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In vitro Micropropagation of Duckweed (*Lemna minor* L.) Plant with Temporary Immersion System Bioreactors

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Abstract

Which is very rich in protein in plant *Lemna minor* L. abundant in Turkey ecologically plays a very important role in protecting the elimination of water pollution and aquaculture environment balance. In this study, in vitro propagation of this plant with Temporary Immersion System Bioreactor and determination of the effects of used plant growth regulators on the protein content of the plant were aimed. With this objective different variant of media with and without sucrose, varying pH and concentrations of BAP, kinetin, TDZ in medium were analyzed. Experiments for micropropagation were performed for 8 hours in the dark and 16 hours in white fluorescent light (150 µ mol photons m-2s-1) under photoperiod and at 24 ± 1 ° C. The highest plant growth was observed at pH 7.23 in sugar free liquid MS medium containing 0.2 mg/L BAP. 50.44 plants per explant were recorded in this medium. In addition, the maximum number of plants per explant in liquid MS medium containing 0.05 mg/L kinetin was calculated as 57,823 and the maximum number of plants per explant in liquid MS medium containing 0.6 mg/L TDZ was calculated as 50.74. As a result of nitrogen determination studies with Kjeldahl method, the protein value of the plant was determined as 25.5%. As a result of hormone application, it was seen that protein content in plant increased to 29.18% with 0.5 mg/L BAP. It was concluded that the aim of the study were fulfilled and positive effects of Temporary Immersion System Bioreactors on plant multiplication were found.

Keywords: In vitro, Temporary Immersion System Bioreactors, *Lemna minor*, Micropropagation

1. Introduction

Aquatic plants, the main producers of the aquatic environment, are chlorophyll-containing organisms of various forms, from one cell to multiple cells. These plants, which are the primary producers, carry out photosynthesis using carbon dioxide and light energy present in the water and form plant sources as the first link in the food chain of the aquatic environment [1].Organic degradation in the aquatic ecosystem is extremely important for the biological cycle. The oxygen supply required for aerobic bacteria and fungi responsible for organic degradation is provided by aquatic plants and indirectly, the plants are important in the aquatic environment for the degradation of organic wastes [2].As is known, aquatic plants play a role in the removal of pathogenic bacteria and are used for phytoremediation and bioaccumulation as they are indicator plants with a large growth potential in industrial wastewater [3]. *Lemna minor* is a very small and simple plant in the Lemnaceae family of Arales, which is free-floating or submerged [4]. This species is a nutritional source of energy, studies on it are gaining momentum in recent years and increasing importance. It is a very rich plant in terms of protein [5]. L. minor is of great ecological importance. In recent years, the use of plants in the elimination of pollution by various factors in natural waters has been increasing. It has a large growth potential in industrial wastewater and is one of the leading plants used for phytoremediation as it is an indicator plant [3].Studies have shown that L. minor is also effective in eliminating the pollution caused by petroleum hydrocarbons. L. minor species synthesize cysteine and other sulfur-containing amino acids by absorbing sulfur from toxic gases such as SO_2 and H_2S. They also have a very high percentage of minerals and essential amino acids such as methionine and lysine [6,7]. *Lemna minor* is also used in proteomic and genomic research. It has been developed for the production of recombinant therapeutic proteins due to

its high protein content. Lemna Expresion System (LEX); it allows rapid clonal growth of transgenic plants, release of recombinant proteins, high protein production [8].Thanks to its rapid propagation and tissue culture techniques, its usage areas are gradually increasing and the studies related to the subject gain momentum.

