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ORIGINAL PAPERS

Duckweed (*Lemna gibba* L.) as a Test Organism for Homeopathic Potencies

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ABSTRACT

Objectives: A bioassay with duckweed (*Lemna gibba* L.) was used to study the effects of homeopathic potencies on the plant's growth rate. Screening included 12 substances: argentum nitricum, copper sulfate, gibberellic acid, 3-indole acetic acid, kinetin, lactose, lemna minor, methyl jasmonate, metoxuron, phosphorus, potassium nitrate, and sulfur. Each substance was tested in the potency range 14x–30x. Controls were unsuccussed and succussed water.

Design: In randomized and blinded experiments, duckweed was grown in either potentized substances or water controls over 7 days. Frond (leaf) growth was measured regularly with a computerized image analysis system and growth rates were calculated for different time intervals (day 0 –7, 0 –3, 3 –7). Additionally, a water control run with unsuccussed water as the only test substance was performed to determine the variability of the bioassay.

Results: For the water control run, the between-group coefficient of variance for groups of five replicates was 0.87% for the frond area–related average specific growth rate $r_{(area)}$ compared to 1.60% for the frond number–related average specific growth rate $r_{(num)}$. Thus, the former is the preferred parameter to be used. Of twelve tested substances, potentized argentum nitricum, phosphorus, and kinetin significantly ($p < 0.05$, analysis of variance F-test) affected the main parameter: frond area–related average specific growth rate (day 0 –7). Segmented area growth rates (day 0 –3 or 3 –7) were affected by potentized argentum nitricum, gibberellic acid, lactose, and phosphorus.

Conclusions: The described experimental set-up with *L. gibba* as test organism appears to be a promising new model system to investigate effects of potentized substances. Yet larger sets of replication experiments with selected test substances and systematic negative controls are necessary to verify the effects found.

INTRODUCTION

Despite the successful use of homeopathic remedies for more than 200 years and several meta-analyses of placebo-controlled clinical trials supporting the notion of specific remedy effects,[1,2] there is still a controversial discussion about specificity or nonspecificity of the homeopathic remedy production procedure.[3] Critics mostly concentrate on the fact that some remedies are diluted beyond the Avogadro number, so that no molecule of the original substances is left (higher than 12c or 24x, respectively). To tackle these problems, studies with high methodological quality in different fields of basic research into homeopathy are required.[4]

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Besides physicochemical investigations,[5] test systems with animals and microorganisms have been used to study the effects of homeopathic remedies.[6–9] Also plants appear to be suitable test organisms, since it has been shown that they are able to react to potentized substances.[10–13] Additionally, botanical test systems are usually fast and simple, allowing large numbers of experimental replications and eliminating disadvantages such as the placebo effect or ethical concerns.

Duckweeds are small, monocotyledonous flowering plants, which occur in standing and slowly flowing waters almost all over the world.[14] Due to their small size and rapid, predominantly vegetative reproduction forming genetically uniform clones as well as their high sensitivity to organic and inorganic substances, duckweeds are used as research organisms for studies in physiology, genetics, ecology, environmental monitoring, and ecotoxicology.[15–18] However, we do not know of any study in which duckweeds have been used as test organisms to assess the effects of homeopathic potencies.

The objective of this study was to develop a simple and stable bioassay to test the effect of potentized substances using *Lemna gibba* L. as model organism. Duckweed's growth rates can be measured based on frond (leaf) area or frond number. Both growth rates were compared regarding their variability. In screening experiments, 12 selected substances were potentized up to 30x and tested for their possible impact on frond growth rate of *L. gibba*.

MATERIALS AND METHODS

Preparation of the test solutions

Among the test substances included in the screening were plant hormones, specific and unspecific toxins, a nutrient, homeopathic remedies, and lactose as trituration medium of one remedy used (Table 1). The homeopathic remedies were chosen in cooperation with physicians due to their relation to human growth processes. Potentized lemna minor was additionally included because of its close botanical relationship with the test species. Water for potentization or controls was distilled (Büchi, Fontavapor 250, Flawil, Switzerland) from tap water and autoclaved before use. All test solutions for one experiment (potencies as well as controls) were freshly prepared according to the multiple glass method[19] before 10 AM on the day of the experiment from the same batch of distilled water. Before use, all potentization vessels were thoroughly rinsed three times with deionized water (<0.5 μS/cm, Christ Milistil P-24, Christ Aqua Ecolife, Aesch, Switzerland) and once with distilled water and then dried at 90°C.

