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Cultivation methods for Lemnaceae.

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Introduction

Representatives from the family of Lemnaceae make ideal experimental material for research into a succession of phytophysiological processes with regard to growth rate and vegetative reproduction. They are also easy to maintain in sterile cultures. Lemnaceae belong to the higher flowering plants (flowers are rarely produced), however they are distinguished by a much simplified morphological and anatomical structure. Reproduction is mainly vegetative. Under favourable conditions vegetative shoots create new descendents unusually quickly through which the increase in area and likewise numbers proceed in a typical exponential manner. Lemnaceae are generally accessible, being found throughout the world, producing a series of physiological differences determined by various environmental factors. As water plants they possess the advantage, that they can be cultivated in synthetic media under laboratory conditions controlled by the application of both a known light intensity and temperature. Another feature of Lemnaceae is the relatively small area they occupy in sterile cultures (in Erlenmeyer flasks), which facilitates the experimentation of the many external factors influencing the various physiological processes. Creation of optimum conditions for the laboratory cultivation of Lemnaceae allows the researcher to be independent from the vegetative season in nature and enables experiments to be conducted throughout the year.

From the four types of Lemnaceae (Lemna, Spirodela, Wolffia and Wolffiella) the most used for physiological research are Lemna and Spirodela. On account of their small size (0.3 - 3mm) Wolffia and Wolffiella are rarely used. A rich review of the literature about the physiological researches for particular species of Lemnaceae is given by Landolt (1957), Hillman (1959 a,b, 1961) and Czopek (1960, 1962).

Method of procuring sterile cultures.

Material collected from the natural environment undergoes sterilization with the aim of obtaining cultures which are completely devoid of micro-organisms. Sterilization is carried out by the submersion of plants in a 0.1% dilution of mercuric chloride and 50% alcohol for 30 - 60 secs. according to the type of plant (Kandeler 1955, Czopek 1959a, Wcislo 1963), or in a 0.05% dilution of sodium hypochlorite (NaOCl) for 60 secs. (Bitcover and Sieling 1951, Landolt 1957). The plant is next washed with sterile distilled water and transferred to the culture flasks containing a sterile synthetic medium with an addition of 1% sucrose and 0.01% asparagine as a criterion for sterility. The above operations are accomplished under aseptic conditions, in special inoculation cells. After several days of cultivation in a luminous thermostat (about 1200 lux, 28°C) descendents regenerate from the dying parent shoots and initiate the sterile cultivation. Thus obtained, sterile cultures can be transferred, aseptically, into fresh medium without repeating the sterilization procedure, making material for physiological research.

Table I.
Number of sterile fronds (after 15 days cultivation) in relation to time applied to sterilze. (Czopek 1959a).

Time of Sterilization Secs.	Mercuric Chloride 45	Alcohol 30	Mercuric Chloride 30	Alcohol 45	Mercuric Chloride 45	Alcohol 45	Mercuric Chloride 60	Alcohol 60
L. gibba	180		115		139		108	
L. minor	138		98		116		82	
L. trisulca	6		6		9		3	
Spirodela	10		7		28		8	
Wolffia	39		51		44		41	

Conditions for Cultivation

The growth rate and its progress is subject to a series of external factors, such as chemical composition of the medium, light intensity and temperature.

I. Medium

The experiments of many authors have shown, that certain species from the family Lemnaceae can grow in different mineral media with a considerable calcium content in comparison to the remaining constituents. This fact is not unexpected considering that Lemnaceae belong to the higher flowering plants, which demand calcium for their development. Lemnaceae have a high demand for micronutrients, particularly boron, manganese, molybdenum and tin. In connection with this the composition of media most used to cultivate Lemnaceae is given by Hutner (Landolt 1957), Pirson and Seidel (Kandeler 1955) and Hoagland (Bonner and Galston 1952).

