Attempts at establishing the culture conditions for *Lemna minor* L.

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Abstract

The influence of the concentration, composition and pH of the substrate as well as of light intensity on the growth and vegetative propagation of *Lemna minor* L. was investigated. The media of Hutner, Hoagland and Pirson and Seidel were used. At first the experiments were carried out under unsterile conditions. Later sterilized duckweed was cultured in aseptic conditions. The dry matter was determined. Surface area increment and an increase in the number of fronds were evaluated by the planimetric method. For total protein determination in *Lemna minor* L. from unsterile and sterile cultures Lowry's method was used.

INTRODUCTION

Species of the family *Lemnaceae* are common and widely spread plants on all continents of our globe, with the exception of the arctic regions (Chudyba, 1969). They are readily available plant material with a very simple morphological and anatomical structure. As aquatic plants they may be cultured in strictly controlled laboratory conditions on synthetic media. In favourable natural (ponds, rivers) or laboratory conditions the vegetative shoots rapidly form daughter fronds, therefore the fresh mass increment of the plants is very intensive. Establishment of optimal conditions of sterile laboratory culture allows investigations independent of the vegetative season in the natural environment and makes possible experiments throughout the year.

Of the 4 genera belonging to the family *Lemnaceae* (*Lemna*, *Spirodela*, *Wolffia* and *Wolffiella*) most frequently *Lemna* and *Spirodela* are used for experiments. These organisms, and particularly *Lemna minor* are characterized by nutritional requirements typical for higher plants

and can grow in light on mineral media. Therefore they have long been utilized in morphogenetic, physiological and biochemical studies as model plants and that is why trials are undertaken to find optimal conditions for their vegetation. A rich source of information on *Lemnaceae* may be found in Hillman's review (1961).

Since *Lemnaceae* also are of practical importance as a source of vegetal protein and are sometimes used as an addition to fodder, the latest investigations concern above all nitrogen metabolism of these plants (Joy, 1969; Ferguson, 1970, Stewart, 1972, Grob et al., 1973; Shepard et al., 1973).

The present studies were undertaken to preliminarily establish the conditions for optimal growth of *Lemna minor* L. in laboratory environment, taking into account the kind, concentration and pH of the medium, the ambient temperature and light intensity, and to obtain sterile duckweed cultures. The influence of the above enumerated physico-chemical factors was evaluated by measuring the surface area increment of the culture and the total protein content. It was endeavoured to establish culture conditions which would ensure large quantities of biomass with a high protein content.

MATERIAL AND METHODS

As material served duckweed (*Lemna minor* L.) collected from a natural habitat. It was cultured in a light incubator on mineral Hutner's Hoagland's and Pirson and Seidel's media (Czopek, 1963).

Unsterile *Lemna minor* L. culture on Hutner's and Hoagland's medium

Duckweed was cultured in unsterile conditions in glass crystallizers with walls and bottom painted black. They were covered with transparent glass plates. Their capacity was 900 ml and they usually contained 500 ml of medium. They were kept at $26 \pm 2^\circ\text{C}$ and illuminated continuously with a mercury lamp.

The first experiments concerned the influence of medium concentration on the intensity of *Lemna* growth. Hutner's medium in 0.1—0.7 and 1.0 and Hoagland's medium in 0.1—1.0 of the basic concentrations were used (Czopek, 1963). Chemical composition of Hutner's medium: K_2HPO_4 400 mg, $\text{Ca}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ 200 mg, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 500 mg, NH_4NO_3 200 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 25 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 65 mg, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 15 mg, H_3BO_3 15 mg, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 25 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 4 mg, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ 1 mg, Na_2EDTA 500 mg, dist. H_2O 1000 mg. Chemical composition of Hoagland's medium: KNO_3 610 mg, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 950 mg, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

490 mg, $\text{NH}_4\text{H}_2\text{PO}_4$ 120 mg, iron tartrate 5 mg and 1 ml of A—Z micro-elements mixture according to Hoagland (Czopek 1963), dist. H_2O 1000 ml.