Water-soluble substances were potentized (i.e., diluted and succussed), in distilled water from 1x to 30x. The herbicide and the plant hormones were potentized in acetone (AppliChem, Darmstadt, Germany) to 1x, then further in distilled water. The homeopathic remedies were obtained in the lowest potency available (4x or 6x) and further potentized in distilled water. To dissolve 1 mg of lactose or trituration in 9 mL of distilled water, solutions were gently heated to 38°C. Substances not usually used as homeopathic dilutions were prepared in concentrations according to their agricultural applications.

Potentization vessels were of Duran® glass (500 mL, Schott, Mainz, Germany). Thirty (30) mL of the potency stock solution was vigorously shaken by hand in a horizontal line for 2 minutes at a rate of approximately 2 Hz at room temperature. Thirty (30) mL of this solution was added to the second potentization vessel containing 270 mL distilled water and shaken in the same manner. This process of successive tenfold dilution steps and vigorously shaking proceeded until the 30th potency step was reached. For the water controls, a potentization vessel containing 300 mL of distilled water remained unsuccussed and was only gently

Table 1. Substances Included in the Screening Experiments

Substance	*Empirical formula*	*Category*	*Source*	*Concentration of potency stock solution*
Argentum nitricum	$AgNO_3$	Homeopathic remedy	Weleda, Arlesheim, Switzerland	4x dilution
Copper(II) sulfate 5-hydrate	$CuSO_4 * 5\ H_2O$	Toxin, unspecific	Merck, Darmstadt, Germany	400 mmol/L
Gibberellic acid	$C_{19}H_{22}O_6$	Plant hormone	Fluka, Buchs, Switzerland	0.029 mmol/L
3-Indole acetic acid	$C_{10}H_9NO_2$	Plant hormone	Riedel-de Haën, Seelze, Germany	0.057 mmol/L
Kinetic (6-furfurylamino-purine)	$C_{10}H_9N_5O$	Plant hormone	Fluka, Buchs, Switzerland	0.010 mmol/L
Lactose	$C_{12}H_{22}O_{11} * H_2O$	Trituration medium	Weleda, Arlesheim, Switzerland	309 mmol/L
Lemna minor	—	Homeopathic remedy	VSM, Alkmaar, The Netherlands	6x dilution
Methyl jasmonate	$C_{13}H_{20}O_3$	Plant hormone	Serva, Heidelberg, Germany	0.050 mmol/L
Metoxuron	$C_{10}H_{13}ClN_2O_2$	Herbicide	Riedel-de Haën, Seelze, Germany	2.200 mmol/L
Phosphorus	P	Homeopathic remedy	Weleda, Arlesheim, Switzerland	4x dilution
Potassium nitrate	KNO_3	Nutrient	AppliChem, Darmstadt, Germany	1000 mmol/L
Sulfur	S	Homeopathic remedy	Weleda, Arlesheim, Switzerland	6x trituration

inverted (c0). From this vessel, 30 mL was added to another potentization vessel with 270 mL of distilled water and succussed as described before (c1).

In every experiment, the potency levels 14x to 30x of one test substance as well as unsuccussed (c0) and succussed (c1) water controls were examined. These controls were chosen according to the considerations of Baumgartner et al.[4]

After preparation of the test solutions, they were randomized and coded (blinded) with a letter code by a person who was not involved in the experiments. The code remained unbroken until statistical analysis was accomplished.

