was evaluated based on sequences without double peaks as the most fitting to carry out experiments designed to reveal a new point mutation generated by low frequency electromagnetic radiation.

Establishing of *L. minor* axenic lines. To establish *L. minor* axenic lines, it is necessary to purify the plants from microbiota left in inner tissues of leaves after surface sterilisation. For this purpose, two-step sterilisation were performed in a laminar-airflow box. In the first step, plants were treated with 0.1% $KMnO_4$ solution. For this, plant fronds were immersed in 15 ml conical tubes with sterilisation solution and mixed by gentle shaking of tubes for 10 s. Then fronds were rinsed with deionised water. In the second step, fronds were immersed in 70% C_2H_5OH solution in conical tubes and mixed by gentle shaking of tubes for 10 s. Finally, fronds were multi-rinsed with deionised water. After sterilisation, the plants were transferred on 60 mm Petri dishes with MS medium reduced to 1/2 of nutrients supplemented or not supplemented with 1% sucrose (Thomson and Dennis, 2013). Plants were maintaining under the conditions described above. Plants were transferred on fresh medium every two weeks.

Verification of the effectiveness of purification. Efficiency of purification was checked by confocal laser scanning microscopy. Four plants of selected clone *Sta2* without sterilisation and four purified plants or the clone were fixed with 96% C_2H_5OH and stained with PI propidium iodide at a concentration of 20 µmol/ml. They were analysed by confocal laser scanning microscope Leica DM RA-2 equipped with a TCS-SL confocal scanning head. The dye (PI) molecules were excited by a 488 nm laser band and the fluorescence was detected between 600 nm and 640 nm wavelengths. The field of view was scanned in three dimensions, using 20 optical sections separated in depth by 10 µm from surface. Objective, 100×; NA, 1.25 Color-coded 3D projections indicate distribution and density of different types of nucleus depending on the species to which each nucleus corresponds.

Cultivation *L. minor* axenic laboratory line. To determine optimal growing conditions for *L. minor* axenic plants purified from algae and bacteria, three different media were tested: a) non-modified Steinberg medium, b) MS medium with 1/2 of nutrients, and c) MS medium with 1/2 of nutrients supplemented with 1% sucrose. In all mentioned media, plants were cultivated in 60 mm Petri dishes and transferred to fresh medium every two weeks.

RESULTS

Level of cell ploidy of *L. minor* lines. Flow cytometry analysis showed that cells of different *L. minor* clones contained nuclei with different amounts of DNA, respectively with different levels of ploidity. Most of the clones were mixoploid and included cells with ploidy of 2n, 3n, and 4n in different proportions. Only two clones (*Sta2* and *Vand19*) were not mixoploid with all plants characterised by (2n) cell nuclei (Fig. 1).

Molecular data analysis. Molecular data analysis based on amplification, sequencing and alignment of DNA sequences of the *CAT* and *GPx* gene fragments revealed differences in sequence purity after comparison of DNA strands corresponding to different clones. It was revealed that the majority of the sequences had clear signals, where all nucleotide positions were represented by a single peak, lacking any noisy or interstitial signals in the line *Sta2*. The majority of the remaining sequences of homologous DNA loci representing other *L. minor* clones belonging to the same or different studied source populations had several to many multicolour peaks and the oddly spaced interstitial peaks, complicating determination of the real nucleotide at a certain position due to possible polyploidy or non-purity of the sample. Figure 2 illustrates the results of the sequencing of the *CAT* gene fragment of *Sta2* and *K2D L. minor* clones. The obtained DNA sequences of *CAT* gene representing specimen *Sta2* are distinguished by clear signals in all nucleotide positions, in contrast to numerous noisy or interstitial signals characteristic for homologous sequence representing specimen *K2D*. The mixed ploidy (2n and 4n) of *K2D* clone resulted in noisy signals or positions with double peaks complicating the determination of the real nucleotide (Fig. 2).

Similar results were obtained after sequencing *GPx* gene fragments in *Sta2 and K2D L. minor* clones (Fig. 3). The diploid line *Sta2* again revealed sequence with clear signals.

