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A Modification of the *Lemna* Test for Phytotoxicity

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INTRODUCTION

Chemical weed controls are employed more and more in the modernization of agriculture. The controls are of increased importance in the economy of an intensively used land area, such as the farming lands of Puerto Rico. Good control of weeds has to be based on a study of the phytotoxic properties of the compounds applied. Field tests alone cannot be used for this purpose because too many extraneous variables, such as the nature of the soil and the variations of climatic conditions, obscure the results. Hence, a number of laboratory methods have been introduced (see *8*, *16* for reviews[3].) Most of these methods are derived from tests on growth-promoting substances; hence, they can be used for this type of weedkiller only. The *Lemna* test, however, is applicable to weedkillers of all types and appears to be the method of choice for studies of relative phytotoxicity.

Since Hessenland, Fromm, and Saalmann (*14*) first used *Lemna minor* Linn. for comparative studies of phytotoxicity, the method has been altered repeatedly (*3*, *9* to *15*). Most of the modifications suggested were based on the fundamental studies of the physiology of *Lemna* by Ashby, Clark, and their coworkers (*1*, *2*, *5*, *6*, *7*). Work carried out with the idea of developing a more satisfactory *Lemna* test is reported here. As the purpose of the test is the evaluation of the toxic action of chemical compounds, but not the production of optimal growth of the plant, the procedure of Ashby and coworkers (*1*, *2*) was not followed strictly by any of the users of *Lemna* as a test plant.

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[2] The author is very much indebted to Chairman B. S. Meyer, Department of Botany and Plant Pathology, Ohio State University, Columbus, Ohio, for his permission to use the facilities of the department for part of the experiments, and to Julio Bird Piñero and Moisés Padilla at this Experiment Station for taking the picture shown as figure 1.

[3] Italic numbers in parentheses refer to Literature Cited, p. 101–2.

PROCEDURES

In recent experiments four 400-ml. beakers, made of resistant laboratory glass, were filled with the pure nutrient [Clark and Roller (*7*)], or the nutrient containing the toxic agent in a given concentration. Twenty plants of *Lemna minor* with two fronds each were planted in each beaker. The plants were all taken from the same strain. In contrast to earlier work done at Pittsburgh, Pa., (*10*, *11*, *12*) and Santurce, P.R., (*9*) for the experiments reported here, strain 2803, now considered the standard clone, was used. It consists exclusively of descendants of one plant, the cultivation of which was started at Columbus, Ohio, on September 11, 1958. The beakers were covered with petri dishes to avoid concentration of the solutions by evaporation of water, and kept at a window sill or in the greenhouse under natural light, and the number of plants and fronds was determined daily. For the control and the sublethal concentrations of a poison the growth rate K was calculated from the equation $\log (N/N_0) = Kt$ according to Clark (*5*) and the percentage growth inhibition established as $100\ (1 - K/K_0)$, K_0 being the growth rate of the control, N_0 the number of fronds at the start of the experiment, and N the number of fronds after t days.

The growth rate K of the controls varied from 0.028 to 0.051 with a standard error of σ 0.001–0.003, *i.e.*, the results are useful qualitatively rather than quantitatively. The various factors which influence the growth of *Lemna minor* were, therefore, examined in the hope that a better control of these variables would yield more accurate values.

GROWTH-RATE STUDIES

A more constant growth rate of the controls than that attained in the laboratory at Pittsburgh could be achieved by more uniform illumination. In the greenhouse at Columbus, Ohio, controls grew generally with K = 0.05–0.06, under artificial light much higher rates, *e.g.*, 0.11 to 0.12 at 500 foot-candles for 24 hours a day were obtained for the growth of fronds.

Even small differences in illumination produced noticeable differences in the growth rate. In experiments in which the four beakers were arranged vertically with respect to the light source, a definite trend for K was observed, while an arrangement strictly parallel to the windows or light source showed random distribution of K (table 1). Table 1 illustrates the growth rate during a hot summer. Illumination immediately at the window was too strong for optimal growth and the growth rate increased, therefore, with the distance from the window. In the winter months the change in growth rate was reversed for the arrangement vertical to the window; the best conditions of illumination existed directly at the window and the growth rate decreased with the distance from the window.