Lemna *bioassay*

Duckweed (*Lemna gibba* L.) was obtained from a laboratory culture of Aachen Technical University, Germany. Cultures were grown in modified Steinberg medium (moStM) according to a drafted standard of the International Organization for Standardization[20] (Table 2). Stock solutions nos. 1 to 3, 8, and 9 were individually prepared with autoclaved distilled water (Büchi, Fontavapor 250, Switzerland), while nos. 4–7 were pooled. Every week and prior to the experiments, the final medium (pH = 5.9 ± 0.1) was freshly prepared with autoclaved distilled water.

Duckweed cultures were grown under controlled laboratory conditions in a plant growth chamber (180 × 75 × 100 cm) illuminated with fluorescent lights (143 ± 3 μmol photons m^{-2} s^{-1} PAR, TL-D 36W/33-640, Philips, Zurich, Switzerland) and a light–dark period of 16:8 hours. During daytime, the mean maximum temperature was 21.5 ± 1°C and the mean maximum relative humidity (RH) was 45 ± 6%, and during nighttime these measures were 17 ± 1°C and 71 ± 6%, respectively.

Prior to the experiments, duckweeds from axenic (pure) stock cultures on 50 mL solid moStM (with 1% (w/v) Bacto® dextrose, (Difco, Detroit, MI) and 1% (w/v) bacteriologic agar No. 1, Oxoid, Basingstoke, GB added) were adapted to 150 mL of liquid autoclaved moStM in Erlenmeyer flasks for at least 4 weeks and in larger glass vessels containing 1.8 L of moStM for another 3 weeks in order to get large amounts of plants. The medium was changed weekly. It was assured that rapid growth near to exponential was maintained and was not restricted (e.g., due to space limitations or nutrient restrictions).

On the day of the experiment, test specimens with bright green color without visible lesions, chlorosis, or necrosis were selected from one vessel. They were sorted according to number of fronds (leaves) of similar size (e.g., three fronds per colony or three big and one small frond per colony, respectively) and were put into petri dishes with medium until used as inoculum for all test beakers.

The screening experiments as well as a water control run with unsuccussed water only were carried out by the corresponding author between January and July 2003. For every experiment in each of the 100 beakers (100 mL, SIMAX,® Kavalier, Sázava, Czech Republic), 3.8 mL of the combined stock solutions of moStM (50-fold concentrated) was pipetted. Then 46.2 mL of sample (potency or control) was added in a blocked randomization scheme. The latter had been successfully tested in pre-investigations with unsuccussed water only (i.e., it did not produce false-positive results). Five beakers were filled with uncoded unsuccussed water due to requirements of the image analysis software. These samples were not included in the statistical analysis. The remaining 95 beakers were filled with 19 coded samples (potencies or controls) in 5 replicates each (i.e., each screening experiment was conducted with 5 beakers of each of the 17 different potency levels of one test substance as well as 5 beakers of each unsuccussed [c0] and succussed [c1] water controls). Afterward, the sorted duckweed colonies were carefully put into the beakers at random, so that every beaker contained the same number of fronds of similar size (usually 10 fronds) at the beginning of the experiment. After dealing with the five replicates of one sample, the used materials (measuring cylinder, lemna transfer wire hook) were carefully rinsed three times with deionized and once with distilled water in order to minimize cross-contamination.

Frond area and frond number in every beaker were measured using an image processing system consisting of video

Table 2. Ingredients of the Modified Steinberg Medium (Onefold Concentrated)

Solution no.	*Substance*	*Concentration*	*Source*
1	KNO_3	3.46 mmol/L	Applichem, Darmstadt, Germany
	KH_2PO_4	0.66 mmol/L	Merck, Darmstadt, Germany
	K_2HPO_4	0.072 mmol/L	Merck, Darmstadt, Germany
2	$MgSO_4 * 7\ H_2O$	0.41 mmol/L	Merck, Darmstadt, Germany
3	$Ca(NO_3)_2 * 4\ H_2O$	1.25 mmol/L	Riedel-de Haën, Seelze, Germany
4	H_3BO_3	1.94 μmol/L	Fluka, Buchs, Switzerland
5	$ZnSO_4 * 7\ H_2O$	0.63 μmol/L	Riedel-de Haën, Seelze, Germany
6	$Na_2MoO_4 * 2\ H_2O$	0.18 μmol/L	Fluka, Buchs, Switzerland
7	$MnCl_2 * 4\ H_2O$	0.91 μmol/L	Fluka, Buchs, Switzerland
8	$FeCl_3 * 6\ H_2O$	2.81 μmol/L	Merck, Darmstadt, Germany
9	EDTA disodium-dihydrate	4.03 μmol/L	Merck, Darmstadt, Germany