A characteristics feature about the medium of Hutner is the high micronutrient content and also the addition of Na_2EDTA (di-sodium salt of ethylenediaminotetra acetic acid). In mineral medium at $pH > 6$ iron

is assimilated better in an organic form (citrate or tart rate). However through the addition of EDTA to the medium it is possible to improve the assimilation of metal ions of salts which are difficult to dissolve, particularly inorganic iron.

Table II

Composition of Chemical Media

HUTNER, mg		PIRSON i SEIDEL, mg		HOAGLAND, mg	
K_2HPO_4	400	KH_2PO_4	200	KNO_3	610
$Ca(NO_3)_2 \cdot H_2O$	200	KNO_3	400	$Ca(NO_3)_2 \cdot 4H_2O$	950
$MgSO_4 \cdot 7H_2O$	500	$CaCl_2 \cdot 6H_2O$	610	$MgSO_4 \cdot 7H_2O$	490
NH_4NO_3	200	$MgSO_4 \cdot 7H_2O$	300	$NH_4H_2PO_4$	120
$FeSO_4 \cdot 7H_2O$	25	Iron citrate	5	Iron tartrate	5
$ZnSO_4 \cdot 7H_2O$	65	$MnCl_2 \cdot 4H_2O$	0,3	Microelements	1ml
$MnSO_4 \cdot H_2O$	15	H_3BO_3	0,5	H_2O dest.	1000 ml
H_3BO_3	15	H_2O dest.	1000 ml		
$Na_2MoO_4 \cdot 2H_2O$	25			Composition of Micronutrient Solution in mg/l	
$CuSO_4 \cdot 5H_2O$	4			H_3BO_3	600
$CoSO_4 \cdot 7H_2O$	1			$MnCl_2 \cdot 4H_2O$	400
Na_2EDTA	500			$ZnSO_4 \cdot 4H_2O$	50
H_2O dest.	1000 ml			$CuSO_4 \cdot 5H_2O$	50
				$H_2MoO_4 \cdot 4H_2O$	20
				H_2O dest.	1000 ml

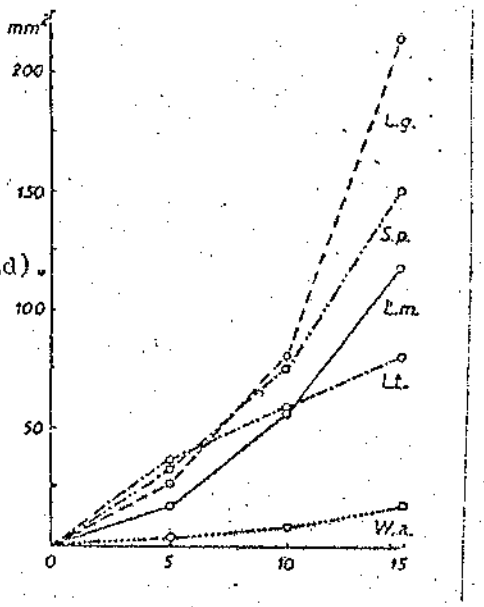

Fig. I. Increase in frond area of Lemna gibba (L.g), Lemna minor (L.m), Lemna trisulca (L.t.), Spirodela polyrrhiza (S.p.), Wolffia arrhiza (W.a) as a function of time (medium Hoagland). Abscissa - days, Ordinate - total area of frond (mm^2) Czopek (1959a).

As experiments of many authors show, the best growth of Lemnaceae is at pH values between 4.8 - 6.1, at which small changes in acidity of the medium during growth do not play a major role. (Henssen 1954, Landolt 1957). In order to get the best vegetative growth and verify the sterlity of the cultures an organic source of carbon is added to the medium. The most used is sucrose at a concentration of 1%, much less is the use of fructose and glucose (Gorham 1945, Henssen 1954, Kandeler 1955, Czopek 1959 b, 1963, Wcisło 1963). Sterile cultures grow well in a clear mineral medium, on the other hand an addition of sucrose accelerates growth considerably.