The proliferation and rooting of plants from organized meristems, somatic cells that have not yet matured or completed maturation, either directly (organogenesis or somatic embryogenesis) or indirect (callus, protoplast, etc.) are generally called micro-propagation [9].Micropropagation is considered to be one of the most advantageous methods for propagation in tissue culture techniques if appropriate nutrients, hormone and culture requirements are required by plants. With this technique it is possible to obtain disease-free plants. Variety is increasing due to somaclonal variation and production is carried out in a shorter time and using less rootstock than other techniques [10]. In tissue culture techniques, solid nutrient media are generally used. In these studies, explants placed in solid nutrient medium begin to darken due to the presence of gelling agents that solidify the nutrient medium and are therefore transferred periodically to new media after 4-6 weeks. Contamination of the material is very high during these transfers. Micropropagation studies in solid nutrient environments where gelling agents are used are higher contamination risk and more costly applications compared to tissue culture studies in liquid nutrient media [11]. For this reason, liquid culture media without solidifying agents are particularly preferred for commercial micropropagation. In liquid culture media, the entire surface of the plant explants is in contact they absorb the components in the nutrient media better. The use of bioreactors used in liquid culture media in micropropagation studies is therefore increasing [12].The most common problem in bioreactor systems in which liquid culture media are used is the observation of hyperhydricity due to the fact that the plant material remains in the nutrient medium continuously. Therefore, recently developed temporary immersion system bioreactors are used which enable the plant to move away from the liquid surface at certain intervals. Temporary immersion system bioreactors were first developed by Haris and Mason [13] and the first successful plant regeneration results were obtained from somatic embryos of Solanum tuberosum and Coffea arabica [11].Unlike other bioreactor systems in temporary immersion system bioreactors, a surface has been developed which can provide temporary contact between explant and liquid nutrient media from time to time. With the air flow, the environment goes up in certain time intervals and ensures that the plant receives the necessary nutrients. During the dipping process, air flow and nutrient media penetrate the plant and pass to the tissues without damaging the material. Temporary immersion system bioreactors are used as suitable systems for somatic embryogenesis and organogenesis for this purpose. In this study, in vitro propagation of the plant with temporary immersion system bioreactor is aimed and with the use of bioreactor, it is aimed to keep the ambient conditions at an optimum level and to reproduce the plant in a faster time and healthier than the natural conditions. Also; The aim of this study is to determine the effects of plant growth regulators used for micro-propagation of plant on growth rate and plant protein quality.

2. Materials and methods

2.1.Plant material

Duckweed (*Lemna minor* L.) obtained from Ankara University Faculty of Agriculture Department of Fisheries were used in this study.

2.2.Growth media and culture conditions in in vitro studies

Basic nutrient medium containing MS (Murashige and Skoog basal medium) mineral salt and vitamins and 3% sucrose and solidified with 0.8% agar (Type A, Sigma-Aldrich Corporation St Lo. Mo) or 0.6–0.65% agar (Duchefa Netherlands) were used in the experiments. Agar was not added in liquid culture experiments. Double distilled water was used in the preparation of the medium and plant growth regulators of different constructions were added to the nutrient medium when necessary. The pH of the nutrient medium was adjusted to 5.6-5.8 by using 1N NaOH or 1N HCl, followed by autoclaving at 120 ° C for 20 minutes under a pressure of 118 KPa. All cultures were kept under photoperiod for 16 hours under white fluorescent light (150 µmol photons $^{m-2s-1}$) at 24 ± 1 ° C [14].

2.3.Plant growth regulators

Plant growth regulators BAP (Benzylaminopurine), TDZ (Thidiazuron) and kinetin used in the study were obtained from Sigma Aldrich and Duchefa. The stock solutions of the growth regulators were prepared in the desired amount and proportion after thawing with suitable solvents as recommended by the company. Prepared stock solutions were stored at 4 ° C for two months. Kinetin was added to the media after autoclaving[14].

2.4.Explant surface sterilization

For surface sterilization, different doses of commercial bleach and H_2O_2 were used in duckweed [14].

2.5.Determination of nitrogen and protein in plant material

Kjeldahl method was used to determine the protein and nitrogen contents of the plants which were micro-propagated and dried [15].

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2.6.Statistical evaluation of data

Regeneration experiments were performed with 3 replications. The data obtained from the studies were analyzed by using SPSS 16 for Windows program on the computer according to factorial experiment pattern. Duncan, LSD or t tests were applied to determine the significance level of the media. Percentage values were subjected to arcsin transformation before statistical analysis [16].

3. Results

3.1. Surface sterilization of the explant

Surface sterilization of the explants in vitro experiments, *Lemna minor* plant explants (20 pieces) were first surface sterilized in 20% commercial bleach, then in 10% and 20% H_2O_2 for 5, 6, 7, 8, 9 and 10 min. Data obtained as a result of surface sterilization was subjected to variance analysis [17] and the results are given in Table 3.1.

Table 3.1. Variance analysis of surface sterilization with bleach and H_2O_2 at different time and concentrations.

Variance resources	Degrees of freedom	Average of squares	Frequency
Time (min)	5	2549,63	86,050
Concentration (%)	2	45029,63	$1,520x10^3$
Time x concentration	10	2549,63	86,050**
Error	36	29,630	
General total	53		

**p<0.05

Table 3.2. Duncan test results of surface sterilization with bleach and H_2O_2 at different time and concentrations.