The growth rate of the plants was not strictly parallel to the increase in the number of fronds and showed a higher standard deviation. Ashby and coworkers (*1*) have already observed a definite rhythm in the growth of *Lemna* plants, but an accurate count of the number of plants appears difficult because during long photoperiods and under continous illumination, *Lemna* tends to form clusters of plants which are difficult to count. Plants with four fronds which are occasionally mentioned in the literature (*1*) are an aggregation of two plants with two fronds each, as can easily be seen by the existence of two roots, and the fact that they separate readily on a light touch or by simple transfer from one solution to another. Even plants with three fronds frequently already have two roots, but they are generally not yet ready to separate.

Figure 1 shows typical forms of the plants. Only the plants with two

TABLE 1.—*Growth rates of fronds of* **Lemna minor** *in Clark's nutrient when beakers containing them were differently arranged*

Vertical to window of greenhouse		Parallel to window of greenhouse	
Position of beaker	$K \pm \sigma$	Beaker No.	$K \pm \sigma$
Nearest to window	0.023 ± 0.003	1	0.069 ± 0.001
1 beaker removed from window	.034 ± .002	2	.070 ± .001
2 beakers removed from window	.033 ± .002	3	.050 ± .002
3 beakers removed from window	.048 ± .002	4	.063 ± .001

fronds at the top right and in the center of the picture are single individuals. All other forms are associations of two plants, as can be seen by the thin dividing lines and, in some cases, by the fact that more than one root is visible. Similar observations on other *Lemnaceae* have also been reported by Yoshimura (*17*). One effect of a toxic agent is also the splitting of the plants into individuals with one frond each, which leads to a remarkable increase in the number of plants, but does not indicate any growth, as the fronds begin to die at the same time (*11*, *15*). The evaluation of the growth of *Lemna* was therefore based on the fronds only.

MINIMUM VOLUME OF NUTRIENT

However, the growth rate of the fronds was not constant during the first 1 to 3 days of the experiment. It was suspected that this deviation was due to the relatively large percentage error which 1 frond more or less represents for 40 fronds. A series of experiments in which 80 fronds were used in petri dishes showed no improvement. The growth rate during the first 2 to 3 days differed greatly from that observed during the following

5 or 6 days. An unexpected result was a significantly lower growth rate in the experiments with 80 plants in petri dishes (60-ml. capacity) than in controls run with 40 plants in 400-ml. beakers (table 2).

The conclusion that the lowering of K was caused by starvation, *i.e.*, an insufficient volume of nutrient, was confirmed in experiments like the second series in table 2, in which the *Lemna* was started on 100 ml. of nutrient, and was transferred to fresh nutrient every day. Under these conditions