The duckweed was cultured for 9 days. Surface area increment (mm^2) was determined by the photographic-planimetric method (Czopek, 1963) after 3, 6 and 9 days of culture. For the measurement a G. Coradi, Zürich planimeter was used. On the days when photographs were taken the plants were transferred to fresh medium.

In further experiments the influence of light intensity on duckweed growth was investigated. It is, namely, known that optimal light intensity may differ at different medium concentrations. According to White (1936, 1937, quoted by Hillman, 1961) the optimal concentration of nitrates in the medium increases with light intensity, this being associated with changes in the intensity of photosynthesis and the possibility of utilization for amino acid synthesis.

The plants were cultured on Hutner medium of 0.4 concentration under the conditions described above. The culture was illuminated with light of 1200, 3000, 4000, 5000, 6000, 7000 and 8000 lux intensity. Light intensity was measured with a type Photo-element 330 lux meter (Czopek, 1968). The increment in the number of fronds and the increase in surface area of the culture were calculated on the 3, 5 and 7th day of culture.

The next step in the investigations consisted in establishing the optimal pH value of the medium. Hutner's medium with the following pH values was used: 4.5, 5.0, 5.5, 6.0 and 6.5. The culture was run for 12 days. Every 3 days the increment in the surface area of the duckweed was determined by the photographic-planimetric method.

Sterile *Lemna minor* L. culture

From the unsterile duckweed culture on Hutner's medium specimens were taken and subjected to sterilization as follows. The plants were immersed in 0.1% mercuric chloride for 45 sec and in 50 per cent ethanol for 30 sec. Then they were washed 4 times with sterile distilled water and 4 times with sterile medium. The sterile specimens were transferred to Erlenmayer flasks of 300 ml capacity containing 150 ml of sterilized medium. In sterile culture Hutner's medium in 0.1—0.4 and 1.0 concentrations, Hoagland's medium in 0.6—1.0 and Pirson and Seidel's medium in 1.0 concentrations were used. Chemical composition of Pirson and Seidel's medium: KH_2PO_4 200 mg, KNO_3 400 mg, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 610 mg, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 300 mg, iron citrate 5 mg, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.3 mg H_3BO_3 0.5 mg, dist. H_2O 1000 ml., pH of the medium was 5.5. The culture was illuminated with continuous mercury light of 1200 and 8000 lux intensity, temperature was $26 \pm 2^\circ\text{C}$.

Dry mass content in the cultured plant material was determined by the weighing method. Total protein content in *Lemna minor* L. cultured under unsterile and sterile conditions on Hutner's Hoagland's and Pirson and Seidel's media was determined by the method of Lowry et al. (1951).

All experiments were replicated 4 times.

RESULTS

First the dependence of the increment of the number of fronds and of the surface area on the concentration of Hutner's and Hoagland's medium was examined. This relation is most evident on the 9th day of culture. Duckweed grew most intensively within 0.3—0.6 concentration of the basic Hutner medium, maximum growth being obtained at a 0.4 concentration (168.3 mm² — Fig. 1). At Hoagland medium concentrations of 0.7 the increment of *Lemna* amounted to 158.6 mm² (Fig. 2). As seen in Fig. 3 the most favourable light intensity within the range applied was 8000 lux. The most suitable pH was 5.0—5.5 (Fig. 4).

Fig. 1. Increase in frond area of *Lemna minor* as related to Hutner medium concentration
a — 3rd day of culturing; b — 6th day of culturing; c — 9th day of culturing

Dry mass content was much higher in unsterile than in sterile duckweed cultures. It was highest reaching 14.4 per cent in cultures in Hoagland's medium of 0.6 concentration. The dry mass content in sterile cultures was 6.5—9.4 per cent (Table 1).

Fig. 2. Increase in frond area of *Lemna minor* as related to Hoagland medium concentration

a — 3rd day of culturing; b — 6th day of culturing; c — 9th day of culturing

Fig. 3. Increase in frond area of *Lemna minor* as related to light intensity (Hutner medium in 0.4 conc)

a — 3rd day of culturing; b — 5th day of culturing; c — 7th day of culturing

Fig. 4. Influence of pH on surface increase of *Lemna minor* fronds (Hutner medium, concentration 0.4)

a — 3rd day of culturing; c — 9th day of culturing; d — 12th day of culturing

Table 1

Per cent content of dry matter in *Lemna minor* cultured in Hutner, Hoagland, Pirson and Seidel media at light intensity 8000 lux.