Time (min)	Ratio of sterile plants		
	%20 Bleach	%10 H_2O_2	%20 H_2O_2
5	100,00	0,00	30,00d
6	100,00	0,00	50,00c
7	100,00	0,00	60,00b
8	100,00	0,00	100,00a
9	100,00	0,00	100,00a
10	100,00	0,00	100,00a

The difference between the means indicated by different letters in the same column is significant at 0.05 level.

As seen in Table 3.1 as a result of the analysis of variance, 5, 6, 7, 8, 9 and 10 minutes with 20% commercial bleach and 20% and 10% H2O2 concentrations in terms of surface sterilization at 0.05 level is important differences were found. Duncan test [18] results to determine the significance level of these differences are given in Table 3.2. As shown in Table 3.2 Duncan test results according to 20% bleach and different time treatment between 0 and 100.00% sterile plants were obtained. Although the sterilization application was carried out with bleach at different times, 100% sterile plants were obtained, but whitening was observed due to chlorophyll disintegration. 30 to 100% sterile plants were obtained by 20% H_2O_2 treatment. However, bleaching and damage of the plants were detected in 20% H_2O_2 treatment for 9 and 10 minutes. No sterile plants were obtained with different time and 10% H_2O_2 treatment. These parameters were used for surface sterilization in the following studies since 100% sterilization was achieved in plants with 8 minutes and 20% H_2O_2 treatment.

3.2. The effect of different pH values on the growth of duckweed in erlenmayer in non-agitated environment

In vitro study, *Lemna minor* plant explants were cultured in Erlenmayer for 1 week in non-agitated medium using distilled water at different pH values. The data obtained after 1 week were subjected to variance analysis and the results are given in Table 3.3. As shown in Table 3.3 as a result of analysis of variance pH 5, 6, 7, 7.23, 8, and 9 values were found to be significant differences at 0.01 level in reproduction rate, number of plants and total plants per explant. Duncan test results to determine the significance level of these differences are given in Table 3.4. The difference between the means indicated by different letters in the same column is significant at the 0.01 level.Plant growth was observed in all pH values. Amplification rate ranged from 3.87% to 280%. The number of plants per explant ranged between 1.47 and 5. The highest increase in the number of plants was obtained at pH 5 with 5 units and the lowest increase was found at pH 9 values with 1.47 units. The total number of plants varied between 7.33 and 25. The maximum number of plant growths with 5 explants starting at pH 7.23 25 units, the least plant formation was recorded as pH 9 7.33 units. In addition, the number of 19 plants at pH 7 and 25 plants at pH 7.23 were recorded. In addition, 8.33 plants were obtained at pH 8 and 7.33 plants were recorded at pH 9. According to this study, the most ideal pH range for the plant is 7–7.23, pH range is lower than pH 7–7.23 (6–5.8) and pH range is higher than pH 7–7.23 (pH 8–9) the percentage of plant formation has been observed to fall (Table 3.4).

3.3.The effect of different pH values on the growth of duckweed in erlenmayer under agitation

In vitro experiments, *Lemna minor* plant explants were cultured in Erlenmayer for 1 week in shaking medium using distilled water at different pH values. The data obtained after 1 week were subjected to variance analysis and the results are given in Table 3.5. As it is seen in Table 3.5, variance analysis showed that

significant differences were found in pH 5, 6, 7, 7.23, 8 and 9 values in terms of reproduction rate, number of plants per explant, total number of plants, wet weight and dry weight changes. Duncan test results to determine the significance level of these differences are given in Table 3.6. Plant growth was observed in all pH values. The amplification rate ranged from -3.33% to 560%. The number of plants per explant ranged between 0.63 and 6.60.

The highest increase in number of plants was obtained in pH 7 with 6.60 and the least increase in number of plants was in pH 5 with 0.63. The total number of plants ranged from 9.67 to 66.00. The highest plant growth was obtained at pH 7 and the lowest plant growth was at pH 5. The heaviest plants in weight were 0.7 g, pH 7.23 and the lightest plants were 0.04 g, pH 5. Dry weight values in weight of 0.55 g pH 7.23, 0.001 g was observed at pH 9 (Table 3.6).

Table 3.3. Analysis of variance of the effect of different pH values on the plant growth of duckweed in Erlenmayer in non-agitated medium using distilled water.