From experiments of Landolt (1957) results show, that a weakened medium of Hutner, even at $\frac{1}{10}$ the basic concentration, only slightly reduced growth. Whereas five times the concentration of the medium completely inhibited growth in Lemna and Wolffia (fig. 2). Altogether other results obtained for the medium of Pirson and Seidel, for which the basic concentration was reduced to $\frac{1}{10}$, showed a strong inhibition of growth in Lemna gibba (Czopek 1959 a)

Table III.

Increase in area, numbers of fronds and plants of Polish species of Lemnaceae in relation to the cultivation time in three media (Czopek 1959a).

Medium	Species	Increase in frond area (mm^2)			Increase in numbers of fronds			Increase in numbers of plants.		
		5	10	15	5	10	15	5	10	15
Pirson i Seidel	*L. gibba*	28,32	93,23	273,45	6	16	51	3	8	22
	L. minor	24,33	92,05	280,62	6	15	45	3	8	20
	L. trisulca	17,00	19,78	22,22	2	3	3	—	—	—
	Spirodela	11,73	37,29	49,30	2	3	4	—	1	2
	Wolffia	5,26	13,05	32,81	6	15	38	3	7	21
Hoagland	*L. gibba*	25,79	80,03	215,19	5	16	43	3	5	20
	L. minor	15,32	56,91	119,48	4	14	32	2	6	17
	L. trisulca	37,62	57,67	81,40	4	6	8	—	—	—
	Spirodela	33,91	76,34	152,14	5	7	13	2	3	6
	Wolffia	3,67	8,41	18,14	5	12	31	2	5	17
Knop	*L. gibba*	11,57	18,03	19,00	3	4	4	2	3	3
	L. minor	12,85	21,89	22,36	5	11	13	2	4	5
	L. trisulca	31,90	51,90	55,24	1	1	1	—	—	—
	Spirodela	3,83	16,33	27,47	2	3	4	1	1	2
	Wolffia	1,71	4,15	6,66	2	8	19	1	4	10

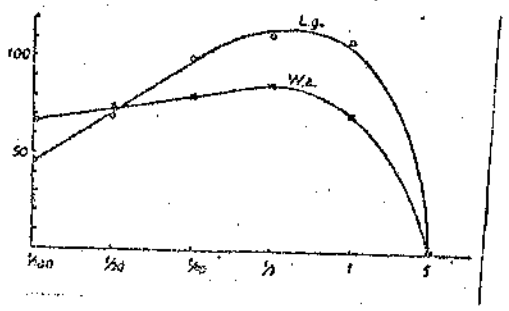

Figure 2. Growth of Lemna gibba (L.g) and Wolffia arrhiza (W.a.) in relation to the concentration of Hutner's medium at 2000 lux. and 24°C. Abscissa - concentration of medium. Ordinate - rate of growth (Landolt 1957)

Wcislo (1963) researching the morphological- physiological differences between clones of Lemna trisulca growingaseptically, stated the best growth was possible in the media of Hoagland and Clark as well as modified media of Rhode and Pfeffer. Lemna trisulca grew rather weakly in the media of Hutner, Knop, Pirson and Seidel.

Lemnaceae which grow adequately in strong light normally do not require growth substances. However growing in the dark they have a demand other than hydrocarbons and amino acids - yeast extract (Gorham 1950, quoted according to Landolt 1957). The addition of humous substances or peat extract to the medium is equally stimulating for some species of Lemnacea. This is however a complex problem demanding further research, since the various results obtained depend upon the culture conditions. The experiments of Hutner et al (1950, quoted according to Landolt 1957) showed, that peat extract like fertilizer extract did not stimulate the growth of Spirodela oligorrhiza. Wcislo (1963) discovered much better growth when cultivating Lemna trisulca in a modified Pfeffer medium prepared in 1%

peat extract. Lemna trisulca cultivated in a sterile 1% peat extract without the addition of the mineral components and sugars, remained viable for a long time but grew very slowly. The addition of 0.04% bacto-peptone to the medium of Pfeffer prodcued abnormalities in Lemna trisulca with coiled leaf shaped shoots. (Wcislo 1963).