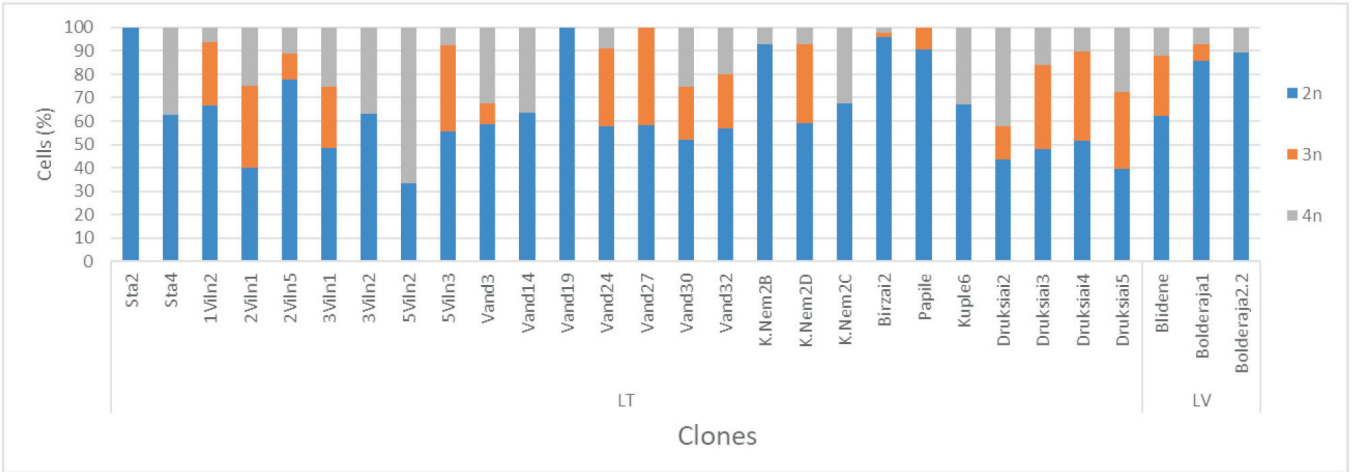


Fig. 1. Mixoploidy level of 29 *L. minor* clones developed from samples collected in different localities of Lithuania and Latvia.