EDTA, ethylenediamine tetraacetic acid.

camera, computer, and image analysis software (Scanalyzer, duckweed analytic software, version 3, LemnaTec, Aachen, Germany). For each recorded image, the quality of the automatic image analysis was checked and corrected by hand if necessary. After the initial measurement of frond area and frond number per beaker at the beginning of the experiment (day 0) each beaker was wrapped in black paper up to the surface of the test solution and put on black paper in the plant growth chamber in order to eliminate any diffused light from the side or the bottom. Additionally, it was covered with a watch glass to avoid excessive evaporation or accidental contamination. Further measurements were taken on days 3, 5, and 7 of the experiment (Fig. 1). From the measured frond area and frond number, the growth rate per day (r) was calculated for the total test period (day 0 –7, average specific growth rate), and for two additional time intervals (day 0 –3 and day 3 –7) according to the equation:

$$r = (\ln x_{t2} - \ln x_{t1}) / (t_2 - t_1) \quad (1)$$

where x_{t1} is the value of observation parameter at day t_1, x_{t2} is the value of observation parameter at day t_2 and $t_2 - t_1$ is the time period between x_{t1} and x_{t2} in days. The main parameter used for statistical analysis was frond area–related growth rate ($r_{(area)}$) since for the water control run the coefficient of variation of the test-system for $r_{(area)}$ was lower compared to the one based on the frond number–related growth rate ($r_{(num)}$).

After every experiment, all beakers were cleaned with hot tap water using a brush, washed in a dishwasher (Renggli, Rotkreuz, Switzerland), and additionally rinsed thoroughly two times with hot deionized water, two times with cold deionized water, once with cold distilled water, and then dried at 90°C.

Statistical analysis

From all experiments, a total of 1235 beaker images were obtained. The data from 8 beakers had to be excluded due to spilling.

Growth rate data ($r_{(area)}$ and $r_{(num)}$) were evaluated for statistical significance based on analysis of variance (ANOVA) F-tests with $\alpha = 5\%$.

For the water control run and the screening experiments, the independent variable was treatment ("pseudo-treatment," or potentized substances and controls, respectively). In addition, data of the unsuccussed and succussed water controls (c0 and c1) of all 12 screening experiments were analyzed regarding a possible succussion effect using a two-way analysis of variance with the independent variables experiment number and succussion (yes/no). This was not done for the water control run since the systematic negative control experiment only included unsuccussed water. All analyses were made using the software STATISTICA Version 6 (Stat Soft, Inc., Tulsa, OK).

RESULTS

Variability of the bioassay

Comparison of the two growth parameters, frond area–related and frond number–related average specific growth rate, measured for the water control run (systematic negative control) yielded small coefficients of variation between the groups of five pseudo-replicates (0.87% for $r_{(area)}$ and 1.60% for $r_{(num)}$ for the main time interval day 0 –7).

Succussion effect

In order to detect any effects of the succussion procedure itself, unsuccussed (c0) and succussed (c1) water controls from all 12 experiments that included potentized substances (no water control run) were compared. In a two-way ANOVA of growth rate raw data, no significant succussion effect (day 0 –7: $p = 0.839$, day 0 –3: $p = 0.881$, day 3 –7: $p = 0.598$) and no significant interaction with experiment number for day 0 –7 and day 3 –7 ($p = 0.104$, $p = 0.4548$, respectively) were observed. However, for day 3 –7 there was a weak significant interaction with the experiment num-