Aeration of Cultures.

Better growth of Lemnaceae can be attained through aeration of the culture medium. However, in sterile cultures it is necessary to employ special filters to sterilize the air. Such a filter can be made by filling a glass tube (about 25cms long, 0.5 cms wide) with tightly packed cotton wool (about 10 cms long) and pieces of broken filter paper (1 cm) used generally for analysis. Thus constructed the filter is connected to culture vessels to prevent the passage of bacteria or types of fungi and can be utilised (after foregoing sterilization) for certain experiments. (Czopek 1963). An aquarium aerator can be used to aerate the medium pumping air through the above mentioned filter.

In certain cases it is better to pass a mixture of air and 5% CO_2 through the medium.

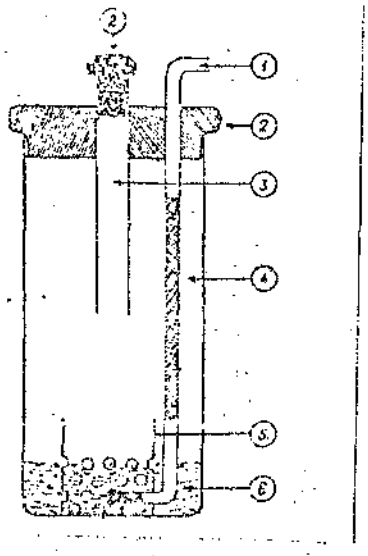

Figure 3. Scheme for the culture vessel using a collar which restricts the growth of Spirodela polyrrhiza to a precise area and filters air to the medium. 1. Flow of air from the cylinder 2. Cotton wool plug 3. Opening for plant inoculation 4. Filter to serilize the air. 5. Collar. 6. Medium (Czopek 1963)

Illumination

Cultivation of Lemnaceae is usually conducted in an illuminated thermostat, in which the light intensity can be freely adjusted. A white light whose spectrum approached that of daylight is the most profitable for the growth of Lemnaceae (like many other higher plants). Measurements oflight intensity are usually taken with photometer (lux) or a thermopile and galvonometer (ergs/cm^2/sec.). The light intensity is measured accurately inside erlenmeyer flasks used for the experiments, since the walls of these vessels and also the cotton wool plugs absorb a certain percent of the light. Photothermostats (fig. 4) with chromatic light illuminated over a considerable area are used for research into the effects of various parts of the spectrum on photobiological processes (germination, flowering). An exact description of this thermostat is found in the work of Czopek (1962).

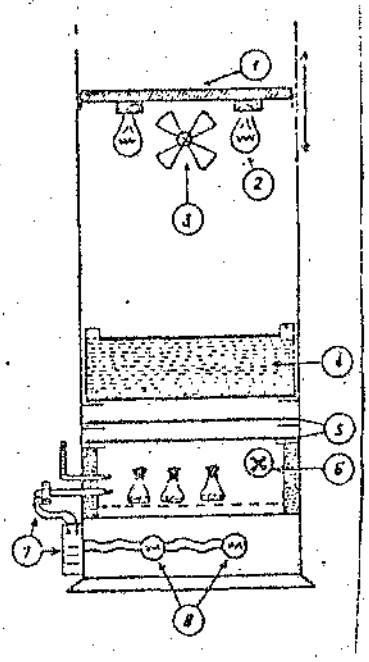

Figure 4. Construction plan of the photothermostat with chromatic light. 1. Moveable plate. 2. Light source (4 x 150w bulbs) 3. Fan to cool the bulbs 4. Glass aquarium with liquid filter, (solution of $CUSO_4$ $5H_2O$ in 0.5%H_2SO_4 or $Fe(NH4)_2$ in 2% H_2SO4) 5. Coloured glass filter. 6 Fan mixing the air 7. Contact thermometer together with a mercury relay 8. Heating thermostat (2 x 25w bulbs). (Czopek 1962).