Medium	Medium concentration	Dry matter in %	
		unsterile culture	sterile culture
Hutner	0.1	12.791	7.657
	0.2	12.111	7.197
	0.3	10.233	6.992
	0.4	10.010	6.896
	1.0	9.442	6.555
Hoagland	0.6	14.427	9.433
	0.7	13.555	9.333
	0.8	13.444	9.000
	0.9	12.681	8.656
	1.0	12.569	8.543
Pirson Seidel	1.0	12.221	7.444

Table 2

Total protein content in *Lemna minor* cultured in Hutner medium

Medium concentration	Unsterile culture		Sterile culture	
	protein in mg/g of fresh matter	protein in mg/g of dry matter	protein in mg/g of fresh matter	protein in mg/g of dry matter
0.1	16.9	131.4	14.3	183.3
0.2	20.6	169.1	14.6	199.1
0.3	19.0	181.9	14.6	206.9
0.4	20.7	198.3	14.6	208.1
1.0	19.2	200.6	14.4	220.3

Table 3

Total protein content in *Lemna minor* cultured in Hoagland medium

Medium concentration	Unsterile culture		Sterile culture	
	protein in mg/g of fresh matter	protein in mg/g of dry matter	protein in mg/g of fresh matter	protein in mg/g of dry matter
0.6	27.04	174.7	18.96	199.4
0.7	26.45	194.4	19.37	211.3
0.8	30.92	228.3	19.99	222.0
0.9	31.44	241.6	21.82	248.3
1.0	30.58	252.0	24.1	264.0

Table 4

Total protein content in *Lemna minor* cultured in Pirson and Seidel medium

Unsterile culture		Sterile culture	
protein in mg/g of fresh matter	protein in mg/g of dry matter	protein in mg/g of fresh matter	protein in mg/g of dry matter
17.5	142.5	17.3	235.0

The highest protein content, 264.0 mg/g of dry mass was found in *Lemna* from sterile cultures in undiluted Hoagland's medium, in Pirson and Seidel's medium it contained 235.0 mg protein and in Hutner's medium 220.3 mg protein per 1 g of dry mass. The protein content of *Lemna minor* L. cultured on Hutner's, Hoagland's and Pirson and Seidel's medium is shown in Tables 2, 3 and 4.

DISCUSSION AND CONCLUSIONS

Investigations of numerous authors demonstrated that the particular species of the family *Lemnaceae* are capable of growing on different mineral media. The composition of the culture medium used for only one species of duckweed is often modified in dependence on the given purpose of the experiment. Much valuable information concerning the optimal conditions for duckweed vegetation may be found in the works of White from the period 1936—1940 (quoted by Hillman, 1961). The investigations of the quoted author concerned the interaction between the nitrogen and potassium level and light intensity. It should be mentioned, however, that these experiments were not carried out under sterile conditions.

In general, media for *Lemnaceae* are characterized by a high calcium content as compared with other components. The media of Hutner, Hoagland and Pirson and Seidel applied in the present experiments are rich in calcium in the form of nitrate or chloride. Another essential component of the medium is nitrogen. Joy (1969) investigating the nitrogen metabolism of *Lemna minor* L. used nitrogen in the form of ammonium or nitrate in mixture with glutamic or aspartic acid and arginine and in mixture of amino acids obtained after casein hydrolysis. He also found that many synthetic amino acids inhibit the growth of *Lemnaceae*.

Such amino acids as glutamic or aspartic acid and arginine are frequently used as nitrogen sources. Iron in organic form (iron tartrate in Hoagland's medium and citrate in Pirson and Seidel's medium) is well assimilated, whereas iron in the form of FeSO_4 (in Hutner's medium) is difficultly assimilable. An addition of Na_2EDTA (ethylenediaminetetraacetic acid disodium salt) to the medium improves metal ions uptake from difficultly soluble salts, particularly those of iron (Czopek, 1963) and calcium (DeKock et al., 1973). The sterile cultures of *Lemna minor* L. obtained in the present experiments grew on mineral media exclusively. Most authors apply in this case 1 per cent sucrose (Joy, 1969; Oota, 1972; Frick and Mohr, 1973; Grob et al., 1973; Shepard and Thurman, 1973). An addition of sucrose prompts the

growth of duckweed and at the same time serves to check the sterility of the cultures.