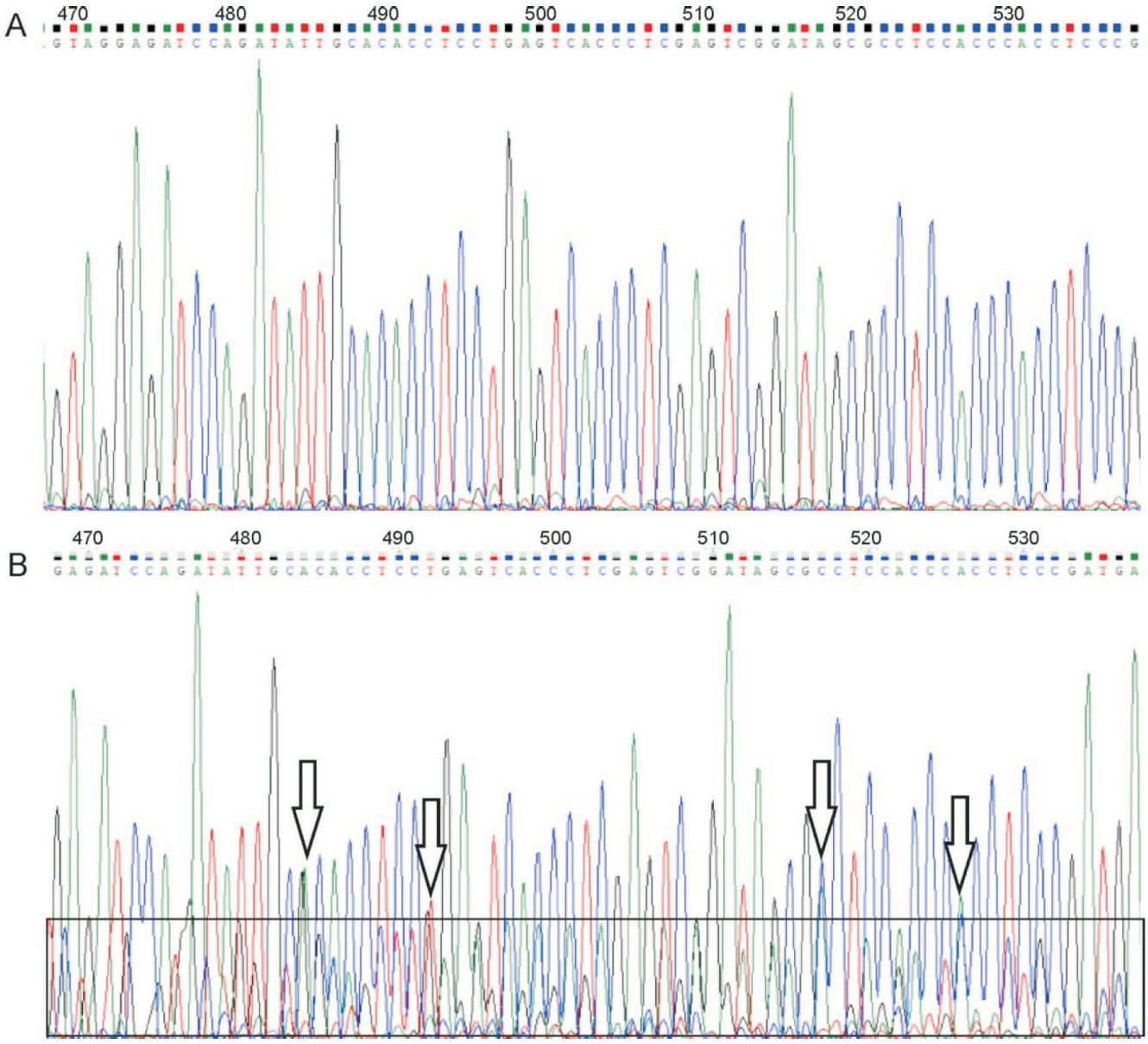


Fig. 2. Chromatograms of DNA sequences of *CAT* gene representing homologous fragments corresponding to *L. minor* clones *Sta2* (**A**) and *K2D* (**B**): outlined zone represents noisy signals, double peaks indicated by arrows.

The mixoploidy line *K2D* showed sequences with high background noise or interstitial signals (Fig. 3).

Consequently, based on the clearly interpretable results of targeted DNA sequences, the line *Sta2* was chosen as one of the most fitting in experiments for identification of new point mutations, generated by electromagnetic radiation.

Verification of the sterilisation method. Comparison of the confocal laser scanning micrographs revealed that the plants of *L. minor* before sterilisation were infected with variable microscopic bacteria and algae (Fig. 4 A, B). After sterilisation, *L. minor* plants were free from microorganisms (Fig. 4 C), which was proved by the fact that only fluorescence of nuclei of plant cells is visible.

Influence of medium on growing of axenic *L. minor* plants. It was found that the examined media influenced the growth rate of *L. minor* plants and the formation of the next vegetative generation. The slowest growth was observed on Steinberg medium. Very rapid growth and multiplication of *L. minor* plants was achieved when 1% sucrose was added to 1/2 MS medium (Fig. 5).

DISCUSSION

Use of convenient model organisms with appropriate genetic and physiological properties can be a way to achieve success in biological and environmental investigations. Small size, possibility to clonal reproduction and high sensitivity to environmental factors are the main reasons to choose of *L. minor* as an object in many fields of research (Brain and Solomon, 2007; Scherr *et al*., 2008; Aliferis *et al*., 2009; Zhang *et al*., 2010; Wang *et al*., 2011; Kostopoulou *et al*., 2020). Nevertheless, there are several restrictions