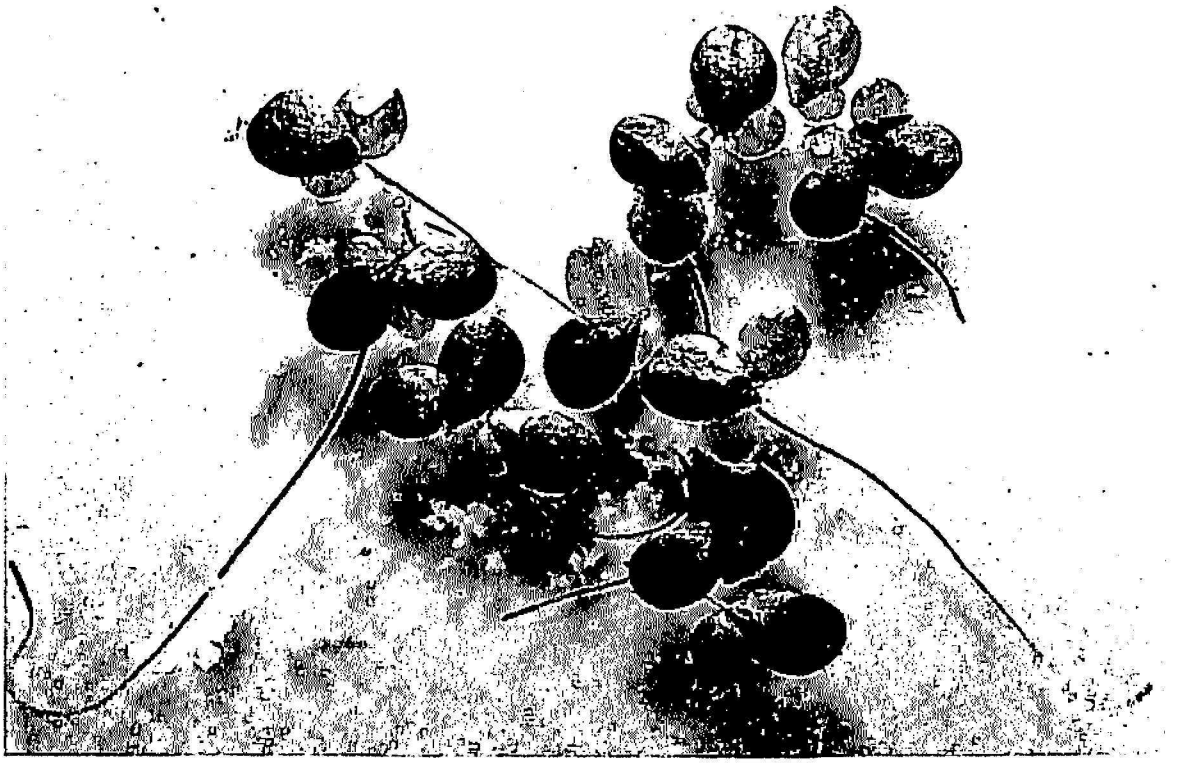

FIG. 1.—Typical plants and clusters of *Lemna minor* enlarged about 2×.

growth in the smaller volume was not significantly different from that of the control. Eventually, a volume of 1 to 2 ml. of nutrient per plant with two fronds per day was established as the minimum requirement for good growth.

Hence, it was concluded that the total volume used must be adjusted to the population size, *i.e.*, the experiment with 20 plants per beaker could be started in 50-ml. beakers, but a larger beaker should be substituted as soon as >50 plants, meaning >100 fronds, have appeared.

Frequent changes of the nutrient solution have already been recommended by Clark (*5*). He introduced the numerous changes to avoid the cumbersome aeration of the solutions and to control the growth of algae

in them. These are not the only advantages obtained by a daily change of solution. It is to be expected that the growth of any plant on a given volume of nutrient will decrease the concentration of the dissolved salts continuously. This decrease can easily be followed by conductivity measurements. Typical data on this change and its effect are shown in table 3. The growth of 20 plants with 2 fronds in each of four 50-ml. beakers, in which the nutrient was changed daily, was compared with that of the same number of plants in four 250-ml. beakers in which the solution was not changed during the 7 days of the experiment. The resistance of the solutions was measured every day.

Generally, the growth rate in the unchanged solution was not significantly different from that of the plants in solutions which were changed daily, but occasionally a significant decrease in growth rate (of about 10 percent) was seen in the unchanged nutrient. More important is the fact that frequently the growth rate in the unchanged nutrient is not constant

TABLE 2.—*Results of growth experiments with* **Lemna minor** *in different volumes of Clark's nutrient*

Volume of nutrient (ml. daily)	Original number of fronds	$K \pm \sigma$	$(100K)/K_0$	T
400	40	0.076 ± 0.004	100	—
60	80	.035 ± .005	46	6.19
400	40	.050 ± .003	100	—
100	40	.048 ± .002	95	.76
60	40	.033 ± .006	66	2.47

at all, but decreases steadily from day to day, *e.g.*, while K for the experiment, with a daily change of nutrient was 0.1091 ± 0.0002, it diminished continuously in the unchanged nutrient from 0.1188 on the third day to 0.1024 on the ninth day.

Similar concentration changes are to be expected for the toxic agent dissolved in the nutrient. Direct evidence is difficult to obtain as most of the toxicants show very little conductance (if any) and their initial concentration is already low.[4]

[4] After completion of this manuscript the paper: The Uptake of Growth Substances, I: Factors controlling the uptake of phenoxyacetic acids by *Lemna minor*, by G. E. Blackman, G. Sen, W. R. Birch, and R. G. Powell, *J. Exp. Bot.* **10**, 33–54 (1959), came to my attention, *C. A.* **54**, 2504 *f* (1960). Their statement that 23 to 25 ml. of nutrient are satisfactory for 115 to 120 fronds for 4 hours is in good agreement with my estimate of 1 to 2 ml./day for 2 fronds. The simultaneous uptake and loss of toxicant by the plant which they describe is a further argument for a daily change of the experimental solution, as a decreasing concentration of this solution would create a concentration gradient, the influence of which on the toxicant concentration in the plant would be difficult to determine.