Variance resources	Degrees of freedom	Number of plants per explant (pcs)		Total number of plants (pcs)	
		Average of squares	F	Average of squares	F
pH	5	5,76	20,56**	143,92	20,56**
Error	12	0,28		7,00	
General total	17				

**p<0.01

Table 3.4. Distilled water using different pH values in the non-agitated environment in the Erlenmayer effect of duckweed on the plant growth of Duncan test results.

pH	Number of plants per explant (pcs)	Total number of plants (pcs)
5	5,00a	10,00cd
6	3,80b	12,67c
7	2,53c	19,00b
7,23	2,00cd	25,00a
8	1,67cd	8,33cd
9	1,47d	7,33d

The difference between the means indicated by different letters in the same column is significant at the 0.01 level.

Table 3.5. Variation analysis of the effect of different pH values on the plant growth of duckweed in Erlenmayer in shaking environment.

V. R	D. F	Number of plants per explant (pcs)		Total number of plants (pcs)		Wet weight (g)		Dry weight (g)	
		A. S	F	A. S	F	A. S	F	A. S	F
pH	5	15,58	62,63 **	1470,32	73,93 **	0,03	65,02 **	0,03	222,42**
Error	12	0,25		19,89		0,00		0,00	
General total	17								

**p<0.01

Table 3.6. Different pH values in the shaking environment of the effect of duckweed plant growth in Erlenmayer Duncan test results.

pH	Number of plants per explant (pcs)	Total number of plants (pcs)	Wet weight (g)	Dry weight (g)
5	0,63e	9,67d	0,04d	0,01c
6	1,26ed	12,67d	0,41d	0,13c
7	6,60a	66,00a	0,12a	0,07a
7,23	4,70b	47,00b	0,70b	0,55b
8	1,77d	17,67d	0,48cd	0,006d
9	2,93c	29,33c	0,05c	0,001d

The difference between the means indicated by different letters in the same column is significant at the 0.01 level.

3.4.Setting the amount of liquid MS (Murashige and Spoog basal medium) suitable for reproduction in temporary immersion system (TIS) bioreactors

After determining the appropriate pH for the plant, the studies were carried out to determine the appropriate MS and sugar concentrations in the liquid culture medium to be used for rapid propagation of the plants in the TIS bioreactor. TIS bioreactors to be used in the study were sterilized twice in each autoclave at 126 °C separately before being used despite any risk of contamination. Afterwards, 20 explants in TIS bioreactors were cultured in medium containing 1/4 MS, 1/2 MS, MS and 30 g sucrose for 1 week. Initially, 350 ml of liquid culture medium was used in the bioreactors, but after 2 weeks the plants in the bioreactor became completely white. It was observed that the plants did not get enough nutrients in 350 ml liquid culture and their development was insufficient.

3.5.Liquid MS medium optimization in TIS bioreactors

Lemna minor plant explants (40 pcs) were cultured in different MS liquid culture medium (400 ml) for 4 weeks. Then the data obtained were subjected to analysis of variance and the results are given in Table 3.7. As seen in Table 3.7, as a result of the analysis of variance in sugar and sugar-free MS, 1/2 MS and 1/4 MS liquid culture media, plant formation rate, number of plants per explant, total number of plants, pH change in terms of significant 0.01 level differences were found. Duncan test results to determine the significance level of these differences are given in Table 3.8. The rate of plant formation in the experiment ranged between 75.00% and 143.33%. The number of plants per explant was observed between 0.51 and 1.90. The maximum number of plant growths was obtained in 1.90, 1/4 MS sugar culture medium and the least plant number was 0.51 in MS 1/4 sugar free culture medium. The highest number of plants (38 pcs) was observed in liquid culture medium with 1/4 MS sugar and the lowest number of plants (15 pcs) was observed in 1/4 MS sugar free liquid culture medium. The effects of cultured explants on the pH of the environment were obtained at the lowest values of 4.88 and 5.66. The lowest change rate pH 4.88 value 1/4 MS sugar culture medium with the highest change rate pH 5.66 value was observed in MS sugar-free environment (Table 3.8).

Table 3.7. Analysis of variance of the effects of plant duckweed on different MS liquid culture media.

V. R	D. F	Number of plants per explant (pcs)		Total number of plants (pcs)		pH end state	
		A. S	F	A. S	F	A. S	F
Different MS medium	5	0,68	16,0**	224,72	61,28**	0,248	424,66**
Error	12	0,043		3,66		0,001	
General total	17						

**p<0.01

Table 3.8. Duncan test results of plant formation effects of duckweed of different MS liquid culture media.