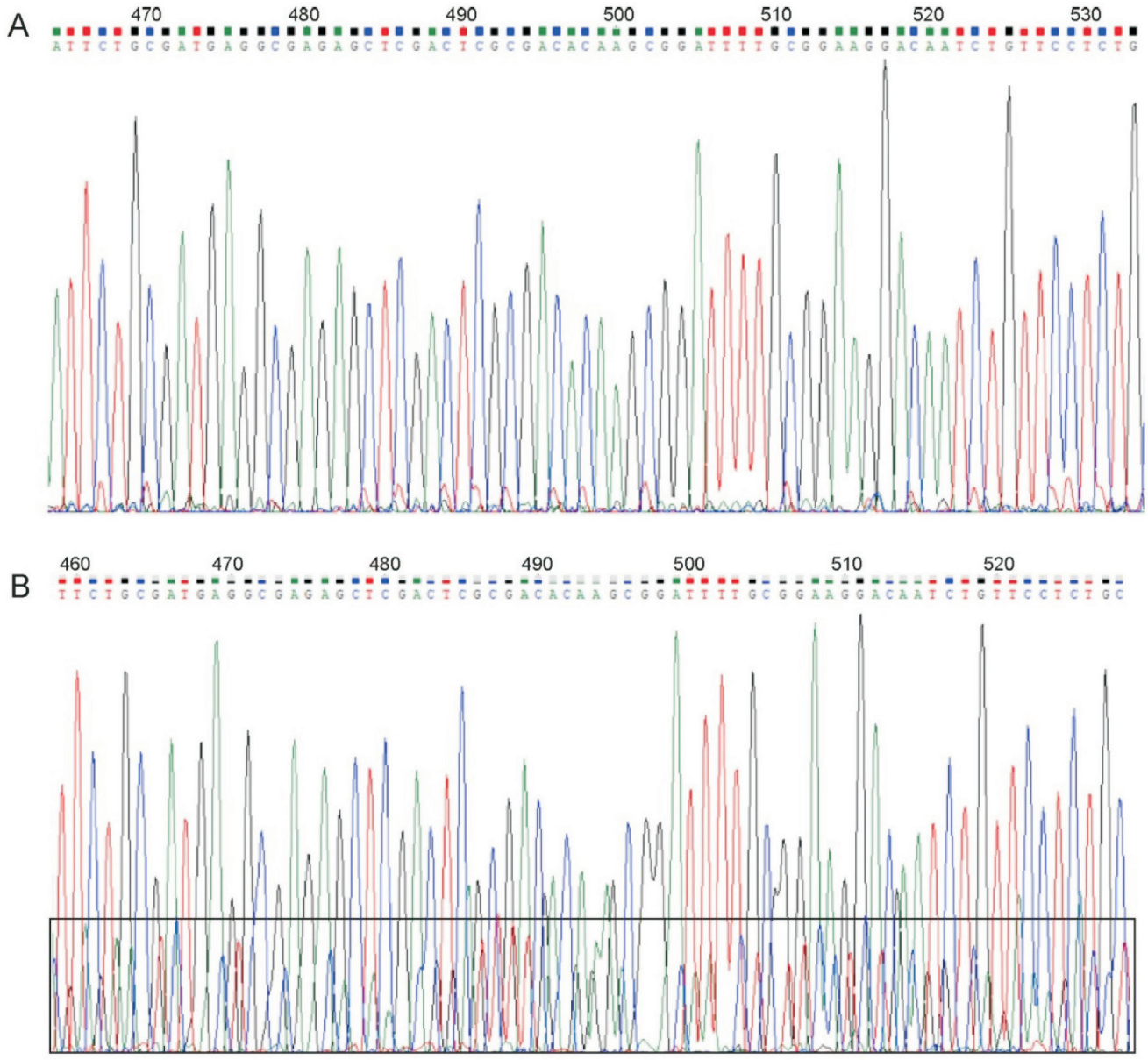


Fig. 3. Chromatograms of the DNA sequences of *GPx* gene representing homologous fragments corresponding to lines *Sta2* (**A**) and *K2D* (**B**): outlined zone represents noisy or interstitial signals.