EFFECTS OF PREVIOUS HISTORY OF PLANT MATERIAL

The daily change to a fresh quantity of at least the minimum volume of nutrient, however, did not explain or resolve the problem of the irregular growth at the start of the experiment. Ashby and Oxley (*2*) have already pointed out that the previous history of the plants influences the outcome of the experiment, but they did not give any data on the intensity or duration of its influence on the growth of the plant.

One of our cultures which suffered a severe Mn deficiency and had a growth rate K of 0.029 when exposed to 400 foot-candles needed 6 days after the addition of the Mn to reach the new constant growth rate of 0.071 ± 0.001. However, in subsequent runs it was proved that the recovery was not yet complete; for the growth rate in individual beakers receiving identical treatments continued to show fluctuations of ± 20

TABLE 3.—*Effects on growth of* **Lemna minor** *of a change of the nutrient and its concentration during 7 days*

Volume of nutrient (ml.) and changes	$K \pm \sigma$	$100K/K_0$	T	Conductance in mho × 10^{-5}		
				0 day	2nd day	Last day
50, daily	0.111 ± 0.003	100	—	122	122	122
250, none	.115 ± .002	104	1.27	122	118	104
50, daily	.123 ± .001	100	—	118	118	118
250, none	.126 ± .001	103	1.86	118	105	98

percent with each other. Only after about 5 weeks of uniform cultivation did parallel beakers give constant growth rates with a standard error of ± 0.003. Similarly, in an experiment in which survivors of poisoning with 0.01 M ethylamine were transferred after 13 days to pure nutrient, the growth rate showed an immediate increase and, after 4 days, was better than that of the control, but the plants and fronds were still exceedingly small when the run had to be terminated on the 19th day of the experiment. Similar periods of adaption were observed when the intensity of illumination was changed.

Even much smaller changes in previous history of test plants produced significant changes in the growth rate. For example, in one series four beakers were planted with stock of clone 2803 which had been growing well, but had then been kept on the same nutrient solution for 22 days, while another four beakers were planted with *Lemna* of standard clone 2803, the nutrient of which had been changed daily. Both sets of beakers were kept in the greenhouse under identical conditions with daily renewal of the nutrient for 11 days. The growth rate of the second set of beakers

was 0.0603 ± 0.0004, while the fronds in the first set grew at $K = 0.051 \pm 0.001$, *i.e.*, 85 percent *as well* (T value 11.04) as that of the second set. In addition, the standard error was more than twice as high, indicating that the growth rates in the individual beakers of the first set fluctuated much more than those of the second set. In another case plants with two fronds each were growing with a value of $K = 0.120 \pm 0.001$. These were being illuminated at 500 foot-candles for 24 hours a day, the solution had pH 6.8 at a temperature of 23° ± 1°. These plants were grown on nutrient which was changed daily. After transplanting these plants to a new series under the same conditions the growth value was found to be $K = 0.123 \pm 0.001$. However, the count on the first day of the new run continued to be irregular.

Therefore, in an attempt to overcome growth irregularity, the plants were passed through a preliminary cultivation under conditions identical with the control of the experiment planned and used only after they had shown a constant growth rate for from 5 to 7 days. Under these conditions the controls would reach a constant growth rate on the second day of the experiment. If the stock of plants was kept on the same, and frequently changed nutrient, as the control, it was found that a preparatory run of about 1 week was sufficient. Often, the controls of the previous experiment can be used directly as material for the next test.