Proportions of MS medium	Number of plants per explant (pcs)	Total number of plants (pcs)	pH end state
1/4 MS with sugar	1,90a	38,00a	4,88e
1/2 MS with sugar	1,43b	28,67b	4,90e
MS with sugar	1,25b	25,00c	5,10c
1/4 MS without sugar	0,51d	15,00d	5,22b
1/2 MS without sugar	1,18bc	23,67c	5,04d
MS without sugar	0,84cd	15,33d	5,66a

The difference between the means indicated by different letters in the same column is significant at the 0.01 level.

3.6.Application of TDZ (Thidiazuron) in different concentrations

In vitro experiment, duckweed plant explants (40) were cultured for 4 weeks in MS liquid culture media (400 ml) by adding different concentrations of TDZ hormone. The data obtained after 4 weeks were subjected to variance analysis and the results are given in Table 3.9. As shown in Table 3.9 as a result of variance analysis of liquid MS culture medium in different concentrations of TDZ hormone application plant formation rate, number of plants per explant, total number of plants, wet weight and dry weight were found to be significant at the level of 0.01. Duncan test results to determine the significance level of these differences are given in Table 3.10. In this experiment, plant growth was observed in liquid culture MS medium treated with all TDZ hormones. Plant formation ranged

between 276% and 2274%. The number of plants per explant was obtained in liquid MS medium containing up to 50,74 units of 0.6 mg/L TDZ and in liquid nutrient medium containing 0.3 mg/L TDZ as minimum number of plants (8,99 units). Cultured explants showed that the least wet plants were 0.36 g in liquid MS medium containing 0.5 mg/L TDZ and the heaviest plants were found in liquid MS medium containing 1.80 g and 0.1 mg/L TDZ. These results reflected similarly in dry weight. The minimum dry weight in exsplant 0.03 g and 0.3 mg / L TDZ containing liquid nutrient medium and the maximum dry weight of 0.68 g 0.1 mg / L TDZ containing liquid medium was determined (Table 3.10). In the experiment, the protein ratios of the plants treated with different concentrations of TDZ were calculated. The highest protein content was 26.96% in liquid MS medium containing 0.2 mg/L TDZ. The lowest protein content was recorded in liquid MS medium containing 16.48% and 0.6 mg/L TDZ.

Table 3.9. The results of variance analysis of the effects of different concentrations of TDZ hormone application on plant growth of duckweed.

V. R	D. F	Number of plants per explant (pcs)		Total number of plants (pcs)	
		A. S	F	A. S	F
TDZ hormone	5	164,225	31510**	262983,033	3,6980 **
Error	12	0,005		7,111	
General total	17				

V. R	D. F	Wet weight (g)		Dry weight (g)		Protein content (%)	
		A. S	F	A. S	F	A. S	F
TDZ hormone	5	0,757	621,896**	0,167	755,001 **	82,112	157,55**
Error	12	0,001		0,00		0,02	
General total	17						

**p<0.01

Table 3.10. Duncan test results of the effects of different concentrations of TDZ hormone application on liquid culture MS medium to duckweed plant growth.

TDZ (mg/L)	Number of plants per explant (pcs)	Total number of plants (pcs)	Wet weight (g)	Dry weight (g)	Protein content (%)
0,1	23,74a	949,67a	1,80a	0,68a	26,25b
0,2	15,59b	624,67b	1,25b	0,51b	26,96b
0,3	8,99d	359,67d	0,99c	0,03f	22,35cd
0,4	9,13c	365,33c	0,90d	0,36c	23,31c
0,5	3,76f	150,67f	0,36f	0,16e	27,72a
0,6	50,47e	219,00e	0,63e	0,24d	16,48e

The difference between the means indicated by different letters in the same column is significant at the 0.01 level.

3.7.Bap (6-Benzylaminopurine) hormone application at different concentrations

In vitro experiments, duckweed plant explants (40 pcs) were cultured in MS liquid culture media (400 ml) for 4 weeks by adding BAP hormone at different concentrations. The data obtained after 4 weeks were subjected to variance analysis and the results are given in Table 3.11. As seen in Table 3.11 variance analysis results of liquid MS culture medium BAP hormone application in different concentrations of plant formation rate, the number of plants per explant, the total number of plants, wet weight and dry weight were found to be significant difference in the 0.01 level. Duncan test results to determine the significance level of these differences are given in Table 3.12. In the experiment, plant growth was observed in liquid culture MS media where all BAP hormone applications were performed. Plant formation ranged between 220 and 4944.2%. The number of plants per explant was determined between 2.78 and 50.44 plants. The maximum number of plants was 50.44 in liquid MS medium containing 0.2 mg/L BAP and the least number of plants (2,78 pcs) in liquid MS medium containing 0.4 mg/L BAP. The total number of plants (2017,7 pcs) was obtained in liquid MS medium containing 0.2 mg/L BAP and at least 111.33 in liquid MS medium containing 0.4 mg/L BAP. Cultured explants had a