The results of Landolt's investigations (1957) indicate that dilution of Hutner's medium to as low a concentration as 0.1 of the basic value only slightly decreases the growth of *Lemnaceae*. On the other hand, fivefold concentration of the same medium completely inhibits the growth of *Lemna* and *Wolffia*. According to Czopek (1963), the optimal concentration of Hutner's medium is 0.33 of the basic value. At present the optimal concentration of Hutner's medium was found to be 0.40. A decrease of the concentration of Pirson and Seidel's medium to 0.1 of the basic value strongly inhibits growth of *Lemna gibba* (Czopek, 1959), therefore in the present studies this medium was used undiluted. Numerous authors used their own modifications or undiluted media for duckweed culture.

It was found in investigations on the influence of light intensity on the increments of surface area and number of fronds in *Lemna minor* L. that most intensive growth occurs at light intensities within the limits of 4000—8000 lux (Fig. 3). Czopek (1963) mentions values ranging from 4000 to 15 000 lux. The development of *Lemnaceae* is favoured by increasing light intensity only within certain limits. Landolt (1957) reports that light of about 15 000 lux damages in some species the upper part of the plant owing to the excessive quantity of thermic rays involved. Wcisło (1963, quoted by Czopek, 1963) claims that for *Lemna trisulca* culture a light of about 500 lux is sufficient.

The growth increment of *Lemna minor* L. was estimated on the basis of dry mass determinations and planimetric measurements of the surface area and number of fronds increment. These methods, particularly the planimetric one, are recommended by Czopek (1963) as particularly suitable for these purposes. Frick and Mohr (1973) showed the increase in the number of daughter fronds of *Lemna minor* L. in logarithmic form ($1000 \times$ decimal logarithm from difference of number of daughter fronds in the time interval $t_1 - t_0$).

It results from the present study that dry mass content in *Lemna minor* L. in unsterile cultures is 9.4—14.4 per cent and in sterile cultures 6.5—9.4 per cent (Table 1). The lower dry mass content in sterile cultures may be explained by the unfavourable influence on the plants of mercuric chloride and alcohol used for antiseptic purposes. Sterilization reduced the initial rate of growth. It is also possible that the increase in mass of unsterile *Lemna minor* L. is due to better aeration of the cultures. *Lemna minor* L. examined by Maciejewska-Potapczyk et al. (1970) contained 7 per cent of dry mass in unsterile conditions.

Protein content in *Lemna cultured* on sterile Hutner's, Hoagland's and Pirson and Seidel's medium was 220.3, 264.0 and 235.0 mg/g dry mass, respectively (in undiluted media, tables 2, 3, 4) and in diluted unsterile media it was 200.6 and 252.0 and 142.5 mg/g dry mass, respectively.

According to the above quoted paper by Maciejewska-Potapczyk et al. (1970) *Lemna minor* L. contains 15.77 per cent protein in relation to dry mass under unsterile conditions.

The protein content in *Lemna minor* L. indicates that this plant is its potential source. The rapid growth and vegetative propagation of duckweed in unsterile and sterile cultures in strictly established conditions allows the obtention of large quantities of the plant material, thus of large amounts of fodder or edible protein.