In the course of these studies widely varying growth rates of *Lemna*, clone 2803, have been used. Hence, the question whether toxicity assays at these different K values are comparable, had to be considered. Blackman and Robertson-Cuninghame (*4*) have shown that the response of *Lemna minor* to 2,4-D increased with increasing temperature, and that a light intensity of 700 foot-candles produced a slightly stronger growth inhibition by 2,4-D than lower intensities. Our data, especially on the action of 10^{-2} M ethylamine and 10^{-3} M aniline at growth rates from 0.015 to 0.110, showed that $K < 0.02$ is liable to give irregular results while at $K > 0.06$ the response to killing concentrations may take several days longer than at $0.02 < K < 0.06$. The different growth rates in these experiments were produced by partial starvation and (or) variations in the temperature from 17 to 27° C.; the illumination was always 500 foot-candles. As the purpose of the test is the determination of the *relative* toxicity, work under as nearly as possible constant conditions is a requisite. For practical reasons, a temperature of 24 ± 1° C. and an illumination of 400 foot-candles is preferred.

THE MODIFIED PROCEDURE

The results and observations described above were incorporated in our present testing process in the following manner:

Ten milliliters of solution containing 10 gm. of $CaHPO_4$ per liter, 8 ml. of *M* KNO_3, 1 ml. of *M* $MgSO_4$, 3 drops of *M* $FeSO_4$, and 4 drops 0.1*M* $MnSO_4$ or $MnCl_2$ were filled up to 1 l. with water which was distilled twice in all-glass equipment. Commercially available c.p. chemicals were used for the stock solutions. Eighty *Lemna minor* plants from clone 2803, each with two fronds, were distributed in four covered 50-ml. beakers containing this nutrient, placed parallel to the light source, and grown at the light intensity, (*e.g.* 400 foot-candles), pH (*e.g.* 6.5), and temperature (*e.g.* 24° C.) planned for the following test. Daily changes of the nutrient and counts of the fronds were continued until K was reasonably constant for at least 5 days.

Then four 50-ml. beakers each for nutrient and each of the concentrations of the toxicant were prepared and each was seeded with 20 plants, 40 fronds (N_0)—only 10 plants are used if the experiment is to be continued for more than 8 days—of the *Lemna* from the prepared test plants. Plants with two roots or a sharp dividing line between fronds were excluded. No clusters of plants were transplanted. If the number of single plants with two fronds was not sufficient for the start of the experiment, the clusters were separated into their components and the suitable individuals used. Daily, all solutions were changed and the number N of fronds was determined at intervals of 24 $\pm$ 1 hour, generally at the time of transfer to fresh solution. The value $\dot{K}$ for each beaker and day was calculated as log (N/N_0)/number of days. From these daily values the mean growth rate K_b per beaker was calculated. From the four K_b of each set (including the control) the average K for each solution was found.

SUMMARY

1. The count of fronds of *Lemna minor* is more suitable for the determination of the growth rate than that of the number of plants.

2. The minimum volume of Clark's nutrient solution for one plant with two fronds is 1 ml. per day.

3. The growth of *Lemna minor* in nutrient solution produces measurable changes in nutrient concentration in 24 hours. A daily change of the solution is, therefore, advisable. This applies also to the nutrient containing the toxic agent, as a decrease in concentration is likely to occur there as well.

4. The previous history of the plants manifests itself in an irregular growth rate during the first days of the experiment, if their history is different from the conditions of the experiment. Plants to be used for a test should, therefore, be cultivated under the conditions to be applied for the control until their growth rate is reasonably constant.

5. The modified method for the *Lemna* test for phytotoxicity is described.

RESUMEN

1. El contaje de las frondas de *Lemna minor* se presta más para determinar el promedio de crecimiento que el número de plantas.

2. El volumen mínimo de la solución nutritiva de Clark para una planta con dos frondas es de 1 ml. diario.

3. El crecimiento de *Lemna minor* en solución de nutrientes produce cambios mensurables en la concentración de nutrientes en un período de 24 horas. Un cambio diario de la solución es, en consecuencia, aconsejable. Esto también se aplica al nutriente que contiene el agente tóxico, así como es posible que ocurra una reducción en la concentración.

4. La historia previa de las plantas se evidencia en un promedio de crecimiento irregular durante los primeros días del experimento, siempre que la historia sea diferente de las condiciones del experimento. Las plantas que van a usarse en una prueba deben cultivarse bajo condiciones que vayan a aplicarse para el control, hasta que el promedio de crecimiento de ellas sea razonablemente constante.

5. Se describe aquí el método modificado para la prueba de fitotoxicidad con *Lemna.*

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