minimum wet weight of 0.15 g and were detected in liquid MS medium containing 0.4 mg/L BAP. The heaviest plants were 3.65 g and were detected in liquid MS medium containing 0.2 mg/L BAP. In the dry weight calculation of explants, the lightest plants (0.15 g) were obtained in liquid MS medium containing 0.1 mg/L BAP and the heaviest plants (0.7 g) were obtained in liquid MS medium containing 0.4 mg/L BAP (Table 3.12). In the experiment, protein ratios of plants with different concentrations of BAP were calculated. The highest protein content was 29.18% in liquid MS medium containing 0.5 mg/L BAP. The lowest protein content was recorded in liquid MS medium containing 20.45% and 0.2 mg/L BAP.

3.8. Application of kinetin in different concentrations

In vitro experiments, plant explants of 40 duckweeds were cultured for 4 weeks in MS liquid culture media (400 ml) by adding different concentrations of kinetin hormone; Data obtained after 4 weeks were subjected to variance analysis and the results are given in Table 3.13.

Table 3.11. The results of variance analysis of the effects of different concentrations of BAP hormone administration on duckweed plant growth.

V. R	D. F	Number of plants per explant (pcs)		Total number of plants (pcs)	
		A. S	F	A. S	F
BAP hormone	5	1072,713	103400**	1716315,689	104000**
Error	12	0,010		16,500	
General total	17				

V. R	D. F	Wet weight (g)		Dry weight (g)		Protein content (%)	
		A. S	F	A. S	F	A. S	F
BAP hormone	5	6,824	20650 **	0,019	264,459**	436,356	51700**
Error	12	0,000		0,000		0,05	
General total	17						

**p<0.01

Table 3.12. Duncan test results of the effects of different concentrations of BAP hormone application on liquid culture MS medium to duckweed plant growth.

BAP (mg/L)	Number of plants per explant (pcs)	Total number of plants (pcs)	Wet weight (g)	Dry weight (g)	Protein content (%)
0,1	3,99d	160,00d	2,89b	0,15d	28.43a
0,2	50,44a	2017,7a	3,65a	0,16d	20.45d
0,3	3,20e	128,33e	0,30e	0,21c	21.00d
0,4	2,78f	111,33f	0,15f	0,70e	27.72b
0,5	5,4c	217,00c	0,48d	0,24b	29.18a
0,6	5,62b	225,0 0b	0,66c	0,30a	26.18bc

The difference between the means indicated by different letters in the same column is significant at the 0.01 level.

As shown in Table 3.13 variance analysis results of liquid MS culture medium kinetin hormone application in different concentrations of plant formation rate, the number of plants per explant, the total number of plants, wet weight and dry weight were found to be significant differences in the 0.01 level. Duncan test results to determine the significance level of these differences are given in Table 3.14. In the experiment, plant growth was observed in liquid culture MS environments where all kinetin hormone applications were performed. Plant formation ranged between 186.67% and 5683.8%. The maximum number of plants per explant was 57,823 and was obtained in liquid MS medium containing 0.05 mg/L kinetin and in liquid MS medium containing at least 2,86 0.15 mg/L kinetin. The total number of plants (2313,3) was observed in MS medium containing 0.05 mg/L kinetin and at least 114.67 in liquid MS medium containing 0.15 mg/L kinetin. The least wet weight of cultured explants was found to be 0.24 g in liquid MS medium containing 0.15 mg/L kinetin and the heaviest plants were detected in liquid MS medium containing 4.06 g 0.05 mg/L kinetin.

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Table 3.13. Variance analysis of the effects of different concentrations of kinetin hormone application on duckweed plant growth.

V. R	D. F	Number of plants per explant (pcs)		Total number of plants (pcs)	
		A. S	F	A. S	F
Kinetin hormone	5	1367,854	468300**	2189274,356	458200**
Error	12	0,003		4,778	
General total	17				

V. R	D. F	Wet weight (g)		Dry weight (g)		Protein content (%)	
		A. S	F	A. S	F	A. S	F
Kinetin hormone	5	6,211	44040**	0,44	282,622**	683,927	234150**
Error	12	0,00		0,00		0,006	
General total	17						

**p<0.01

In the calculation of dry weight of explants, the lightest plants were observed in nutrient medium containing 0.02 g and 0.15 mg/L kinetin and the heaviest plants were observed in liquid MS medium containing 0.39 g and 0.1 mg/L kinetin. In the experiment, protein ratios of kinetin treated plants were calculated. The highest protein content was 26.96% and was observed in liquid MS medium containing 0.15 mg/L kinetin. The lowest protein content was recorded in liquid MS medium containing 0.2 mg/L kinetin at 23.18%.

