Plant Cell, Tissue and Organ Culture (PCTOC)
https://doi.org/10.1007/s11240-018-1494-6

ORIGINAL ARTICLE

Creation of culture media for efficient duckweeds micropropagation (*Wolffia arrhiza* and *Lemna minor*) using artificial mathematical optimization models

Pavel Khvatkov[1,2] · **Mariya Chernobrovkina**[1] · **Anna Okuneva**[1] · **Sergey Dolgov**[1,2,3]

Received: 20 June 2018 / Accepted: 16 September 2018

Abstract

Recently, computer technologies have provided the researchers with the new approaches for modeling and better understanding the role of the factors that are involved in plant growth in vitro. To develop new models for the optimization of growth conditions, it is reasonable to use plants with a high speed of vegetative in vitro reproduction, such as duckweed (*Lemnaceae* family). This article focuses on the trophic levels of the two types of duckweeds (*Wolffia arrhiza* and *Lemna minor*). Using the development of the optimal modeling of the biological processes we have obtained the prescriptions for individually-balanced culture medium that enable 3.0 higher yields of the total soluble protein from each of the populations for both types of *Lemnaceae*.

Keywords Duckweed · Ion nutrients · *Lemna minor* · Micropropagation · Model · *Wolffia arrhiza*

Abbreviations

B_5	Gamborg medium
DT	Doubling time
FW	Fresh weight
Go	Gorham medium
HA	Hoagland & Arnon medium
Kn	Knop medium
MS	Murashige & Skoog medium
RGR	Relative growth rate
RY	Relative yield after 1 week
SH	Schenk & Hildebrandt medium
St	Steinberg medium
TSP	Total soluble protein

Communicated by Silvia Moreno.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11240-018-1494-6) contains supplementary material, which is available to authorized users.

✉ Pavel Khvatkov
khvatkov1987@gmail.com

[1] All-Russia Research Institute of Agricultural Biotechnology, Moscow, Russia

[2] Nikita Botanical Gardens, Yalta, Russia

[3] Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Puschino, Russia

Introduction

Biological processes are highly complex and dynamic, and are often influenced by genetic and environmental factors. These highly variable factors are largely responsible for nondeterministic and non-linear nature of developmental processes of biological entities (Gallego et al. 2011; Prasad et al. 2016). Recently, computer technologies have provided to the researchers new approaches for modeling and better understanding the role of the factors involved on in vitro plant growth (Zielinska and Kepczynska 2013). Artificial neural, mathematical, physical or other modeling can utilize and process a set of multi-dimensional data as inputs and produce outputs indicating the relationship between the data (Patnaik 1999; Prasad et al. 2016). Due to this property, modelling is applicable to a wide area of scientific advances including health, agro-technology, genetic engineering, and plant biotechnology (Jimenez et al. 2008; Gago et al. 2010). From the inception of plant tissue culture (leaf mesophyll and hair cells) by Austrian botanist Gottlieb Haberlandt (1902) in nutritive media, numerous researches have been done on the optimization of various culture media to provide explants favorite propagation conditions (Jamshidi et al. 2016). In plant biotechnology, with particular reference to plant tissue culture research, are modelling generally used to manipulate tissue growth to enhance biomass yield in lesser time or to derive useful metabolites/compounds by

Published online: 19 September 2018

various approaches optimization of physical and nutritional parameters under in vitro conditions is a regular practice (Jamshidi et al. 2016; Prasad et al. 2016). Hence, study on the relationship between media nutrients and explant proliferation may result to design a more effective medium (Jamshidi et al. 2016).

The *Lemnaceae* (duckweeds) are a widespread family of free-floating monocotyledonous plants growing in freshwater habitats (Landolt 1986; Kuehdorf and Appenroth 2012). *Lemnaceae* reproduce primarily by vegetative means with high rates of biomass accumulation (Landolt and Kandeler 1987). A protein content as high as 45% of the dry weight (Yuan and Xu 2017), an excellent tolerance for a variety of toxic substances (Landolt and Kandeler 1987) and a rapid uptake of nutrients make *Lemnaceae* an ideal choice for the development of efficient, plant-based, gene-expression systems (Li et al. 2004). A significant increase in the demand for therapeutic proteins has been observed in the pharmaceutical industry nowadays (Daniella et al. 2001; Mett et al. 2008).To date, *Lemna* and *Spirodela* species of *Lemnaceae* family are the only species that have been used to produce recombinant proteins for pharmaceutical and veterinary needs (Stomp and Rajbhandary 2000; Gasdaska et al. 2003; Rival et al. 2008.; Firsov et al. 2015, 2018). α-2β-interferon and monoclonal antibodies that are produced in the tissues of transgenic *Lemna minor* (L.) (Gasdaska et al. 2003; Stomp et al. 2005) which is grown in photobioreactors with the surface fermentation (Branson et al. 2007) are already used in commercialized products of Bayer. Colony of *L. minor* fronds has 3–5 oval light-green fronds with a single rhizoid each (Wolff 1992). The more promising biopharming model is a rootless duckweed *Wolffia arrhiza* (L.) Horkel ex Wimm., which is the most simply organized species among the *Lemnaceae* family. The absence of roots enables its cultivation in bioreactors using a submerging technique (Khvatkov et al. 2013), which in turn can significantly increase recombinant protein production profitability.

Duckweed production open systems for farmers fodder needs (mostly *Lemna* spp.) are commonly used in Asian countries (Bangladesh, Vietnam, Taiwan). It is possible to affect the accumulation of the crude protein in plants by regulating the amount of nitrogen compounds in the nutrition substrates (Leng 1999; Roche et al. 2016). Applying of duckweed as a source of fodder or supplemental food has been tested on milk cows, bulls, pigs, sheep, ducks, turkeys, rabbits, coypus, muskrats and pond fish. Most of the species have showed a rapid weight gain. It was demonstrated that duckweed can account for up to 80% of ducks ration, while replacing soybeans (Men et al. 1995), and 100% of fish ration (Leng et al. 1995). For chickens the maximum percentage is 20 of the total food (Akter et al. 2011). Thus, integration of duckweeds into agricultural production gives the chance to receive cheap forage.

Commercial implementation or increase in profitability of such projects as biopharming and forage production depend on the availability of a high-efficiency technology for duckweed cultivation, part of which is the optimization of culture media for plant- producers growth and accumulation of the synthesized product (Gasdaska et al. 2003; Khvatkov et al. 2015, 2018).

The aim of the present study is to develop the balance of nutrients in the culture medium as well as optimize duckweed populations growth parameters. Studying of duckweed trophic levels using the optimized model of biological processes results in prescriptions for individually-balanced cultivation medium trebling the yield of total protein for two species of the *Lemnaceae* family (*W. arrhiza* and *L. minor*).

Materials and methods

Plant material

Our study was carried out using an aseptic population of whole *W. arrhiza* plants (RDSC Clone *Wolffia* 5564), cultivated on Schenk & Hildebrandt (SH) medium (Schenk and Hildebrandt 1972), supplemented with 2% (w/v) sucrose and 0.7% (w/v) agar (Panreac, EU), and whole aseptic *L. minor* plants [in vitro culture originally collected from Oka river at Puschino town (54.848922, 37.638381), Russia (Firsov et al. 2018)], cultivated on Steinberg (St) medium (Steinberg 1946), supplemented with 2% (w/v) sucrose (molar concentrations of aforementioned media are shown in Table 1). *W. arrhiza* plants were placed in Petri dishes containing culture medium, 10 plants per Petri dish. *L. minor* plants were placed into culture vessels containing liquid culture medium, 10 plants per culture vessel. All plants were cultivated at 21 ± 1 °C and light intensity of 65 µmol m^{-2} s^{-1} during 16-h day photoperiod, and transferred onto a fresh medium every 30 days. pH of media was set to 5.8 prior autoclaving (121 °C, 1 kg cm^{-2} s^{-1} for 20 min).

Study design and data acquisition

The first step was the identification of the most effective culture medium for propagation of both duckweeds (Table 1). The second step—optimization of each medium component, based on the composition of mineral nutrients in the media for *W. arrhiza* and *L. minor* propagation, as it was determined in the first step of the study (Tables 2, 3). For this, experiments Nos. 1–6, 9, 10, 12, 13 were carried out independently of each other. After obtaining the results of experiments No. 1 and No. 2, we carried out the experiment No. 7, and after its completion the experiment No. 8 was executed. Upon receipt of the research results from the experiment No. 10, the experiment No. 11 was performed. The results of experiment No. 13 gave

Table 1 Culture medium composition according to the author's prescriptions

Components	Hoagland & Arnon medium	Schenk & Hildebrandt medium	Knop medium	Gamborg medium	Murashige & Skoog medium	Stein-berg medium	Gorham medium	BOi2Y medium
Inorganic salts (mM)								
Ammonium nitrate	0.0	0.0	0.0	0.0	20.6	0.0	0.0	12.5
Ammoniun phosphate monobasic	1.0	2.6	0.0	0.0	0.0	0.0	0.0	0.0
Ammonium sulfate	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0
Calcium chloride (anhydrous)	0.0	1.4	0.0	1.0	3.0	0.0	0.0	0.0
Calcium nitrate·4 H_2O	3.8	0.0	1.4	0.0	0.0	1.3	0.4	3.4
Magnesium sulfate (anhydrous)	2.1	1.6	0.5	1.0	3.2	0.4	0.4	0.3
Potassium nitrate	6.0	24.8	0.0	24.8	18.8	3.5	0.0	9.9
Potassium phosphate monobasic	0.0	0.0	0.4	0.0	1.25	0.7	0.2	0.0
Potassium chloride	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0
Sodium phosphate monobasic (anhydrous)	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0
Inorganic salts (µM)								
Boric acid	46.0	80.0	0.0	49.0	100.0	1.9	45.5	25.8
Cobalt chloride·6 H_2O	0.0	0.4	0.0	0.1	0.1	0.0	0.0	0.0
Cupric sulfate·5 H_2O	0.32	0.8	0.0	0.1	0.1	0.0	0.3	0.0
EDTA acid, Na_2·2 H_2O	80.5	54.0	0.0	100.0	100.0	54.0	20.0	86.0
Ferrous sulfate·7 H_2O	80.5	54.0	0.0	100.0	100.0	54.0	20.0	86.0
Manganese sulfate·H_2O	9.2	59.0	0.0	59.0	132.0	1.3	13.4	0.0
Molybdic acid (sodium salt)·2 H_2O	0.2	0.4	0.0	1.0	1.0	0.2	0.4	0.0
Potassium iodide	0.0	6.0	0.0	4.8	5.0	0.0	0.0	4.8
Zinc sulfate·7 H_2O	0.8	3.5	0.0	7.0	30.0	0.6	0.8	5.2
Organics (mM)								
Sucrose	58.4	58.4	58.4	58.4	58.4	58.4	58.4	58.4