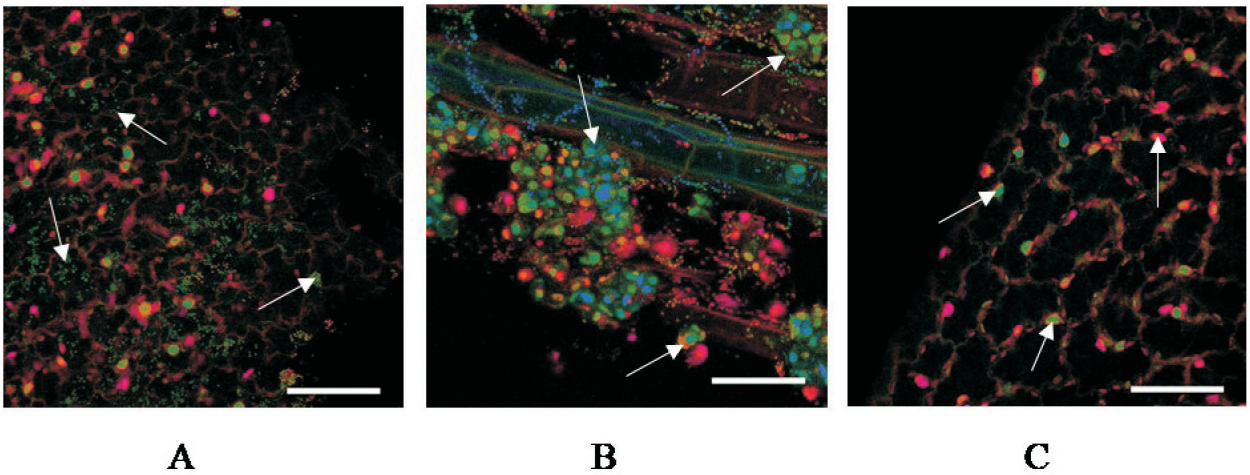


Fig. 4. Confocal laser scanning micrographs of different *L. minor* samples stained by propidium iodide. A – epidermis of plants before sterilisation covered with road shaped bacteria. B – epidermis of plants before sterilisation covered with unicellular algae. C – epidermis with large nuclei of plants and with no traces of bacteria and algae. Bar 30 μm.