Table 3.14. Duncan test results of the effects of different concentrations of kinetin hormone application on liquid culture MS medium duckweed plant growth.

Kinetin hormone (mg/L)	Number of plants per explant (pcs)	Total number of plants (pcs)	Wet weight (g)	Dry weight (g)	Protein content (%)
0,05	57,82a	2313,3 a	4,06a	0,19d	24.68b
0,1	9,11b	364,67 b	0,90b	0,39a	25.44a b
0,15	2,86f	114,67 f	0,24f	0,02e	26.96a
0,2	6,90c	276,33 c	0,70d	0,24c	23.18c
0,5	3,56e	142,67 e	0,32e	0,19d	26.18a
0,6	6,67d	267,00 d	0,79c	0,29b	26.84a

The difference between the means indicated by different letters in the same column is significant at the 0.01 level.

4. Discussion

The production of aquarium plants in Turkey is made in limited quantities. Aquatic plants in tissue culture work is done in a limited number of countries including Turkey. In this thesis, *Lemna minor*, one of the aquatic plants, is aimed to be propagated with temporary immersion system bioreactors. In this direction, the production limits of the plant are increased in a faster time than the natural conditions. Turkey from *Lemna minor* plant previously made on the very few studies about the availability of temporary system does not have to work with the micropropagation bioreactors. Generally, the studies have been carried out on the elimination of water pollution causing substances such as heavy metal wastes by using *Lemna minor* plant from water sources [19, 20]. Surface sterilization is one of the most important steps in tissue culture studies. When working with the green part of the plant, the chemicals to be applied to the explant for surface sterilization are very likely to damage the plant. Therefore, the chemicals to be used must be treated in the least amount and as soon as possible. Although there are many studies in the literature on the sterilization of land plants, there are not many studies on surface sterilization of aquatic plants. In this study, commercial bleach and H_2O_2 were used for sterilization and the best results were obtained by sterilizing 20% H_2O_2 for 8 minutes. Cook et al. [21] have used sulfuric acid and ethanol in surface sterilization of Kosteletzkya virginica seeds. Straub et al. [22] used bleach or ethanol for surface sterilization of Phragmites australis seeds. Agrawal and Ram [23] studied in vitro germination and micropropagation of water chestnut (Trapa sp.). After surface sterilization, the embryos were placed in NBS (basic semi-solid medium of Nitsch) to obtain plants. Simon and Helliwel [24] found that when the level of chlorophyll a with pure water is lower than pH 8, chlorophyll a rapidly converts to fiophytine a, but in the case where it is greater than pH 8, hydrolysis is found to

leave the phytol group. Studies were carried out to determine the appropriate pH range in surface sterilized plants. pH optimization for the plant took a long time. In order to make the optimization, the plants were placed in the distilled water in different pH levels and were observed in shaking and non-shaking conditions for 1 week. The material was placed in the shaker in the dark medium at 190 rpm and 28 ° C for shaking media. For the shaking environment, it was kept in the climatic chamber in the flasks for 1 week. As a result, the optimum pH range for the plant was determined to be 7.23 in shaking environment. In the experiment, it was observed that the number of plants in pH 8 and 9 was less than that of pH 7.23, and at pH values less than 7.23, it was observed that the plant could not develop and the growth did not occur. Therefore, it was determined that optimum pH range had an effect on plant growth. Similarly, Kim and Jang [25] used Drosera peltata micropropagation studies of pH 3.7, 4.7, 5.7 and 6.7 values and 3% (w/v) sucrose containing 1/2 MS medium used. They determined the optimum value for the micropropagation of the plant as pH 5.7. Perica and Berljak [26] found that pH is an effective factor in the micropropagation of Drosera spatulata Labill. Simola [27] found that the most effective value of Drosera rotundifolia L. in micropropagation is pH 6.00. After determining the appropriate pH for the plant, the studies were carried out to determine the appropriate MS and sugar