The table shows single strength molar concentrations of media as described by Hoagland and Arnon (1938), Schenk and Hildebrandt (1972), Knop (1865), Gamborg et al. (1968), Murashige and Skoog (1962), Steinberg (1946), Gorham (1950) and Blaydes (1966)

rise to experiment No. 14. The third step of the study was the conversion of the obtained data from the experiments Nos. 1 to 14 into the graphs of the reconstructed functional impact and obtaining regression equations for those graphics (Tables 4, 5). In the fourth step of the study, two mathematical models (one for each of two duckweeds) were compiled to comprehensive obtainment of the optimal solutions for every nutritive element and the subsequent assembly of the optimal media (Table 6). Finally at the fifth step were carried out the experiments on practical verification of the effectiveness of the developed media for duckweed cultivation (Tables 7, 8).

Identification of the most effective culture medium for duckweed micropropagation (the first step)

A number of researchers have recommended the following medium for duckweed plants cultivation and propagation (different species *Lemna, Wolffia, Spirodella*): Knop, Hoagland & Arnon, Gorham, Steinberg and Schenk & Hildebrandt medium (Boehm et al. 2001; Li et al. 2004; Friedrich 2005), and for the plants with the high protein levels Murashige & Skoog, Gamborg and BOi2Y medium were recommended (Santarem et al. 1998; Mariza et al. 2007). We used 8 different medium: Murashige & Skoog (MS) (Murashige and Skoog 1962), Gamborg (B5) (Gamborg et al. 1968), Schenk & Hildebrandt (SH) (Schenk and Hildebrandt 1972), BOi2Y (Blaydes 1966), Knop (Kn) (Knop 1865), Hoagland & Arnon (HA) (Hoagland and Arnon 1938), Gorham (Go) (Gorham 1950) and Steinberg (St) (Steinberg 1946) to identify the most effective culture medium for propagation of both duckweeds. All of the media contained various concentration of mineral components (quarter, half, single, double, triple and quadruple strength media relative to the original compositions are shown in

Table 2 The scheme for optimizing the balance of the medium for cultivation *W. arrhiza*

Experiment number	Test substances	Tested concentrations of substances	Number of options in the experiment	Number of plants involved in the experiment
1	NO_3^- (KNO_3)	7.49, 8.74, 11.24, 16.24, 18.74 mM_{eq}	90	2700
	$CuSO_4 \cdot 5H_2O$	0.045, 0.09, 0.18, 0.36, 0.90, 1.80 µM		
	$CoCL_2 \cdot 6H_2O$	0.0, 0.42, 1.05 µM		
2	NO_3^- (KNO_3)	8.74, 11.24, 13.74 mM_{eq}	120	3600
	$FeSO_4 \cdot 7H_2O$ (from Iron EDDHA Chelate)	0.0, 0.022, 0.034, 0.067, 0.134, 0.268, 0.536, 0.804 mM		
	$Na_2MoO_4 \cdot 2H_2O$	0.047, 0.093, 0.186, 0.372, 0.744 µM		
3	KH_2PO_4	0.0, 0.075, 0.125, 0.25, 0.50, 1.0, 2.0, 2.50 mM	24	720
	$MnSO_4 \cdot 5H2O$	2.303, 4.606, 9.212 µM		
4	$MgSO_4 \cdot 7H_2O$	0.0, 0.15, 0.25, 0.50, 1.0, 2.0, 4.0, 6.0 mM	8	240
5	KJ	0.0, 1.004, 1.506, 3.012, 6.024, 12.048, 24.096, 36.145 µM	8	240
6	H_3BO_3	5.96, 11.92, 23.83, 47.67, 95.33 µM	40	1200
	$ZnSO_4 \cdot 7H_2O$	0.0, 0.095, 0.192, 0.383, 0.767, 1.533, 2.300, 3.066 µM		
7	K^+/Ca^{2+}	0.15, 0.76, 1.28, 2.57, 3.3, 4.67, 5.75	7	210
8	Total saturation of mineral nutrients	0.75, 1.0, 1.25, 1.5, 1.75	5	150
9	Sugars[a]	0.0, 1.0, 2.0, 3.0% (w/v)	20	600
10	Amino acids[b]	0.0, 50.0, 100.0, 150.0, 200.0 mg l^{-1}	75	2250
11	Combination of amino acids			
	Asparagine	0.0, 50.0, 100.0, 150.0 mg l^{-1}	16	480
	Glutamine	0.0, 50.0, 100.0, 150.0 mg l^{-1}		
12	Myo-inositol	0.0, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 g l^{-1}	8	240
13	Vitamins[c]	0.0, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 15.0 mg l^{-1}	72	2160
14	Combination of vitamins			
	Ascorbic acid	0.0, 4.0, 8.0, 15.0 mg l^{-1}	64	1920
	Thiamine	0.0, 0.1, 0.25, 0.50 mg l^{-1}		
	Pyridoxine	0.0, 1.0, 2.0, 4.0 mg l^{-1}		

[a]Were tested effect different concentrations of sucrose, glucose, maltose, galactose, fructose on the growth of population *W. arrhiza*

[b]Were tested effect different concentrations of leucine, methionine, lysine, isoleucine, asparagine, glycine, cysteine, proline, serine, arginine, glutamine, valine, histidine, phenylalanine, alanine on the growth of population *W. arrhiza*

[c]Were tested effect different concentrations of thiamine, pyridoxine, ascorbic acid, biotin, nicotinic acid and folic acid on the growth of population *W. arrhiza*

Table 1) and were supplemented with 2% sucrose. *W. arrhiza* plants were placed on an agar-gelled medium (10 plants per Petri dish, in triplicates). *L. minor* plants were placed into a liquid medium (10 plants in each of the 50 ml culture vessels, in triplicates). The values of plant growth (the number of fronds) were determined every 5 days. For each of the samples the total number of visible fronds was quantified (the frond number comprised both mother and daughter fronds). In a month after inoculation fresh weight (FW) of each sample was determined through drying the surfaces of the fronds by patting with paper towels, and weighing. After and the weight of one plant in each variant was calculated dividing the mass of the population by the number of plants in it. FW of each count (every 5 days) was calculated based on the number of fronds multiplied by their weight.

Optimization of each media component (the second step)

Based on the composition of the mineral nutrition in the medium that enabled the maximal efficiency of vegetative reproduction of *W. arrhiza* and *L. minor* (basal medium) we performed experiments to determine the optimal impact of each of the minerals (Tables 2, 3, experiments 1–6). In one

Table 3 The scheme for optimizing the balance of the medium for cultivation *L. minor*

Experiment number	Test substances	Tested concentrations of substances	Number of options in the experiment	Number of plants involved in the experiment
1	NO_3^- (KNO_3)	5.33, 7.06, 10.52, 17.44 mM_{eq}	100	3000
	$CuSO_4 \cdot 5H_2O$	0.045, 0.09, 0.18, 0.36, 0.72 µM		
	$CoCL_2 \cdot 6H_2O$	0.105, 0.21, 0.42, 0.84, 1.68 µM		
2	NO_3^- (KNO_3)	5.06, 8.79, 10.52 mM_{eq}	48	1440
	$FeSO_4 \cdot 7H_2O$ (from Iron EDDHA Chelate)	0.017, 0.034, 0.067, 0.134 mM		
	$Na_2MoO_4 \cdot 2H_2O$	0.045, 0.09, 0.18, 0.36 µM		
3	KH_2PO_4	0.099, 0.165, 0.33, 0.66, 1.32, 2.64, 3.30 mM	21	630
	$MnSO_4 \cdot 5H2O$	0.46, 0.91, 1.83 µM		
4	$MgSO_4 \cdot 7H_2O$	0.062, 0.103, 0.205, 0.41, 0.82, 1.23, 1.64 mM	7	210
5	KJ	0.0, 1.004, 1.506, 3.012, 6.024, 12.048, 18.072, 31.41 µM	8	240
6	H_3BO_3	0.485, 1.94, 2.91, 3.88 µM	16	480
	$ZnSO_4 \cdot 7H_2O$	0.156, 0.630, 0.945, 1.260 µM		
7	K^+/Ca^{2+}	0.15, 0.76, 1.28, 2.57, 3.3, 4.67, 5.75	7	210
8	Total saturation of mineral nutrients	0.75, 1.0, 1.25, 1.5, 1.75	5	150
9	Sugars[a]	0.0, 1.0, 2.0, 3.0% (w/v)	20	600
10	Amino acids[b]	0.0, 50.0, 100.0, 150.0, 200.0 mg l^{-1}	75	2250
11	Combination of amino acids			
	Glycine	0.0, 50.0, 100.0, 150.0 mg l^{-1}	16	480
	Glutamine	0.0, 50.0, 100.0, 150.0 mg l^{-1}		
12	Myo-inositol	0.0, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 g l^{-1}	11	330
13	Vitamins[c]	0.0, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 15.0 mg l^{-1}	72	2160
14	Combination of vitamins			
	Folic acid	0.0, 10.0, 15.0, 20.0 mg l^{-1}	64	1920
	Thiamine· HCl	0.0, 8.0, 10.0, 12.0 mg l^{-1}		
	Pyridoxine· HCl	0.0, 1.0, 2.0, 3.0 mg l^{-1}		

[a]Were tested effect different concentrations of sucrose, glucose, maltose, galactose, fructose on the growth of population *L. minor*