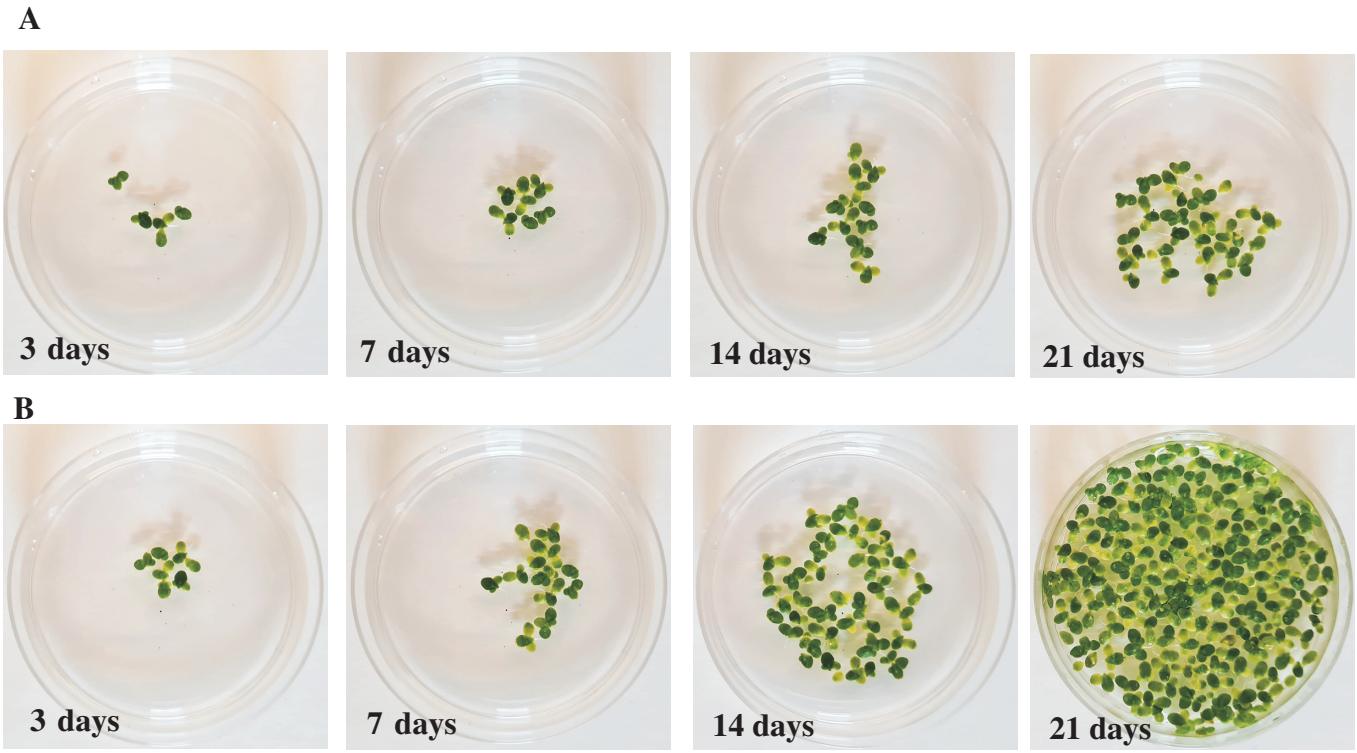


Fig. 5. L. minor fronds of the line *Sta2* after different days of growth on 1/2 MS medium (A), and on 1/2 MS medium supplemented with 1% sucrose (B).

which cause difficulty in using this species as a model plant.

Undeniably, the ploidy level of model organisms is a very important factor. The analysis of polyploid genetic data is difficult because theory and tools that have been developed for diploids not always work correctly for polyploids. In this case, interpretation of the experimental results may be not appropriate. At the same time, the presence of an uncertain number of allelic copies could also create problems in DNA sequence based analyses and interpretation of obtained results (Cole and Voskuil, 1996; Wang et al., 2011; Dufresne *et al*., 2014; Cao and Vu, 2020). For these reasons, diploid (2n = 2×) model plants in comparison with tetraploid (2n = 4×) or other ploidy plants is more appropriate. Particularly problematic interpretation of the results arises in the case of mixoploidy when different cells of the same plant have different levels of ploidity. Mixoploid lines may vary in the percentage of cells of different ploidy and this can affect the obtained results.

There are some reports on genome sizes of *L. minor* measured by flow cytometry. Wang *et al.* (2011) found that the *L. minor* genome contains about 481 Mbp. Dufresne *et al.* (2014) found that genome sizes in 26 samples of *L. minor* ranged from 356 to 604 Mbp, and that ploidity of separate cells varied even from 20 to 126. This indicates that the use of *L. minor* samples obtained from natural conditions will involve unknown levels of ploidity in most experiments.

In our work for estimation of the ploidity level of the collected *L. minor* samples we used the flow cytometry method (FCM), which is a widely used method for detection of quantity of cell DNA. FCM gives the possibility to evaluate a large number of cell nuclei in a very short time, and therefore the obtained results are statistically significant and representative of the whole cell population. In FCM, DNA amount detection is based on relative fluorescence of the particular cell (Doležel *et al*., 2007; Galbraith, 2010). On the basis of information on ploidy of collected samples of *L. minor*, the diploid clone *Sta2* without mixoploidy was selected to establish the necessary axenic line.

Many properties of *L. minor* useful in different fields of environmental science have been described. For example, floating macrophyte can accumulate more metals from its surroundings in water through completely submerged roots in comparison with leaves that float on the water surface, Therefore, *L. minor* can be successfully used as an indicator plant for understanding the quality of the environment (water, sediment) because it can constantly accumulate metals during the growing season and tolerates large amounts of metal without adverse impact on its growth and development (Ater *et al*., 2006; Kastratović *et al*., 2015). The bioremediation potential of *L. minor* to remove phenol from aqueous environments at extremely high initial phenol concentrations has been shown in phytoremediation experiments (Radulović *et al*., 2019a; Radulović *et al*., 2020). It was found also that application of *L. minor* is efficient for the reduction of inorganic and organic pollutants from wastewater (Kamyab *et al*, 2017; Daud *et al*., 2018).