The development of Lemnaceae is improved by a rise in the light intensity up to a certain limit, Landolt (1957) states that particular species and varieties of Lemnaceae achieve maximum growth at intensities

between 4000 and 15000 lux. However for certain species an intensity around 15000 lux. produces damage to more than half the plants, in the opinion of Landolt (1957) this is caused by a harmful excess of warm irradiation. Czopek (1959 a, b, 1962, 1963) successfully cultivated Lemnaceae at a lower intensity - about 1200 lux. Whereas Wcislo was satisfied that an intensity around 500 lux was sufficient to cultivate Leman trisulca. For the growth of the majority of Lemnaceae continuous illumination is an advantage. Illumination for less than 24 hrs. per day reduces and even inhibits growth, particularly at low intensities (Landolt 1957). At a reduced light intensity about 1000 lux for 12 hrs daily, the fresh weight of Lemna trisulca, a plant liking the shade, is reduced in the region of 10% - 30%. For this species the most fronds and also the highest amount of fresh and dry weight are attained under continuous illumination (Wcislo 1963).

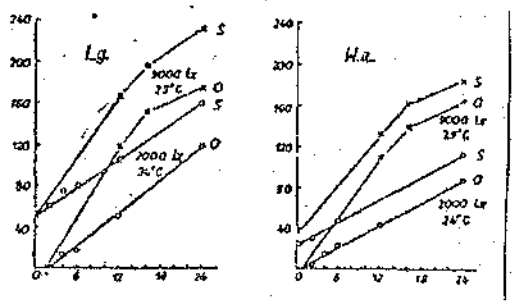

Figure 5. Growth of Lemna gibba (L.g) and Wolffia arrhiza (W.a.) in relation to the illumination time (9000 lux., 29°C and 2000 lux, 24°C) in media with (S) and without (O) sucrose, Abscissa - illumination time (hrs.) Ordinate - rate of growth (Landolt 1957).

Temperature

Particular species of Lemnaceae have different thermal requirements. Maximum temperatures for the growth of Lemnaceae (relating to temperatures at which the plants survive over a long period of time) are in the region 26 - 37°C and minimum between 4 - 18°C. These are more or less similar to the range of temperature for other flowering plants. The turions (organs of

sporulation) of Spirodela polyrrhiza are unaffected by low and high temperatures. They resist temperatures of 50°C for 24 hrs, 45°C for a week, -4°C for two weeks without any harm (Jacobs 1947). For the growth of Lemnaceae in a medium without sucrose Landolt (1957) showed that the optimum temperature was in the region 20 - 30°C, however with sucrose it is a little higher, between 23 - 32°C. Raising the temperature reduces the area of shoots in the medium without sucrose at the optimum regions, whereas the addition of sucrose increases the area at these temperatures (Table IV). Czopek (1959 a, b, 1963) shows the cultivation of Polish species of Lemnaceae at a stable temperature 28°C(± 1°C) giving perfect results.

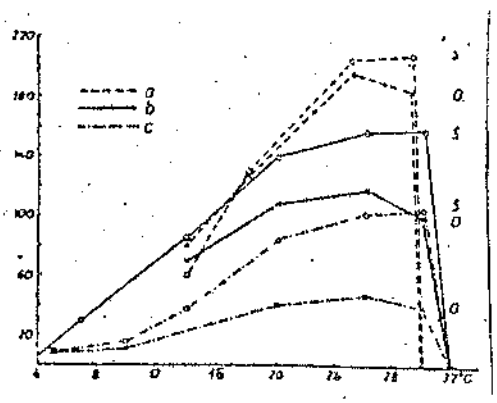

Figure 6. Growth of Lemna gibba (L.g.) in relation to temperature at various light intensities a. -9000 lux (continuous), b. - 2500 lux (for 16 hrs.), c - 1000 lux (18 hrs). Medium with sucrose (S), without sucrose (o). Abscissa - temperature Ordinate - rate of growth (Landolt 1957).