[b]Were tested effect different concentrations of leucine, methionine, lysine, isoleucine, asparagine, glycine, cysteine, proline, serine, arginine, glutamine, valine, histidine, phenylalanine, alanine on the growth of population *L. minor*

[c]Were tested effect different concentrations of thiamine, pyridoxine, ascorbic acid, biotin, nicotinic acid and folic acid on the growth of population *L. minor*

of the experiments we studied the impact of the synergetic mineral elements (exp.1—$NO_3^- + Cu^{2+} + Co^{2+}$; exp.2—$NO_3^- + Fe^{2+} + Mo^{2+}$; exp.3—$PO_4^{3-} + Mn^{2+}$; exp.6—$B^{3+} + Zn^{2+}$). Since NO_3^-concentration depends on both the amounts of KNO_3 and $Ca(NO_3)_2 \times 4H_2O$, as a part of which there are ions antagonists (K^+ and Ca^{2+}), we started with the investigation of the optimal concentration of NO_3^- by changing the concentration of KNO_3 in the medium followed by a separate experiment performed at the optimal concentration of NO_3^- (mM_{eq}) to determine the optimal ratio between K^+/Ca^{2+} (Tables 2, 3, experiments 7). In experiments 1–7 all the media contained the remaining mineral components [according to their reference basal medium prescriptions (Table 1)] in addition to the tested compounds. Based on the data obtained in these 7 experiments (Tables 2, 3, experiments 1–7), modeling of the mineral balance was performed as described below. Since the ion balance in the medium is not the only factor affecting the trophic feeding of the plants, we also tested the fraction concentration of the obtained medium on the efficiency of the vegetative reproduction of duckweed (Tables 2, 3, experiment 8). The media in experiments 1–8 contained 2% sucrose as organic supplement.

Optimization of organic components to ensure the effective vegetative reproduction of duckweeds was performed in a similar way (Tables 2, 3, experiments 9–14). The study included testing for different concentrations of myoinositol (Sigma, USA) (Tables 2, 3, experiment 12), 5 carbohydrates (D-sucrose, D-glucose, D-maltose, D-galactose, D-fructose; Panreac, EU) (Tables 2, 3, experiment 9), 15 amino acids (leucine, methionine, lysine, isoleucine, asparagine, glycine, cysteine, proline, serine, arginine, glutamine, valine, histidine, phenylalanine, alanine) (all anhydrous L-form; Sigma, USA) (Tables 2, 3, experiment 10) and 6 vitamins (thiamine, pyridoxine, ascorbic acid, biotin, nicotinic acid and folic acid; Sigma, USA) (Tables 2, 3, experiment 13). Amino acids and vitamins that facilitated the efficient vegetative reproduction of duckweeds were tested to work synergistically in different combinations (Tables 2, 3, experiments 11 and 14). In those experiments, in addition to the rates of biomass growth, the total amount of protein was determined according to the Bradford method (Bradford 1976).

All experiments were terminated in a month after inoculation, and the results were evaluated. To obtain the data required for modeling, 18,150 *W. arrhiza* plants and 14,850 *L. minor* plants were used. At the end of experiments the number of plants was 2,689,349 and 1,644,799 for *W. arrhizal* and *L. minor*, respectively.

Conversion of experimental data (the third step)

The significance of differences between the variants was estimated using analysis of variance (ANOVA) followed by multiple comparisons of individual averages and evaluation by Duncan's test ($P < 0.05$ was considered statistically significant). When statistically significant differences between the tested variants were found, regression curves for the compounds impact were plotted by straightening of the empirical variation curves based on the normal distribution (De Groot 1970). Those plots of the restored functional impact were tested for correspondence with the experimental results by using a determination coefficient of the areas on the plot that included zones of optimum and repression—1 standard deviation zone (from $\mu - \sigma$ to $\mu + \sigma$, where μ—the mean of the distribution, σ—standard deviation). Determination coefficient was set to be $0.95 \leq X \leq 1$ (where X is the ratio of quadratic sum of the deviation to the total quadratic sum of the data). Based on the regression curves that were successfully validated, we calculated the regression equation to determine the maximal y-value that should be as close as possible to the optimal impact factor (Tables 4, 5).

This method is convenient in that it does not require additional verification of experimental values distribution on homoscedasticity, since each value is represented by means of a number of biological replicas from the experiment and is characterized by statistical reliability according to ANOVA of exponent σ^2 (Kjersem et al. 2014). Also an additional test for the normal distribution is not required, so both the parabola and the normal distribution (in the first standard deviation zone) have similar properties (symmetry, continuity, etc.), and the correspondence of approximately normally distributed experimental data and appropriate points of the parabola to the graphic has already been confirmed by using the coefficient of determination (Bozorgmehr and Sebastian 2014). At present similar decisions are often used in clinical epidemiology and population studies (Rabe-Hesketh and Skrondal 2008; Bozorgmehr and Sebastian 2014; Kjersem et al. 2014).

Mathematical optimization model (the fourth step)

Using the obtained equations and the "solution search" tool in MS Excel 2003, we have applied the method for optimization modeling of biological processes to determine the mineral balance of the medium. The well containing the sum of the y-values from all the equations for regression curves was selected to be the target well of modeling. The goal of the modeling was to determine the x-values that would ensure that the target well would reach the maximum value (the maximal value of the sum can be reached only if the values of individual terms are maximal). This should be done in compliance with the number of software and biological limitations for the measured wells that contain x-values for each of the regression curve equations. The software limitations were the scalar limitations for the equation values based on the confidence interval of the regression curve (Tables 4, 5). Because of the presence of antagonistic ions, biological limitations had to be included. Both models had 3 biological limitations: K^+/Ca^{2+} ratio (antagonists of membrane transport into the cell), antiporter/simporter ratio [antagonists that regulate transport inside of plasmalemma and tonoplast—$(Ca^{2+} + Mg^{2+})/(NO^{3-} + PO_4^{3-} + K^+)$], and $Fe^{2+}/(Cu^{2+} + Mo^{6+})$ ratio (active electron carriers and important participants of the basic physiological processes in a plant cell) (Zanin et al. 2015; Gao et al. 2016). The best value of K^+/Ca^{2+} ratio was determined experimentally (Tables 2, 3, experiment 7), while the values of antiporters/simporters and $Fe^{2+}/(Cu^{2+}+Mo^{6+})$ ratios were established based on the most balanced in terms of their mineral composition media (SH, HA and St). To obtain the numerical expression of the antiporter/simporter ratio, we made toting of molarities of all antiporters ($Ca^{2+} + Mg^{2+}$) and divided them into the sum of molarities of all simporters ($NO^{3-} + PO_4^{3-} + K^+$).

Similarly, the Fe/(Cu + Mo) index was calculated. Thus, the restriction of "antiporter/simporter" was expressed by the interval [0.2, 0.5], and $Fe^{2+}/(Cu^{2+}+Mo^{6+})$ by [50.0; 100.0].

Since neither of biological constraints for the values of organic compounds were determined, each compound was modeled independently within its own scalar limitation (Table 4, experiments 9, 11, 12, 14 and Table 5, experiments 9, 11, 14).

Bioassay of the model media (the fifth step)

To verify the results of modeling, we carried out the studies to compare the basal media and created on their basis model media. The goal of the first experiment was to determine the most balanced medium for each duckweed in terms of its mineral composition, and to establish the value of each organic additive when they were added step-wise (each subsequently tested medium contained all the components of the previous medium plus one new ingredient) (Fig. 4).

The efficiency of the modeled medium was evaluated based on the main valuable qualitative parameters of the cultivated populations (population biomass, yield of the dry mass, yield of the total water-soluble protein, the amount of protein in the dry mass). For this evaluation, the second experiment was performed to compare the modeled medium (W3M or L4M) to either the basal medium (HA, SH, St, Table 1) or the basal medium with the modified organic compounds composition. In addition, medium MS and B_5 containing modified organic compositions were tested