concentrations in the liquid culture medium to be used for rapid propagation of the plants in the TIS bioreactor. Plants were cultured in medium containing 1/4 MS, 1/2 MS, MS with 30 g sucrose and without sucrose in TIS bioreactors. According to the optimization results of the liquid culture medium, the best plant growth percentage (90%) and the total number of plant formation (38.00) were determined in the liquid culture medium with sugar 1/4 MS. It was determined that the chemical substances and concentrations of the liquid culture medium to be used were effective in the micropropagation of the plant. Similarly, Houllou-Kido et al. [28] *Nopalea cochenilifera* heterotrophic and photootrophic plant micropropagation have made. They added 3% (w/v) sucrose, 0.8% (w/v) agar, 1.0 mg/L BAP and 0.1 mg/L NAA to the MS medium to be used for micropropagation. At the end of their experiments, they found that in sucrose-containing media 10 times more micropropagation occurs than in non-sucrose-containing media. After determining the sugar and MS concentrations of the liquid culture medium, the effects of different doses of TDZ, BAP and kinetin on the rapid growth of the plant were determined. In the studies, it was observed that the number of plants per explant (57,823 units) and the total number of plants (2313,3 units) were obtained more than the BAP and TDZ applications in the application of kinetin at different rates (0.05-0.6 mg/L). Although the number of plants and reproduction rate were high, the plants were found to be dull and unhealthy, and the plants were found to have hyperhydricity. In another study, TDZ was applied at different doses (0.1–0.6 mg/L) and the maximum number of plants (949.67 pcs) was recorded in liquid culture medium containing 0.1 mg/L TDZ. In the rapid amplification study with the addition of TDZ, although the number of plant propagations and percentage of plants per explant were high, it was observed that plants with lesser doses and smaller leaf sizes were formed than BAP at different doses. Then, BAP hormone was applied to the liquid culture media at different doses (0.1–0.6 mg/L). The maximum number of plants per explant (50.44 units) and the maximum total number of plants (2017.7 units) were obtained at a BAP concentration of 0.2 mg/L. All plants are green and healthy. In addition, no hyperhydricity was observed in plants. Obviously, different doses of plant growth regulators (BAP, kinetin and TDZ) applied in the scope of the study have been found to have effects on plant micropropagation. Micropropagation experiments without hormone supplementation showed no hyperhydricity, whereas hyperhidricity was observed in different concentrations of TDZ and kinetin in plants. This is due to the presence and concentration of some plant growth regulators. In this study, in order to determine the effects of different cytokinin concentrations on the protein content of the plants, microcoproduced dried plants were determined protein and nitrogen by Kjeldahl method. In literature studies, it was not found that protein and nitrogen were determined in *Lemna minor* plant by this method. In the determination of protein with the plant samples taken from the medium where plant growth regulators were added in the experiments, the amount of 29.18% protein in the plants obtained as a result of 0.5 mg/L BAP application, the amount of protein as 27.72% as a result of 0.5 mg/L TDZ application and as a result of the application of 0.15 mg/L kinetin protein amount was determined as 26.96%. Stewart et al. [29] carrot (*Daucus carota* L.) in the in vitro study carried out in plants developed in the bioreactor due to lack of oxygen have found a negative effect on root elongation. In order to overcome this problem and maintain the O_2 balance in the environment, the explants were cultured in auxophyton containing bioreactors and continuous air intake was provided and positive effects were observed in root elongation. It was recorded that the weight of the plant increased 2.6 times after 20 days. Escalant et al. [30] carried out micropropagation of banana plant with temporary immersion system bioreactors and compared the results obtained with the micropropagation test results of banana plant in solid food medium using agar. In the explants cultured in TIS bioreactor, the number of somatic embryos formed after 2 months was recorded as 1375 and the number of embryos in agar containing solids was 450. As a result, the percentage of plant growth in micropropagation by bioreactor application was 3.05 times higher than the percentage of plant growth in micropropagation in agar medium.

As a result, the propagation of *Lemna minor* plant with temporary immersion system bioreactors was carried out successfully and the plant was produced in a shorter time and more number than the natural environment. The negative risks that may occur during the production phase have been completely eliminated under laboratory conditions and production has been carried out in a controlled manner. It was determined that plant growth regulators used during micropropagation did not have a negative effect on plant protein ratio and it was seen that it caused an increase in the protein ratio of the plant. Even though in recent years has accelerated studies on aquatic plants are considered to be especially important open about the work in this field in Turkey. Therefore, especially tissue culture studies with aquatic plants are very important. It is thought that this study carried out with *Lemna minor* plant will contribute to the rapid and intensive production of aquatic plants and will contribute to future aquatic plant biotechnology studies.

Author's Contributions

Ethics

There are no ethical issues after the publication of this manuscript.

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