Table IV. Average area of mature shoots (mm^2) at 1000 lux for 18 hrs, at various temperatures. Results from 4 repeats on 50 shoots.

Species	Medium	Temperature			
		10°	20°	26°	32°
Lemna minor	without sucrose	4,4	5,7	4,0	2,6
	with sucrose	4,2	6,8	6,5	4,2
Spirodela polyrrhiza	without sucrose	turions	16,5	15,2	13,2
	with sucrose	(2,7)	22,3	35,0	38,7

Cultivation of Lemnaceae in the dark

For research into some photobiological processes, it is necessary to make etiolated shoots of Lemna and Spirodela in darkness. Under these conditions Lemnaceae require (apart from mineral medium) not only an organic source of carbon and nitrogen (sucrose and amino acid), but also yeast extract. From 23 amino acids tested (Gorham 1950, quoted according to Landolt 1957) only d, 1-isoluecine, d, 1-aminobutyric acid and β - alanine stimulate heterotrophic growth, while other amino acids have a rather harmful effect. In experiments with Lemna minor Gorham used a mixture of 1% sucrose, 0.08% hydrolised casein and 0.004% yeast extract. Wcislo (1963) using a medium prepared in 1% peat extract with an addition of 1% sucrose noticed only a minimum increase in the fresh weight of Lemna trisulca when grown in darkness, whereas she showed an increase in the numbers of descendents coming from a single parent. A similar phenomenon was observed for vegetative shoots of Spirodela polyrrhiza by Czopek (1959b).

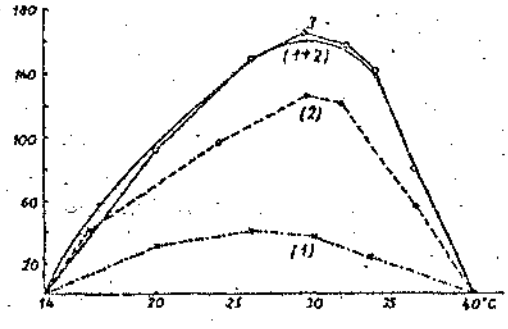

Figure 7. The growth of Spirodela polyrrhiza stimulated by sucrose in light and darkness. Graph shows the dependence of growth on temperature, illuminated at 1000 lux for 18 hrs. 1. (1000 lux, without sucrose). Abscissa - temperature. Ordinate - rate of growth. (Landolt 1957).

Apart from heterotrophic growth in complete darkness in medium with the addition of organic components(sucrose, hydrolised casein and yeast

extract) growth exists «non-photosynthetic» which is insufficient at a low intensity of light to synthesize chlorophyll and photosynthesize. This type of growth demands an addition of sucrose to the medium. The influence of this light replaces the action of organic substances (hydrolised casein, yeast extract) essential for positive growth in complete darkness. In the opinion of Hillman (1961) heterotrophic growth can be considered as a subdivision «non-photosynthetic» of growth. As experiments of Hillman showed, the rate of vegetative reproduction for Lemna minor in darkness in a medium with 1% surcrose can be much improved by disrupting the dark period with several minutes action of red light during three to four days. The effect of red light can be reversed by direct action of far red (730mμ) light. Here the role played is probably the same absorptive arrangement as in photoperiodicity, deetiolation, germination and other photobiological processes. The effect of low intensities of red light upon the rate of vegetative reproduction in Lemna minor can be replaced by a stimulative activity of certain chemical substances, particularly kinetin(6-furfurylaminopurine) at a concentration of 3×10^{-6}M (Hillman 1957). In connection with this Hillman put forward the supposition that yeast extract served the medium as a source of kinetin. Czopek (1959b) stated, that etiolated turions of Spirodela polyrrhiza formed in the medium of Pirson and Seidee with 1% sucrose also in darkness; they are however rather smaller in comparison with those formed in the light. Etiolated turions possess a similar ability for growth as the olivegreen ones from the light.