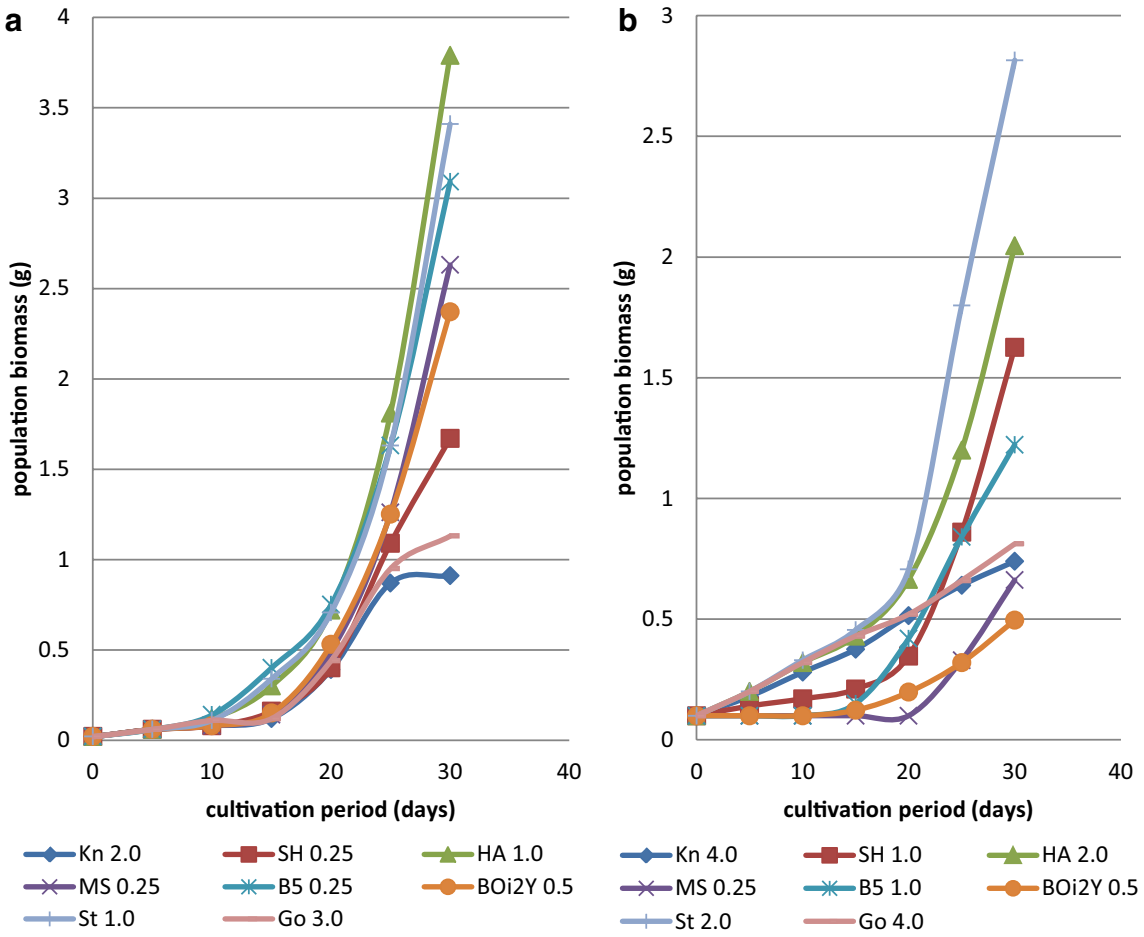

Fig. 1 Biomass growth of duckweed cultivated on the maximal yield medium **(a)** for *W. arrhiza* (Kn 2.0—double strength Knop media, SH 0.25—quarter strength Schenk & Hildebrandt media, HA 1.0—single strength Hoagland & Arnon media, MS 0.25—quarter strength Murashige & Skoog media, B5 0.25—quarter strength Gamborg media, BOi2Y 0.5—half strength BOi2Y media, St 1.0—single strength Steinberg media, Go 3.0—triple strength Gorham media) and **b** for *L. minor* (Kn 4.0—quadruple strength Knop media, SH 1.0—single strength Schenk & Hildebrandt media, HA 2.0—double strength Hoagland & Arnon media, MS 0.25—quarter strength Murashige & Skoog media, B5 0.5—single strength Gamborg media, BOi2Y 0.5—half strength BOi2Y media, St 2.0—double strength Steinberg media, Go 4.0—quadruple strength Gorham media)

(Table 7A). Modified media HA, SH, St, MS and B5 to be compared with W3M medium (part A in Table 7) contained 2% sucrose, 150 mg l^{-1} casein hydrolysate [the most widely used source of amino acids (Banerjee 2001; Ma et al. 2003; Friedrich 2005; Iantcheva et al. 2005)] (concentration was determined based on the amounts of amino acids asparagine + glutamine in W3M medium), 100 mg l^{-1} myo-inositol [the most commonly used concentration for the medium supplementation (Murashige and Skoog 1962; Gamborg et al. 1968)] and vitamin complex for MS (Murashige and Skoog 1962) (HA^+, SH^+, St^+ in part A of Table 7); modified medium HA, SH, St, MS and B5 to be compared with L4M medium (part B in Table 7) contained 2% of fructose, 200 mg l^{-1} casein hydrolysate (concentration was determined based on the amounts of amino acids glycine + glutamine in L4M medium) and vitamin complex for MS (HA^+, SH^+, St^+ in part B of Table 7).

Wolffia arrhiza was placed into liquid nutrient medium (100 plants/500 ml flask, in triplicates). The medium volume in each flask was 300 ml, cultivation was performed using a shaker (90 rpm). *L. minor* was placed into liquid nutrient medium (10 plants/300 ml culture vessel, in triplicates). The medium volume in each culture vessel was 100 ml. Both experiments were terminated in a month after inoculation. Fresh weight (population biomass) of each sample was determined by drying the surfaces of the fronds by patting with paper towels and weighing.

The use of organic additives in culture medium is too expensive for the industrial production of duckweed. Therefore for definition of the conditions similar to the industrial production, 10 fronds of both duckweeds were randomly selected as the inoculum for measuring growth rate parameters (RGR, DT and RY) and placed into 300-ml individual culture vessels covered with a disk of filter

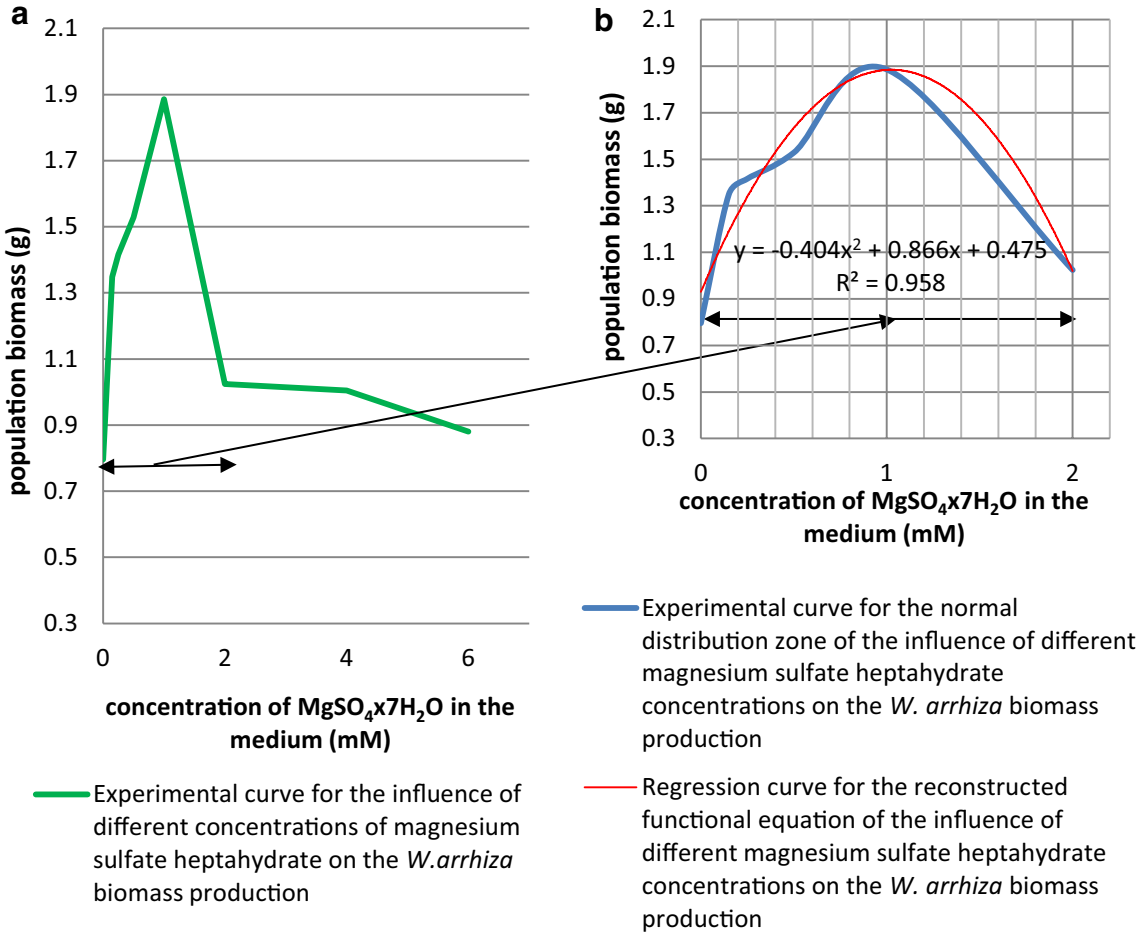

Fig. 2 The example of developing regression curve and derivation of the quadratic equation for describing this curve, and the influence of different concentrations of $MgSO_4 \cdot 7H_20$ on *W. arrhiza* biomass yield is shown. **a** The total experimental curve (in the interval 0.0—6.0 mM $MgSO_4 \cdot 7H_20$); **b** the curves of the influence of different concentrations of $MgSO_4 \cdot 7H_20$ in the interval of normal distribution (0.0–2.0 mM). The figure demonstrates the results in a month of cultivation. HA medium was used as a basic medium for variation

paper and containing 100 ml autoclaved medium (W3M, St and HA; all without organic components in triplicate for each duckweed). Cultivation lasted for 7 days, during which the size of the inoculum ensured that the fronds never completely covered the surface of the medium, which would have led to growth limitation. The values of all parameters were determined at the onset of the experiment and 7 days later. Growth rate parameters were calculated according to the method described by Ziegler et al. (2015). All plants were cultivated at 21 ± 1 °C with the light intensity 65 µmol m^{-2} s^{-1} during 16-h day photoperiod.

Results and discussion

The experiments on determination of the cultivation medium mineral balance ensuring the maximal reproduction of *W. arrhiza* have shown that the population reaches the peak of biomass productivity in the medium poor in mineral composition (such as HA and St). The most effective medium for *W. arrhiza* cultivation is HA medium (Fig. 1a) containing nutritious elements according to the original prescriptions (Hoagland and Arnon 1938). This medium allows to achieve good results of cultivation for a wide range of plant species, but in view of its relative universality this medium is incapable of meeting the maximal trophic needs of *W. arrhiza*. Individual optimization of mineral and organic bases of HA medium can lead to increased biomass yield.