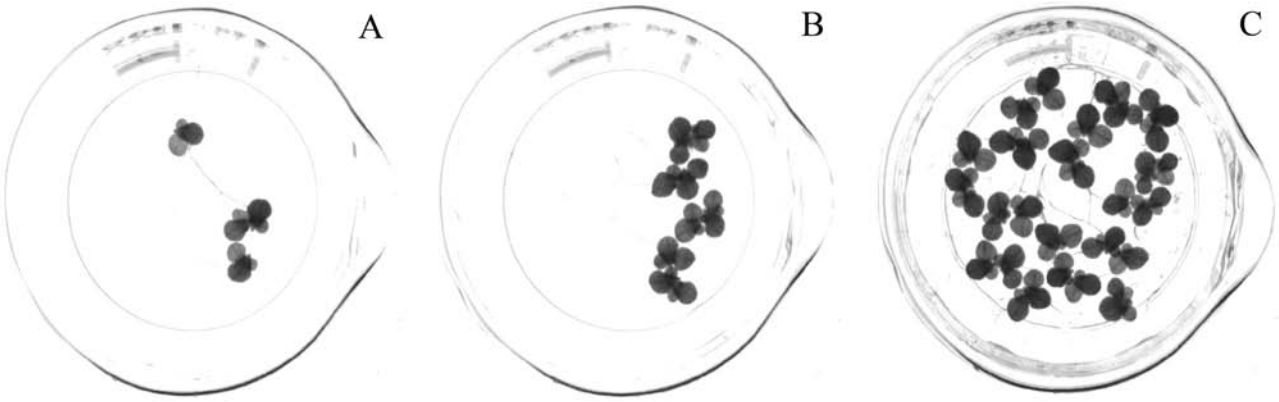

FIG. 1. Growth of *Lemna gibba* in one representative beaker of the water control run at day 0 **(A)**, day 3 **(B)**, and day 7 **(C)**.

ber ($p = 0.046$). Hence, a succussion effect could be excluded for the main time interval (day 0 –7), but not for day 3 –7. Therefore, succussed water (c1) was regarded as the adequate control.[4]

Screening experiments

Homeopathic decimal potencies (14x–30x) of 12 different substances were investigated for their effect on frond area–related growth rate of *L. gibba*. The main parameter, average specific growth rate, was assessed over 7 days, relying on the data measured on day 0 and 7. Additionally, segmented growth rates for shorter time intervals (day 0 –3 and day 3 –7) were calculated because some test substances may primarily affect the metabolism of old fronds rather than the development of new fronds.

It was observed that *L. gibba* performed differently in the presence of potentized substances (Table 3). Three test substances—argentum nitricum, kinetin, and phosphorus—significantly affected the main parameter average specific growth rate (ANOVA F-test). Compared to the succussed water control (c1), potencies of argentum nitricum and kinetin decreased $r_{(area)}$, whereas potencies of phosphorus increased $r_{(area)}$. Statistically significant effects (least significant difference test) were observed for the following potency levels: argentum nitricum 24x (decrease of −6% compared to c1, $p = 0.001$), 28x (−6%, $p < 0.001$), 29x (−7%, $p < 0.001$); kinetin 14x (−3%, $p = 0.027$), 16x (−4%, $p = 0.012$), 20x (−6%, $p < 0.001$), 23x (−3%, $p = 0.032$), 26x (−5%, $p = 0.002$), 27x (−5%, $p = 0.002$), 30x (−3%, $p = 0.023$); phosphorus 21x (+6%, $p = 0.021$), 25x (+7%, $p = 0.005$), 29x (+5%, $p = 0.046$). Segmented growth rates for the shorter time intervals were significantly affected by argentum nitricum, gibberellic acid, lactose, and phosphorus. Whereas the former three substances affected $r_{(area)}$ during day 3 –7, the latter affected $r_{(area)}$ during day 0 –3. All other substances tested showed no significant effect on $r_{(area)}$.

DISCUSSION

Duckweeds are used as test organisms in ecotoxicologic and environmental studies since they represent small aqueous macrophytes that are highly sensitive to low concentrations of various substances (e.g., herbicides, pharmaceuticals, heavy metals).[21–25] Here it is shown that duckweeds also seem to be able to react to homeopathic potencies.