Measurements of Growth

With regard to the flattened lens shape of Lemnaceae the most positive and applicable indication of growth is the increase in area, frond and plant numbers. The fresh and dry weights are calculated much less. Various methods are used to measure the area of the shoots for Lemna and Spirodela. One of these is the projection of a picture of the floating fronds onto a relatively dull screen (Ashby, Bolas, and Henderson 1928, Ashby and Oxley 1935).

A condition of this method is that the fronds do not overlap. A further perfection is the method of photographic planimetry (Gorham 1941, Czopek 1959 a,b). At the beginning of an experiment and every 24 hrs. thereafter an enlarged photograph is made of the plants (upto termination of growth) and the area determined by a planimeter. Over large areas the error is slight (± 0.2%). Besides, from photographs it is possible to determine a factor for the shape of an individual frond (relation of length to breadth), the number of fronds and the number of plants. The area in cultures, like the total number of developing fronds increases exponentially. As shown by Czopek (1959b) the growth of area of an individual frond of Spirodela polyrrhiza terminates during 10 - 15 days.

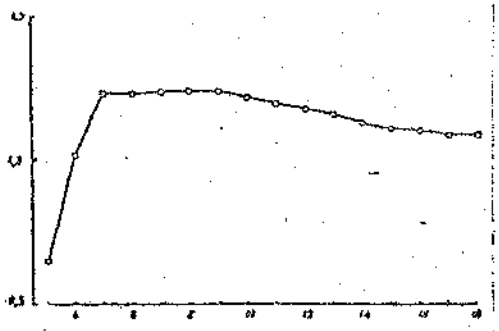

Figure 8. Factor for the shape (length to breadth) of an individual frond of Spirodela polyrrhiza as a function of time. Abscissa - days. Ordinate - factor (Czopek 1959b).

Table V. Formation of offshoots, progress of their growth and total increase in area of fronds, which developed from a single germinated turion of Spirodela polyrrhiza, in relation to cultivation time (Czopek 1959b).

Day of cultivation after germination	Area of fronds (mm^2)				Total area of fronds I - IV (mm^2)	Total increase in area of all fronds which developed from one turion (mm^2)
	I.	II.	III	IV		
3	5	—	—	—	5	5
4	9	—	—	—	9	9
5	11,9	1,7	—	—	13,6	13,6
6	13,8	6,6	1,2	—	21,6	21,6
7	16,6	12,2	2,5	—	31,3	31,3
8	19,8	16,2	5,4	2,3	43,7	43,7
9	20,8	17,1	9,9	7,8	55,6	58,4
10	23,7	18,5	16,5	13,1	71,8	82,6
11	25,4	21,4	18,9	16,6	82,3	103,5
12	28,6	23,0	20,6	18,1	90,3	132,9
13	19,6	26,1	23,4	22,2	101,3	190,5
14	30,8	26,9	25,2	23,6	106,5	249,1
15	32,0	28,3	26,9	25,9	113,1	313,2
16	32,5	30,5	28,6	27,7	119,3	402,3
17	32,5	32,0	31,0	28,5	124,0	—
18	32,5	32,5	32,4	31,8	129,2	—

Experiments concerned with the growth of Lemnaceae in the dark demand the removal of measurements in the light not influencing physiological activity. In experiments with Spirodela Gorham (1945) used dark green light, which only to a small degree influenced growth. Like results of Withrow and Price (1957) green light in the region 500 - 550 mμ had a minimum effect on photomorphogenesis. Because the eyesight adapted to darkness possesses a maximum sensitivity at 510 mμ it is possible to use low intensities of green light for the observation of growing material developing in darkness. The rates of vegetative reproduction in sterile cultures in light and darkness and also the easiness by which Lemnaceae are cultivated in accurately determined laboratory conditions, allows the use of material for photophysiological research independent of the time of year.

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Notice

Please note that these translations were produced to assist the scientific staff of the FBA (Freshwater Biological Association) in their research. These translations were done by scientific staff with relevant language skills and not by professional translators.