The maximal biomass production of *L. minor* population was achieved at St medium with doubled amount of mineral nutrition elements as compared to the original prescriptions (single strength concentrations) (Fig. 1b). Despite of the fact that Steinberg developed his cultivation medium according to the needs of *L. minor* mineral nutrition (Steinberg 1946), the necessity of doubling the amount of mineral elements compared to the original prescriptions can be explained by the high ecological flexibility of this duckweed. Unlike other representatives of *Lemna* genus, *L. minor* is capable of

Table 4 Analysis results of experiments to determine the effect of various nutritional elements on the productivity of *W. arrhiza* under in vitro conditions

Experiment number	Test substances	Limits of concentrations of test substances	Regression equations	Interval of reliability of regression equation	Estimated optimal solution
1	NO_3^- (from KNO_3)	7.49–18.74 mM_{eq}	$y = -0.062x^2 + 1.559x - 7.887$	[8.74, 16.24] mM_{eq}	11.07 mM_{eq}
	$CuSO_4 \cdot 5H_2O$	0.045–1.80 µM	$y = -26.53x^2 + 7.101x + 0.957$	[0.045, 0.180] µM	0.132 µM
	$CoCL_2 \cdot 6H_2O$	0.0–1.05 µM	$y = -0.748x^2 + 0.810x + 1.192$	[0.0, 1.05] µM	0.56 µM
2	NO_3^- (from KNO_3)	8.74–13.74 mM_{eq}	$y = -0.008x^2 + 0.199x - 0.283$	[8.74,13.74] mM_{eq}	12.10 mM_{eq}
	$FeSO_4 \cdot 7H_2O$	0.0–0.804 mM	$y = -298.6x^2 + 26.32x + 0.362$	[0.0, 0.067] mM	0.047 mM
	$Na_2MoO_4 \cdot 2H_2O$	0.047–0.744 µM	$y = -0.914x^2 + 0.779x + 0.785$	[0.045, 0.744] µM	0.43 µM
3	KH_2PO_4	0.0–2.5 mM	$y = -0.182x^2 + 0.507x + 0.37$	[0.0, 2.5] mM	1.39 mM
	$MnSO_4 \cdot 5H2O$	2.303–9.212 µM	$y = -0.024x^2 + 0.297x - 0.178$	[2.303, 9.212] µM	6.02 µM
4	$MgSO_4 \cdot 7H_2O$	0.0–6.0 mM	$y = -0.404x^2 + 0.866x + 0.475$	[0.0, 2.0] mM	1.09 mM
5	KJ	0.0–36.15 µM	$y = -0.011x^2 + 0.108x + 0.550$	[0.0, 12.05] µM	4.68 µM
6	H_3BO_3	5.96–95.33 µM	$y = -0.0001x^2 + 0.002x + 0.839$	[5.96, 95.33] µM	40.71 µM
	$ZnSO_4 \cdot 7H_2O$	0.0–3.066 µM	$y = -30.67x^2 + 7.266x + 0.63$	[0.0, 0.383] µM	0.143 µM
7	K^+/Ca^{2+}	0.2–2.2	$y = -0.758x^2 + 1.29x + 0.031$	[0.2, 1.1]	0.98
8	Total saturation of mineral nutrients	0.5–1.75	$y = -1.371x^2 + 3.463x - 1.292$	[0.75, 1.75]	1.23
9	Sucrose	0.0–3.0% (w/v)	$y = -0.149x^2 + 0.595x + 0.068$	[0.0, 3.0] %	2.0% (w/v)
11	Combination of amino acids				
	Asparagine	0.0–150.0 mg l^{-1}	$y = -0.0001x^2 + 0.068x + 5.43$	[0.0, 150.0] mg l^{-1}	55.5 mg l^{-1}
	Glutamine	0.0–150.0 mg l^{-1}	$y = -0.0001x^2 + 0.147x + 0.909$	[50.0, 150.0] mg l^{-1}	89.4 mg l^{-1}
12	Myo-inositol	0.0–6.0 g l^{-1}	$y = -14.77x^2 + 8.807x + 0.034$	[0.1, 0.5] g l^{-1}	0.3 g l^{-1}
14	Combination of vitamins				
	Ascorbic acid	0.0–15.0 mg l^{-1}	$y = -0.002x^2 + 0.051x + 0.644$	[0.0, 15.0] mg l^{-1}	10.0 mg l^{-1}
	Thiamine· HCl	0.0–0.5 mg l^{-1}	$y = -3.225x^2 + 2.086x + 0.671$	[0.0, 0.5] mg l^{-1}	0.28 mg l^{-1}
	Pyridoxine· HCl	0.0–4.0 mg l^{-1}	$y = -0.624x^2 + 3.759x - 4.127$	[1.0, 4.0] mg l^{-1}	2.97 mg l^{-1}

growing in ponds either rich or poor in mineral and organic nutrition (Wolff 1992). Leng (1999) pointed out that when growing *L. minor* using liquid manure or manure recycle waste from producing biogas as nutrition, the amount of raw protein in duckweed tissues increased from 15 to 37% with the increase of nitrogen concentration in water from 1–4 to 10–15 mg l^{-1}, correspondingly. When the concentration of nitrogen in water reached 50 mg l^{-1}, the ammonium compounds led to intoxication and as a result to inhibition of population growth.

Most likely different geographical isolates of *L. minor* have different needs in the elements of mineral nutrition in the medium. Our data suggest that the highest productivity of *L. minor* may be achieved when the content of mineral nutrition per unit is changed, while the ion balance of the medium corresponds to the original Steinberg media (Fig. 1b).

Based on the curves shown in Fig. 1, we can conclude that in trophic terms duckweeds prefer media that are quite poor in mineral composition, lack of NH_4^+ ions (HA, St and SH).

After determining the most balanced media for duckweed cultivation, we have performed the experiments to optimize HA (Table 2) and St (Table 3) media to increase duckweed growth in vitro. Based on the experimental data, graphs of the impact of the tested compounds within the given concentration intervals were plotted (Fig. 2a). These curves are based on discrete values and thus are unable to represent the exact optimal impact of the tested compound. The other disadvantages of such curves are their dependence on the experimental mistakes, measurement errors, heterogeneity of the subjects' physiological conditions and possible random factors that lead to the skewing of the data. It is known that despite of the errors in the certain experimental values, a lot of empirical distributions, particularly in the field of plant physiology, obey the rules of the normal distribution (De Groot 1970), meaning that they have the binominal distribution. Thus, while creating the probability model, we assumed the distribution of each observed value to be close to normal. The observed values that have the binominal distribution properties should obey the rules of multinomial distribution in the multidimentional case (the

Table 5 Analysis results of experiments to determine the effect of various nutritional elements on the productivity of *L. minor* under in vitro conditions

Experiment number	Test substances	Limits of concentrations of test substances	Regression equations	Interval of reliability of regression equation	Estimated optimal solution
1	NO_3^- (from KNO_3)	5.33–17.44 mM_{eq}	$y = -0.017x^2 + 0.244x - 0.2$	[5.33, 10.52] mM_{eq}	7.04 mM_{eq}
	$CuSO_4 \cdot 5H_2O$	0.045–0.720 µM	$y = -27.11x^2 + 5.95x + 0.284$	[0.045, 0.180] µM	0.103 µM
	$CoCL_2 \cdot 6H_2O$	0.105–1.680 µM	$y = -6.160x^2 + 3.577x + 0.115$	[0.105, 0.420] µM	0.282 µM
2	NO_3^- (from KNO_3)	5.06–10.52 mM_{eq}	$y = -0.004x^2 + 0.055x + 0.328$	[5.06,10.52] mM_{eq}	7.10 mM_{eq}
	$FeSO_4 \cdot 7H_2O$	0.017–0.134 mM	$y = -31.27x^2 + 6.012x + 0.223$	[0.034, 0.134] mM	0.107 mM
	$Na_2MoO_4 \cdot 2H_2O$	0.045–0.36 µM	$y = -1.112x^2 + 0.452x + 0.397$	[0.045, 0.36] µM	0.201 µM
3	KH_2PO_4	0.099–3.3 mM	$y = -1.247x^2 + 1.892x + 0.974$	[0.099, 2.64] mM	0.78 mM
	$MnSO_4 \cdot 5H2O$	0.46–1.83 µM	$y = -0.150x^2 + 0.342x + 1.015$	[0.460, 1.830] µM	1.138 µM
4	$MgSO_4 \cdot 7H_2O$	0.062–1.64 mM	$y = -1.236x^2 + 2.111x + 1.119$	[0.062, 1.64] mM	0.85 mM
5	KJ	0.0–31.41 µM	$y = -0.634x^2 + 2.589x - 1.17$	[0.1004, 3.012] µM	2.146 µM
6	H_3BO_3	0.485–3.880 µM	$y = -0.151x^2 + 0.755x - 0.21$	[0.485, 3.880] µM	2.559 µM
	$ZnSO_4 \cdot 7H_2O$	0.156–1.260 µM	$y = -0.595x^2 + 0.758x + 0.483$	[0.156, 1.260] µM	0.651 µM
7	K^+/Ca^{2+}	0.15–5.75	$y = -0.164x^2 + 0.915x + 2.047$	[0.15, 5.75]	2.74
8	Total saturation of mineral nutrients	0.75–1.75	$y = -13.28x^2 + 33.04x - 17.18$	[1.0, 1.5]	1.24
9	Fructose	0.0–3.0% (w/v)	$y = -3.872x^2 + 14.28x + 0.510$	[0.0, 3.0] %	2.0% (w/v)
11	Combination of amino acids				
	Glycine	0.0–150.0 mg l^{-1}	$y = -0.001x^2 + 0.235x - 6.468$	[50.0, 150.0] mg l^{-1}	112.34 mg l^{-1}
	Glutamine	0.0–150.0 mg l^{-1}	$y = -0.001x^2 + 0.158x - 3.071$	[50.0, 150.0] mg l^{-1}	105.11 mg l^{-1}
14	Combination of vitamins				
	Folic acid	0.0–20.0 mg l^{-1}	$y = -0.006x^2 + 0.197x + 0.190$	[10.0, 20.0] mg l^{-1}	15.75 mg l^{-1}
	Thiamine· HCl	0.0–12.0 mg l^{-1}	$y = -0.032x^2 + 0.640x - 1.520$	[8.0, 12.0] mg l^{-1}	10.34 mg l^{-1}
	Pyridoxine· HCl	0.0–3.0 mg l^{-1}	$y = -0.121x^2 + 0.265x + 1.477$	[0.0, 2.0] mg l^{-1}	0.92 mg l^{-1}

number of dimensions corresponds to the number of the observed values) (De Groot 1970). Thus, the optimization of the compound concentrations (values) in the media for plant cultivation becomes the unified multinomial problem.