Many studies have shown that microorganisms co-existing with duckweed have significant effects on growth in natural conditions, and some can enhance host plant growth by in-

creasing nutrient acquisition or by alleviating biotic and abiotic stresses (Glick, 2012; Ishizawa *et al*., 2017). It was found that 36 taxa of algae from *Cyanophyta*, *Chlorophyta*, *Euglenophyta,* and *Bacillariophyta* can co-exist with *L. minor* in natural habitats (Pala *et al*., 2019). The rhizosphere of *L. minor* can be inhabited with various bacterial strains; 60 species of rhizosphere-associated bacteria belonging to 10 genera (*Pseudomonas*, *Hafnia*, *Serratia*, *Enterobacter*, *Micrococcus*, *Stenotrophomonas*, *Xanthomonas*, *Bacillus*, *Staphylococcus,* and *Klebsiella*) were identified in the rhizosphere of *L. minor* (Radulović *et al*., 2019a). Additionally, it is known that the influence of high frequency EMF in frequencies of 900 and/or 1800 MHz even during a relatively short time (2 hours) of exposure can cause genetic changes in the genome of bacteria (Salmen *et al*., 2018) that naturally inhabit the *L. minor* rhizosphere.

As microbiota in various collected samples can differ substantially, the effects on the growth of *L. minor* also differs, resulting in biased comparison of results obtained from *L. minor* samples obtained from different habitats. To avoid an effect of possible microbiota in experiments, it is preferable to use axenic lines of *L. minor* free from contamination with bacteria and algae. It was established that the line *Sta2* provides such possibility and can be used as a model object in a wide spectrum of biological and environmental experiments.

CONCLUSIONS

From samples of *L. minor* collected in natural conditions, the line *Sta2* with predominantly diploid cells was selected and cloned. By appropriate sterilisation, an axenic line with plants free from microbes and algae was obtained. The best conditions both for maintenance and micropropagation of the plants were identified. The established axenic line *Sta2* is suitable for a wide range of biological and environmental investigations where elimination of the influence of microbiota is crucial.

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AKSĒNISKAS *LEMNA MINOR* LĪNIJAS IZVEIDOŠANA KĀ MODEĻORGANISMU PLAŠAM BIOLOĢISKO UN VIDES PĒTĪJUMU SPEKTRAM

Mazo ūdensziedu *Lemna minor* L. var vienkārši audzēt Petri platēs un ātri pavairot laboratorijas apstākļos, tāpēc šo augu var izmantot kā modeļorganismu dažādu vides faktoru ietekmes pētīšanai. Tomēr *L. minor* dabā veido simbiozi ar daudzām baktēriju un aļģu sugām, kas būtiski ietekmē augu attīstību un augšanu. Tāpēc, lai varētu veikt salīdzinošus pētījumus ar šo modeļobjektu, nepieciešams izveidot aksēniskas līnijas, kas ir brīvas no simbiozi veidojošas mikrobiotas. No 29 Lietuvā un Latvijā ievāktiem *L. minor* paraugiem, veģetatīvi pavairojot, izveidoti ģenētiski viendabīgi kloni. Ar plūsmas citometrijas palīdzību noteikts, ka lielākā daļa no tiem ir miksoploīdi un tikai divi kloni no tiem bija viendabīgi dipoloīdi. Izstrādāti sterilizācijas paņēmieni, kas ļauj atbrīvoties gan no virsmas mikroorganismiem, gan arī no mikrobiotas dziļākos augu audos. Izvēlēta diploīdā līnija *Sta2*, kuras ģenētiskā viendabība noteikta, sekvencējot trīs dažādus lokusus. Līnijas atbrīvošana no mikrobiotas tika pārbaudīta ar konfokālās lāzerskenēšanas mikroskopijas palīdzību. Izstrādātas barotnes līnijas ilgstošai uzturēšanai un straujai pavairošanai.