During the preparation process of homeopathic potencies, physicochemical alterations of the potentization medium (water) occur, such as dissolution of glass ions, radical formation through cavitation, pH alterations, etc. These effects are not related to the substance potentized and are therefore unspecific. Any influences of such succussion effects on a test system can be determined by comparison of the unsuccussed (c0) and succussed (c1) water controls. In case of the Lemna bioassay investigated, unspecific succussion effects could be excluded for the average specific growth rate (day 0 –7) as well as for the segmented growth rate day 0 –3; however, for day 3 –7 it could not. Consequently, effects of different potency levels were compared with the succussed water control (c1), since these controls were prepared analogously to the potentized substances.

Of the 12 substances selected for the randomized and coded (blinded) screening experiments argentum nitricum, phosphorus, and kinetin significantly affected the average specific growth rate. This does not necessarily exclude the possibility that substances that show no effect in the screening might have an effect in future experiments with higher statistical power; however, one might get a useful hint about which substances will be appropriate to investigate in more

Table 3. Statistical Analysis of the Water Control Run and Screening Experiments with *Lemna gibba* for All Potentized Substances Tested and Three Time Intervals

Substances	$r_{(area)}$ *Day 0 –7*	$r_{(area)}$ *Day 0 –3*	$r_{(area)}$ *Day 3 –7*
Water control run	0.933	0.983	0.495
Argentum nitricum	**0.003**	0.292	**0.003**
$CuSO_4$	0.119	0.211	0.566
Gibberellic acid	0.336	0.203	**0.047**
3-Indole acetic acid	0.460	0.058	0.239
Kinetin	**0.036**	0.197	0.056
Lactose	0.097	0.124	**0.011**
Lemna minor	0.477	0.451	0.065
Methyl jasmonate	0.273	0.272	0.188
Metoxuron	0.561	0.132	0.629
Phosphorus	**0.027**	**0.019**	0.099
Potassium nitrate	0.561	0.132	0.629
Sulfur	0.754	0.983	0.733

Includes p values of F-tests of the corresponding analyses of variance; significant values ($p < 0.05$) are shown in bold.

detail in several replication series with the test organism chosen. Besides the two mineral substances, argentum nitricum and phosphorus, which are well-known homeopathic remedies, also endogenous substances, plant hormones such as kinetin or gibberellic acid, might affect duckweed growth when being potentized. Reactions to potentized hormones have previously been observed in other test systems with animals and plants.[6,7,13,26]

Although *L. gibba* is not able to use lactose as energy supplier under nonsaturating light conditions[27] and though lactose is used as a (supposedly inert) carrier for homeopathic remedies, it is somewhat astonishing that lactose potencies did show an effect on the segmented growth rate $r_{(area)}$ on day 3 –7. However, an absolutely inert carrier substance would be hard to find. In *Materia Medica*, lactose is a proven remedy (*Saccharum lactis*).[28]

It is clear that the results of the present investigation cannot be directly transferred to humans or animals. Nevertheless, plant models can be used to answer the basic question of the specificity of homeopathic remedies. Facing the well-known problem of reproducibility in homeopathic basic research[29–33] it is obvious, however, that larger series of independent internal and external replications are needed before any definite statement can be made.

To facilitate reproduction experiments, we want to highlight some critical points of the setup chosen. For the *Lemna* bioassay, strictly controlled laboratory conditions are needed, especially regarding even distribution of light, temperature, and humidity. Careful handling of plants is required, avoiding any injury. *L. gibba* needs sufficient time (several weeks) to acclimatize from plant stock cultures to the experimental conditions. Systematic negative controls are recommended to determine test-system stability.

CONCLUSIONS

We conclude that the experimental setup described with *L. gibba* as test organism might be a promising new model system to demonstrate effects of potentized substances and that it is worthwhile to further investigate selected test substances in larger sets of replication experiments. Several series of internal and external replications are necessary to obtain reliable information about effects of homeopathic potencies on duckweed growth. Furthermore, systematic negative control experiments should be performed to document the stability of the experimental setup.

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