The simplest approach to solve such problems is to establish a system of differential equations and deciding them. However, such a method results in the unacceptable complexity when it is attempted to use it for solving more complex models (Bailey 1967). Thus, we attempted to establish another approach that is based on simplifying the system: every value that obeys the normal distribution can be represented as a parabola within the optimal range of concentrations (Fig. 2b). This parabola is described by the standard quadratic equation with the strict limitation of confidence interval. As a result of this simplification, two multinomial problems have been established in a form of a complex of quadratic equations (Table 4: a problem of nutrient optimization of *W. arrhiza* and Table 5: a problem of nutrient optimization of *L. minor*). All of the equations in these tables describe the impact of some compounds on the duckweed growth, except for the impact of amino acids. Because of the fact that adding of amino acids [asparagine and glutamine for *W. arrhiza* (Fig. 3a), glycine and glutamine for *L. minor* (Fig. 3b)] enabled the increase in the protein composition along with the increment in duckweed growth, the equations based on the value of the total water-soluble protein concentration derived from the resulting duckweed population, were established. Based on the ranged curves of the combined impact of amino acids (Fig. 3a,b, green line), we can conclude that the addition of amino acids at optimal concentrations can dramatically increase the productivity of protein production (3.6-fold increase for *W. arrhiza* and > 10-fold increase for *L. minor* compared to the control).

We used the method of optimization modeling of the biological processes using "solution search" tool in MS Excel 2003 to deal with such multinomial tasks on determination of the optimal concentrations of compounds in the media. We have created one cell for the sum of y-values

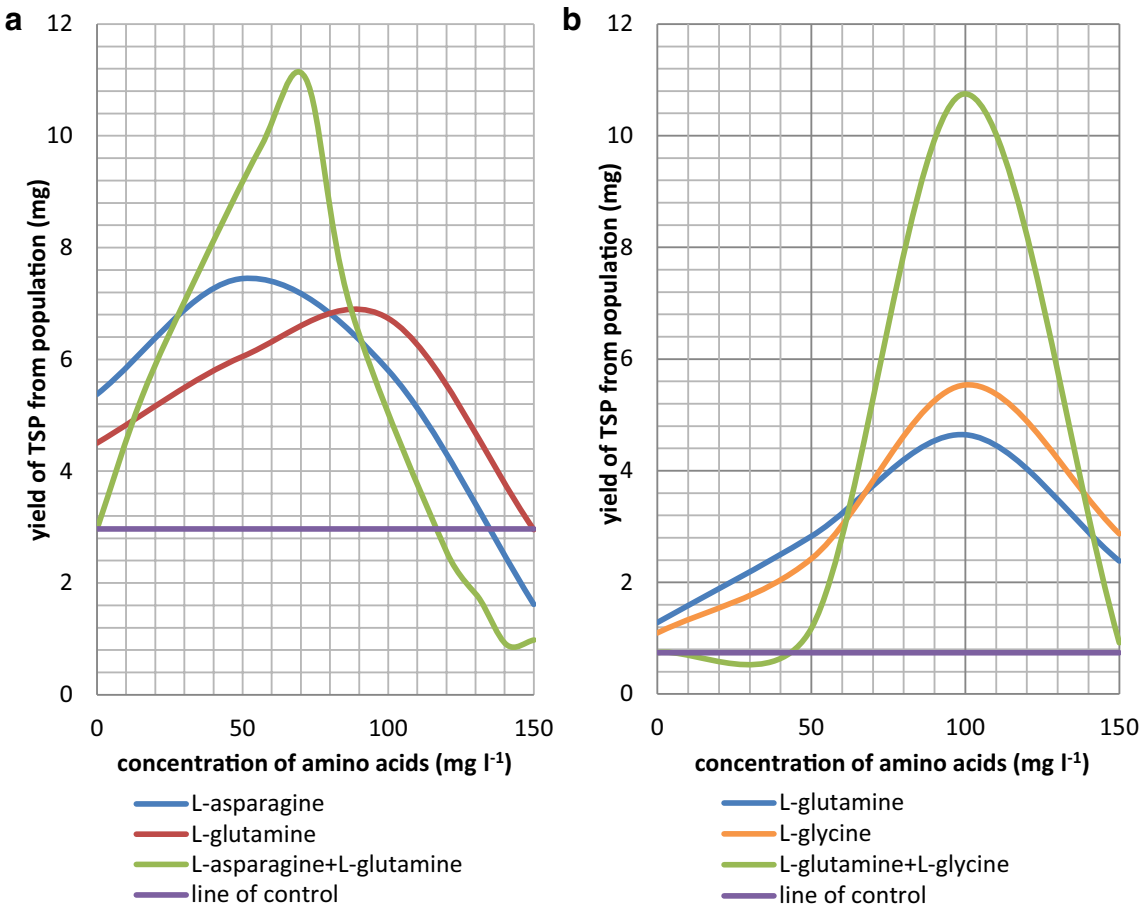

Fig. 3 The effect of individual amino acids (**a** asparagine and glutamine on *W.arrhiza*, **b** glutamine and glycine on *L.minor*), as well as their combined effect (separately vs. combined—ranked curve) on the total protein amount obtained from the population of both duckweeds. (Color figure online)

Table 6 The composition of the culture media according to the modeling results

Components	W3M	L4M
Inorganic salts (mM)		
Calcium nitrate·4 H_2O	3.7	1.85
Magnesium sulfate·7 H_2O	1.09	0.85
Potassium nitrate	3.68	5.07
Potassium phosphate monobasic	3.64	0.78
Inorganic salts (μM)		
Boric acid	40.71	2.559
Cobalt chloride·6 H_2O	0.56	0.282
Cupric sulfate·5 H_2O	0.132	0.103
EDTA acid, Na_2·2 H_2O	47.0	107.0
Ferrous sulfate·7 H_2O	47.0	107.0
Manganese sulfate·H_2O	6.02	1.138
Molybdic acid (sodium salt)·2 H_2O	0.43	0.201
Potassium iodide	4.68	2.146
Zinc sulfate·7 H_2O	0.143	0.651
Organics (mM)		
Sucrose	58.4	0.0
Fructose	0.0	111.0
Myo-inositol	1.67	0.0
L-Glycine (sodium glycinate)	0.0	1.16
L-Glutamine (anhydrous)	0.61	0.72
L-Asparagine (anhydrous)	0.42	0.0
Organics (μM)		
Folic acid	0.0	35.68
Thiamine · HCl	0.83	30.66
Pyridoxine · HCl	14.45	4.47
Ascorbic acid	50.51	0.0

of all quadratic equations of multinomial task regression to establish a unified target cell of modeling within each task. The goal of modeling was set up to be the calculation of the maximal value for this cell because, according to the mathematical rules, the maximal value of such target cell can be reached only when the individual y-values are maximal. As a result of modeling we have obtained the solutions for each equation of the multinomial complex (Tables 4, 5) that were used to calculate the concentrations of all nutrition elements in the media (Table 6) and to develop the prescriptions for the optimal media. The modeled cultivation medium for *W. arrhiza* was called W3M (*Wolffia arrhiza* model multiplying medium) (Dolgov et al. 2013—Patent RU2472338C1), and for *L. minor*—L4M (*Lemna minor* model multiplying medium) (Khvatkov et al. 2016—Patent RU2578394C1).

After testing the modeled media and determining the actual significance of each of the modeled organic component considering its step-wise addition (every next version of the medium contains all of the ingredients of the previous one plus one new component) it has been determined that the use of the modeled media without adding any organic components results in 1.5- to twofold increase in the duckweeds biomass [93.3% of increase in *W. arrhiza* (Fig. 4a) and 76.9% increase in *L. minor* (Fig. 4b)] compared to the cultivation in HA and St media. Adding carbohydrates into the modeled non-organic media was beneficial for both increase in biomass (48.5% of increase in *W. arrhiza* and 72.8% increase in *L. minor*) and protein concentration (6.7% of increase in the dry mass of both duckweeds). Adding the vitamins was beneficial only for biomass increase (21.9% of increase in *W. arrhiza* and 40.9% increase in *L. minor*). Addition of amino acids into the media resulted in the 1.5-fold increase in the biomass (53.8% of increase in *W.arrhiza* and 64.3% increase in *L.minor*) while the protein concentration was more than 10% increased (40.1% of TSP in the dry mass of *W. arrhiza* and 37.2% of TSP in the dry mass of *L. minor*) (Fig. 4). In general, the data presented in Fig. 1, make it possible to conclude that organic components of the culture media make a significant contribution to the duckweed harvest. Addition of carbohydrates, vitamins and amino acids to the optimized mineral nutrition media resulted in the 2.8-fold increase in biomass production of *W. arrhiza* and fourfold increase of that of *L. minor*. The protein concentration was 1.4- to 2.0-fold increased as well (1.4-fold increase of TSP in the dry mass of *W. arrhiza* and 2.0-fold increase of in *L. minor*). Thus, enrichment of the nutrition media with the optimal concentrations of the organic components enabled to increase the yield of the total protein amount 3.9-fold and eightfold for *W. arrhiza* and *L. minor*, respectively.

After establishing the significance of the impact of organic compounds on the duckweed productivity, we have performed an experiment to determine the ability of duckweed to realize the capacity of productivity in the media with different mineral composition with the fixed organic components background (Table 7).