REFERENCES

- Chudyba H., 1969, Rośliny wodne bogate w białko, *Wszechświat* 10: 253-255.
- Czopek M., 1959, Cultivation of Polish *Lemnaceae* species in laboratory conditions, *Acta Biol. Crac. — Ser. Bot.*, vol. II: 13-20.
- Czopek M., 1963, Metody hodowli *Lemnaceae*, *Wiadomości Botaniczne* 7(2): 153-164.
- Czopek M., 1968, Metody pomiaru światła w ekofizjologii, *Wiadomości Botaniczne*, 16 (1): 4-22.
- De Kock P. C., Oota Y., Inkson R.H.E. and Knight A. H., 1973, *Physiol.-Plant.*, 28: 379-382.
- Ferguson A. R., 1970, Nitrogen metabolism of *Spirodela oligorrhiza*, III — Amino acids and the utilization of Nitrate, *Planta* (Berlin) 90: 365-369.
- Frick H., Mohr H., 1973, Phytochrome-mediated growth responses in green and etiolated *Lemna minor*, *Planta* (Berl.), 109: 281-292.
- Grob E. C., Hodler M., Eichenberger W., 1973, Beiträge zum Stickstoffmetabolismus grüner Pflanzen. II. Der Einfluss exogener Glucose auf die Aufnahme von Nitrat und Ammonium aus der Nährlösung und auf den Proteingehalt von *Spirodela oligorrhiza*, *Experientia* 29: 401-402.
- Hillman W. S., 1961, The *Lemnaceae*, or Duckweeds, *The Botanical Review*, 27(2): 221-287.
- Joy K. W., 1969, Nitrogen metabolism of *Lemna minor*. I. Growth, nitrogen sources and amino acid inhibition, *Plant Physiol.*, 44: 845-848.
- Landolt E., 1957, Physiologische und ökologische Untersuchungen an *Lemnaceen*, *Ber. Schweiz. Bot. Ges.* 67: 269-410.
- Lowry O. H., Rosenbrough N. J., Ferr A. L., Randall R. J., 1951, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193: 265-275.
- Maciejewska-Potapczyk W., Konopska L., Narzymska E., 1970, Proteins in duckweed (*Lemna minor* L.), *Acta Soc. Bot. Pol.* 2: 251-255.
- Oota Y., 1972, The response of *Lemna gibba* G3 to a single long day in the presence of EDTA, *Plant Cell. Physiol.* 13: 575-580.
- Shepard D. V., Thurman D. A., 1973, Effect of nitrogen sources upon the activity of L-glutamate dehydrogenase of *Lemna gibba*, *Phytochem.* 12: 1937-1946.

- Stewart G. R., 1972, The regulation of nitrate reductase level in *Lemna minor* L., J. Exp. Bot. 23: 171—183.
- White H. L., 1936—1940, cyt. Hillman W. S., 1961, The Lemnaceae, or Duckweeds, The Botanical Review 27 (2): 221—287.

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Próby ustalenia warunków hodowli Lemna minor L.

Streszczenie

Badano wpływ składu, stężenia i pH pożywki oraz intensywności oświetlenia na wzrost i rozmnażanie wegetatywne *Lemna minor* L. Zastosowano pożywkę Hutnera o stężeniu 0.1—0.7 i 1.0 stężenia podstawowego, pożywkę Hoaglanda o stężeniu 0.1—1.0 stężenia podstawowego i nierozcieńczoną pożywkę Pirsona i Seidela. Następnie rośliny hodowano na 0.4 stężenia podstawowego pożywki Hutnera w różnych warunkach oświetlenia (światło rtęciowe o intensywności 1200—8000 lx) i przy odmiennych wartościach pH (pH 4.5—6.5).

Początkowo doświadczenia przeprowadzano w warunkach niesterylnych. Następnie rzęsę wodną hodowano w warunkach aseptycznych. Oznaczano zawartość suchej masy materiału roślinnego. Sucha masa *Lemna minor* L. z hodowli niesterylnych wynosiła 9.4—14.4% a z hodowli sterylnych 6.5—9.4%. Rzęsa rosła najintensywniej na pożywce Hutnera o stężeniu 0.4, przy oświetleniu rtęciowym o natężeniu 8000 lx.

Najwyższą zawartość białka, 264.0 mg/g suchej masy, posiadała rzęsa hodowana sterylnie na nierozcieńczonej pożywce Hoaglanda, rzęsa hodowana na pożywce Pirsona i Seidela zawierała 235.0 mg/g suchej masy a na pożywce Hutnera 220.3 mg/g suchej masy.

Badania miały na celu opracowanie warunków hodowli *Lemna minor* L. zapewniające otrzymanie dużej ilości biomasy o wysokiej zawartości białka.