The significant differences among all of the factors of productivity were achieved by using the modeled media (W3M and L4M) and their closest analogs (HA^+ and St^+) compared to other media (SH, MS and B_5). It is worth noting that after the alignment of the levels of organic compounds in HA and St media (the versions HA^+ and St^+) the parameters of productivity in duckweed populations [*W. arrhiza* (Table 7A) and *L.minor* (Table 7B)] did not differ

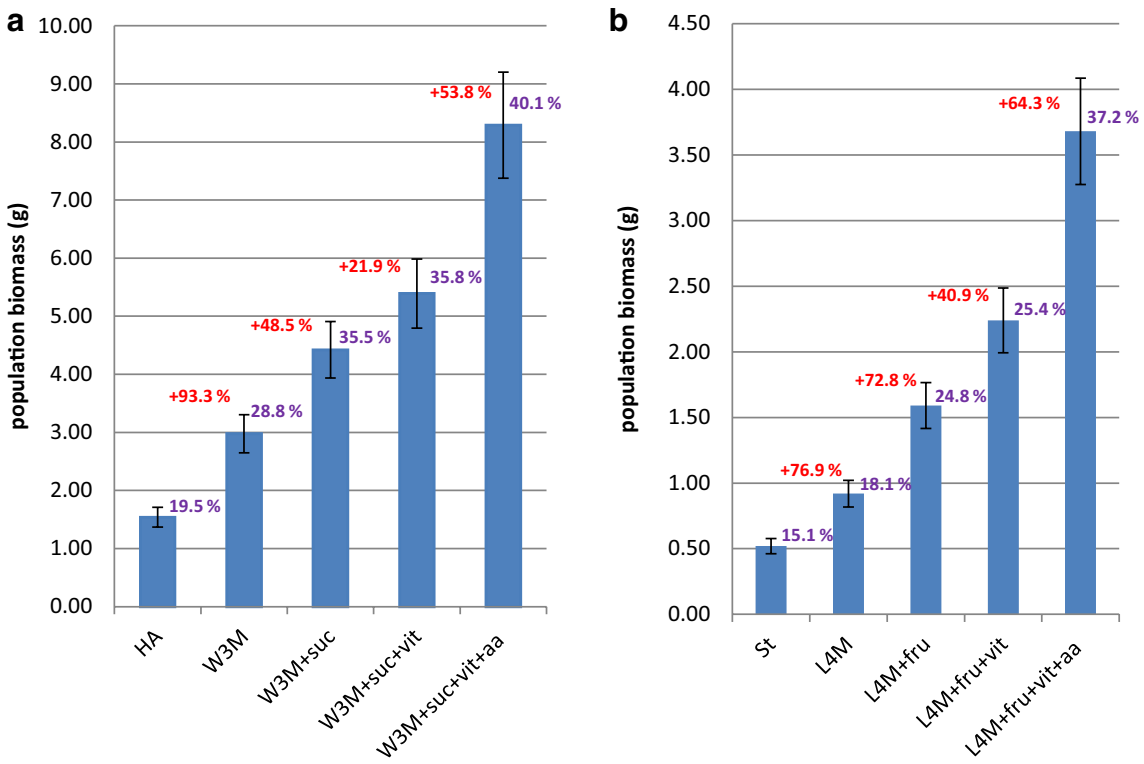

Fig. 4 Biomass production for **a** *W. arrhiza* and **b** *L. minor* obtained from incremental modelled nutrition elements. HA—classic Hoagland & Arnon medium lack of organic components; St—classic Steinberg medium lack of organic components; W3M—modelled medium (lack of organic components), designed for *W.arrhiza*; L4M—modelled medium (lack of organic components), designed for *L. minor*; W3M + suc—mineral background of the modelled W3M medium enriched with 2% of sucrose; L4M + fru—mineral background of the modelled L4M medium enriched with 2% of fructose; W3M + suc + vit—the W3M + suc medium with the vitamin supplementations (modelled concentrations of 0.83 µM thiamine, 14.45 µM pyridoxine, 50.51 µM ascorbic acid and 1.67 mM myoinositol); L4M + fru + vit—the L4M + fru medium with the vitamin supplementations (modelled concentrations of 30.66 µM thiamine, 4.47 µM pyridoxine and 35.68 µM folic acid),W3M + suc + vit + aa—the W3M + suc + vit medium with the addition of amino acids (modelled concentrations of 4.42 mM asparagine and 0.61 mM glutamine); L4M + fru + vit + aa—the L4M + fru + vit medium with the addition of amino acids (modelled concentrations of 1.16 mM glycine and 0.72 mM glutamine). The red markings, to the left of the column, indicate the percentage of the biomass growth on the new medium compared to the previous one. The purple markings, to the right of the column, indicate the amount of protein in the dry mass calculated relative to the dry mass for every medium

statistically. In those versions of the media the impact of the differences in mineral balance was negated by high levels of organic nutrients. The most statistical significance in productivity of duckweed growth was achieved using the modeled media for in vitro duckweed cultivation (Table 7). The modeled media (W3M and L4M) enabled obtaining 1.5- to 2.0-fold increase in duckweed biomass production having higher protein content in dry mass (1.5-fold increase) as compared to the analogous media (HA^+ and St^+).

The use of model culture media for industrial production of duckweed is proved to be effective. The data shown in the Table 8 represent true growth potential of our clones. In all cases by the end of the 7-day cultivation, the surface of the culture medium was far from being covered by even the most rapidly propagating fronds. pH of the medium was between 5.5 (typical for HA and St media) and 5.1 (for W3M media), and so differed slightly from the initial value of 5.80. The increase in FW never deviated from an exponential progression within 7-day period, and the proportions of differently sized fronds remained constant. As a result, it was found that when using model media for growing duckweed RGR was 1.2 to 1.3 higher (1.2 for *L. minor* and 1.3 for *W. arrhiza*), the DT was 17 to 28% less (17% for *L. minor* and 28% for *W. arrhiza*), and RY was 1.5 to 1.7 times more (1.5 for *L. minor* and 1.7 for *W. arrhiza*) as compared with HA and St media.

Table 7 The influence of the composition of the culture medium on the main actual significant indicators of cultivated populations *W. arrhiza* and *L. minor*

Culture medium	A. *W. arrhiza* (W3M)					B. *L. minor* (L4M)				
	Biomass population (g)	Total soluble protein (μg/100 mg fresh weight)	Total yield of TSP (mg)	Dry weight (%)	Protein in dry weight (%)	Biomass population (g)	Total soluble protein (μg/100 mg fresh weight)	Total yield of TSP (mg)	Dry weight (%)	Protein in dry weight (%)
W3M/L4M	19.05 g	1964.04 h	374.15 f	4.9 cd	40.1 f	3.77 d	8643.50 c	325.86 e	23.3 cd	37.1 d
HA	6.99 de	860.94 d	60.18 bc	3.9 c	22.1 bc	1.31 bc	5265.29 b	68.91c	28.9 d	18.2 a
HA^+	8.36 ef	1444.74 fg	120.78 de	4.6 cd	31.4 def	1.76 c	6981.82 b	122.88 d	30.3 e	23.0 ab
St	6.64 cde	1101.66 e	73.15 c	4.3 cd	25.6 cde	1.40 bc	3687.55 ab	51.49 c	17.9 ab	20.6 ab
St^+	8.93 f	1460.81 g	130.45 e	4.5 cd	32.5 ef	1.67 c	6188.02 b	103.34 cd	21.1 bcd	29.3 b
SH	0.93 a	403.23 a	3.75 a	5.9 e	6.8 a	0.91 bc	2413.40 a	21.99 bc	13.3 a	18.1a
SH^+	2.04 ab	947.06 de	19.32 a	4.8 d	19.7 bc	1.56 c	3299.36 ab	51.47 c	10.8 a	30.5 bc
MS^+	1.42 a	866.90 d	12.31 a	4.9 d	17.7 bc	0.58 b	4994.82 ab	28.97 bc	25.6 d	19.5 ab
B_5^+	3.58 b	901.68 cd	32.28 ab	4.2 cd	21.5 bc	1.58 c	3042.41ab	48.07 c	9.1 a	33.4 cd

W3M and L4M mediums were used in the compositions shown in Table 6. HA, SH, St, MS and B_5 mediums (not marked with a "+") were used according to the author's prescriptions shown in the Table 1. The sign "+" indicates culture media with a modified organic composition

Different letters in a column indicate significant differences in variant data according by Duncan's test

The table shows the results after 1 month of cultivation

Table 8 Growth rates of *W. arrhiza* and *L. minor* on different culture media

Species	Culture medium	RGR	DT	RY
W. arrhiza	W3M	0.395 ± 0.041	1.77 ± 0.09	11.5 ± 2.1
	HA	0.267 ± 0.028	2.73 ± 0.12	5.9 ± 0.8
	St	0.325 ± 0.032	2.16 ± 0.15	7.8 ± 1.3
L. minor	L4M	0.476 ± 0.024	1.48 ± 0.11	16.9 ± 2.2
	HA	0.366 ± 0.015	1.89 ± 0.09	9.6 ± 1.6
	St	0.414 ± 0.020	1.67 ± 0.08	12.5 ± 1.8

The values of RGR, DT and RY quoted here were calculated from fresh weight measurements. Errors are SE of means (n = 3). All used culture media are lack of organic components

RGR relative growth rate (day^{-1}), *DT* doubling time (day), *RY* relative yield after 1 week ($week^{-1}$)

Conclusion

HA medium and St medium are the most balanced medium in terms of the mineral composition for *W. arrhiza* and *L. minor*, respectively. Based on the mathematical optimization model, we have developed individually-balanced cultivation medium (W3M and L4M) which enabled us to obtain 1.5–2.0 times more duckweed biomass with the 1.5 times higher concentration of protein in their dry mass. Thus, we have demonstrated that the method of the optimization modeling of the biological processes based on solving multinomial task from the series of quadratic equations can be used for optimization of trophic needs of plants, specifically for micro-propagation of duckweeds in vitro.

Author contributions The authors have made the following declarations regarding their contributions: Conceived and designed the experiments: PK. Performed the experiments: PK, MC, AO. Analyzed the data: PK, MC, SD. Contributed reagents/materials: SD. Contributed to the writing of the manuscript: PK, MC, SD.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Akter M, Chowdhury SD, Akter Y, Khatun MA (2011) Effect of duckweed (Lemna minor) meal in the diet of laying en and their performance. Bangladesh Res Publ J 5:252–261

Bailey NTJ (1967) The mathematical approach to biology and medicine. Wiley, London, pp 174–183

Banerjee AK (2001) In vitro regeneration and genetic transformation studies in indian cultivars of cotton (*Gossypium hirsutum* L.). PhD thesis, University of Pune, plant tissue culture division national chemical laboratory, India. January 2001, p 183

Blaydes DF (1966) Interaction of kinetin and varius inhibitors in the growth of soybean tissue. Physiol Plant 19:748–753

Boehm R, Kruse C, Veste D, Barth S, Schnabl H (2001) A transient transformation system for duckweed *Wolffia columbiana* using Agrobacterium-mediated gene transfer. J Appl Bot 75:107–111

Bozorgmehr K, Sebastian MS (2014) Trade liberalization and tuberculosis incidence: a longitudinal multi-level analysis in 22 high burden countries between 1990 and 2010. Health Policy Plan 29(3):328–351

Bradford MM (1976) A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Branson RE, Everett K, Hester B, Vickers TB (2007) Biorector for growing biological materials supported on a liquid surface. USA Patent Publication US7176024B2

Daniella H, Streatfieldb SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci J 6:219–226

De Groot MH (1970) Optimal statistical decisions. McGraw-Hill series in probability and statistics, New York, pp 45–347

Dolgov SV, Chernobrovkina MA, Khvatkov PA (2013) Composition of medium for culturing duckweed plants (*Wolffia arrhiza*) in vitro. Russian Patent Publication RU2472338C1

Firsov A, Tarasenko I, Mitiouchkina T, Ismailova N, Shaloiko L, Vainstein A, Dolgov S (2015) High-yeld expression of M2e peptide of avian influenza virus H5N1 in transgenic duckweed plants. Mol Biotechnol 57:653–661

Firsov A, Tarasenko I, Mitiouchkina T, Shaloiko L, Kozlov O, Vinokurov L, Rasskazova E, Murashev A, Vainstein A, Dolgov S (2018) Expression and immunogenicity of M2e peptide of avian influenza virus H5N1 fused to ricin toxin b chain produced in duckweed plants. Front Chem 6:22

Friedrich AS (2005) Transformation und fermentation von Wolffia spec. Dissertation, Untersuchungen zu Kultivierung. Vorgelegt. p 154

Gago J, Martinez-Nunez L, Landin M, Gallego PP (2010) Artificial neural networks as an alternative to the traditional statistical methodology in plant research. J Plant Physiol 167:23–27

Gallego PP, Gago J, Landin M (2011) Artificial neural networks technology to model and predict plant biology process. In: Suzuki K (ed) Artificial neural networks—methodological advances and biomedical applications. In tech Open Access Publisher, Rijeka. pp 197–216

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gao JS, Wu FF, Shen ZL, Meng Y, Cai YP, Lin Y (2016) A putative molybdate transporter LjMOT1 is required for molybdenum transport in Lotus japonicas. Physiol Plant 158:331–340

Gasdaska JR, Spenser D, Dickey L (2003) Advantages of therapeutic protein production in the aquatic plant Lemna. Bio Process J 2:49–56

Gorham PR (1950) Heterotrophic nutrition of seed plants with particular reference to *Lemna minor* L. Can J Res 28:356–381

Haberlandt G (1902) Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber K Preuss Akad Wiss Wien. Math Naturwiss 111:69–92

Hoagland DR, Arnon DI (1938) The water-culture method for growing plants without soil. Circular/University of California, College of Agriculture, Agricultural Experiment Station, Berkeley, pp 347–353

Iantcheva A, Vlahova M, Gvetoslavova S, Evtimova M, Atanassov A (2005) Somatic embryogenesis of the model legume—*Medicago truncatula* and other diploid medics. Biotechnol Biotechnol Equip 19:41–47

Jamshidi S, Yadollahi A, Ahmadi H, Arab MM, Eftekhari M (2016) Predicting in vitro culture medium macro-nutrients composition for pear rootstocks using regression analysis and neural network models. Front Plant Sci 7(suppl 274):1–12

Jimenez D, Perez-Uribe A, Satizabal H, Barreto M, Damme VP, Tomassini M (2008) A survey of neural network-based modeling in agroecology. In: Prasad B (ed) Soft computing applications in industry, STUDFUZZ, vol 226. Springer, Berlin, pp 247–269

Khvatkov PA, Chernobrovkina MA, Sinyov VV, Dolgov SV (2013) Study on conditions for *Wolffia arrhiza* (L.) Horkel ex Wimm submerged culturing in a modified bioreactor. Biotekhnologiya 6:51–56. (**In Russian, with English Abstract**)

Khvatkov P, Chernobrovkina M, Okuneva A, Pushin A, Dolgov S (2015) Transformation of *Wolffia arrhiza* (L.) Horkel ex Wimm. Plant Cell Tiss Org Cult 123:299–307

Khvatkov P, Chernobrovkina M, Okuneva A, Dolgov S (2016) Composition of medium for cultivation of Lemnaceae family plants (*Lemna minor*) under in vitro conditions. Russian Patent Publication RU2578394C1

Khvatkov P, Firsov A, Shvedova A, Shaloiko L, Kozlov O, Chernobrovkina M, Pushin A, Tarasenko I, Chaban I, Dolgov S (2018) Development of *Wolffia arrhiza* as a producer for recombinant human granulocyte colony-stimulating factor. Front Chem 6:304

Kjersem JB, Skovlund E, Ikdahl T, Guren T, Kersten C, Dalsgaard AM, Yilmaz MK, Fokstuen T, Tveit KM, Kure EH (2014) FCGR2A and FCGR3A polymorphisms and clinical outcome in metastatic colorectal cancer patients treated with first-line 5-fluorouracil/folinic acid and oxaliplatin +/- cetuximab. BMC Cancer 14:340

Knop W (1865) Quantitative Utersuchungen Über den Ernährungensprozeß der Pflanze. Landw Versuchssat 7:93

Kuehdorf K, Appenroth KJ (2012) Influence of salinity and high temperature on turion formation in the duckweed *Spirodela polyrhiza*. Aquat Bot 97:69–72

Landolt E (1986) The family of Lemnaceae—a monographic study. Veroff Geobot Inst ETH Stiftung Rubel Zurich 1:417–435

Landolt E, Kandeler R (1987) The family of Lemnaceae—a monographic study. Veroff Geobot Inst ETH, Stiftung Rubel, Zurich 2:65–69

Leng RA (1999) Duckweed: a tiny aquatic plant with enormous potential for agriculture and environment. FAO, Rome (Italy). Animal Production and Health Div., University of Tropical Agriculture Foundation, Phnom Penh (Cambodia), p 108

Leng RA, Stambolie JH, Bell R (1995) Duckweed—a potential high-protein feed resource for domestic animals and fish. Livest Res Rural Dev 7:36–51

Li J, Jain M, Vunsh R, Vishnevetsky J, Hanania U, Flaishman M, Perl A, Edelman M (2004) Callus induction and regeneration in *Spirodela* and *Lemna*. Plant Cell Rep 22:457–464

Ma JKC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805

Mariza M, Beatriz AG, Maria JV, Carlos AO, Maria LC (2007) Plant regeneration from protoplasts of alfalfa (*Medicago sativa*) via somatic embryogenesis. Instituto de Zootecnia de Nova Odessa, Nova Odessa. pp 683–689

Men BX, Ogle B, Preston TR (1995) Use of duckweed (*Lemna* spp.) as replacement for soya bean meal in a basal diet of broken rice for fattening ducks. Livest Res Rural Dev 7:3

Mett V, Musiychuk K, Hong B, Horsey A, Ugulava N, Shoji Y, Patricia DLR, Palmer GA, Rabindran S, Streatfield SJ, Boyers A, Russell M, Mann A, Lambkin R, Oxford JS, Schild GC, Yusibov V (2008) A plant-produced influenza subunit vaccine protects ferrets against virus challenge. Influenza Other Respir Viruses J 2:33–40

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Patnaik PR (1999) Applications of neural networks to recovery of biological products. Biotechnol Adv 17:477–488

Prasad A, Prakash O, Mehrotra S, Khan F, Mathur AK, Mathur A (2016) Artificial neural network-based model for the prediction of optimal growth and culture conditions for maximum biomass accumulation in multiple shoot cultures of *Centella asiatica*. Protoplasma. https://doi.org/10.1007/s00709-016-0953-3

Rabe-Hesketh S, Skrondal A (2008) Multilevel and longitudinal modeling using Stata, 2nd edn. Stata Press, College Station

Rival S, Wisniewski JP, Langlais A, Kaplan H, Freyssinet G, Vancanneyt G, Vunsh R, Perl A, Edelman M (2008) Spirodela (duckweed) as an alternative production system for pharmaceuticals: a case study, aprotinin. Transgenic Res 17:503–513

Roche J, Love J, Guo Q, Song J, Cao M, Fraser K, Huege J, Jones C, Novák O, Turnbull MH, Jameson PE (2016) Metabolic changes and associated cytokinin signals in response to nitrate assimilation in roots and shoots of *Lolium perenne*. Physiol Plant 156:497–511

Santarem ER, Trick HN, Essig JE, Finer JJ (1998) Sonication assisted Agrobactenum-mediated transformation of soybean immature cotyledons: optimization of transient expression. Plant Cell Rep 17:752–759

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199–204

Steinberg R (1946) Mineral requirement of *Lemna minor*. Plant Physiol J 21:42–48

Stomp AM, Rajbhandary N (2000) Genetically engineered duckweed. US Patent Publication US6040498A

Stomp AM, Lynn D, Gasdaska J (2005) Expression of biologically active polypeptides in duckweed. US Patent Publication US20050060776A1

Wolff P (1992) Les lentilles d'eau de l'Alsace. Bull. Assoc. Amis Jard. bot. Col de Saveme, 60^{e} anniversaire 1932/1992, pp 25–33

Yuan J, Xu K (2017) Effects of simulated microgravity on the performance of the duckweeds *Lemna aequinoctialis* and *Wolffia globosa*. Aquat Bot 137:65–71

Zanin L, Tomasi N, Rizzardo C, Gottardi S, Terzano R, Alfeld M, Janssens K, De Nobili M, Mimmo T, Cesco S (2015) Iron allocation in leaves of Fe-deficient cucumber plants fed with natural Fe complexes. Physiol Plant 154:82–94

Ziegler P, Adelmann K, Zimmer S, Schmidt C, Appenroth K-J (2015) Relative in vitro growth rates of duckweeds (*Lemnaceae*)—the most rapidly growing higher plants. Plant Biol 17(1):33–41

Zielinska A, Kepczynska E (2013) Neural modeling of plant tissue cultures: a review. Biotechnologia 94:253–268