Forschende Komplementärmedizin
Wissenschaft • Praxis • Perspektiven

Original Article · Originalarbeit

Forsch Komplementärmed 2006;13:298–306
DOI: 10.1159/000095302

Published online: September 29, 2006

Effects of Potentised Substances on Growth Kinetics of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*

Claudia Scherr[a,c] Stephan Baumgartner[a,b] Jörg Spranger[c,e] Meinhard Simon[d]

[a] Verein für Krebsforschung, Institut Hiscia, Arlesheim, Schweiz
[b] Kollegiale Instanz für Komplementärmedizin (KIKOM), Universität Bern, Insel-Spital, Imhoof-Pavillon, Bern, Schweiz
[c] Forschungsinstitut für biologischen Landbau, Frick, Schweiz
[d] Institut für Chemie und Biologie des Meeres (ICBM), Universität Oldenburg, Deutschland
[e] Institut für anthroposophische Veterinärmedizin, Frick, Schweiz

Key Words
Homeopathic potencies · Yeast · *Saccharomyces cerevisiae* · *Schizosaccharomyces pombe* · Test system stability

Summary
Background: Homeopathic potencies are used as specific remedies in complementary medicine. Since the mode of action is unknown, the presumed specificity is discussed controversially. **Objective:** This study investigated the effects of potentised substances on two yeast species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, in a stable and reliable test system with systematic negative controls. **Materials and Methods:** Yeast cells were cultivated in either potentised substances or water controls in microplates and their growth kinetics were measured photometrically. Water control runs were performed repeatedly to investigate the stability of the experimental set-up (systematic negative controls). **Results:** 4 out of 14 screened substances seem to have affected the growth curve parameters slope or yield. Out of these substances, azoxystrobin and phosphorus were chosen for 8 further replication experiments, which partly confirmed the results of the screening. On the average of all experiments, azoxystrobin affected the slope of the growth curve of *Saccharomyces cerevisiae* ($p < 0.05$), and phosphorus affected the slope of the growth curve of *Schizosaccharomyces pombe* ($p < 0.05$). No effects were seen in the water control runs. In addition, significant interactions between treatment with potentised substances and experiment number were observed in all experiments with potentised substances ($p < 0.01$), but not in the water control runs. **Conclusions:** Both yeast species reacted to certain potentised substances by changing their growth kinetics. However, the interactions found point to additional factors of still unknown nature, that modulate the effects of potentised substances. This stable test system with yeasts may be suitable for further studies regarding the efficacy of homeopathic potencies.

Schlüsselwörter
Homöopathische Potenzen · Hefen · *Saccharomyces cerevisiae* · *Schizosaccharomyces pombe* · Testsystem, Stabilität

Zusammenfassung
Hintergrund: Homöopathische Potenzen werden als spezifische Heilmittel in der Komplementärmedizin eingesetzt. Da der Wirkmechanismus unbekannt ist, wird eine spezifische Wirksamkeit jedoch kontrovers diskutiert. **Fragestellung:** Diese Studie untersuchte die Wirkung von potenzierten Substanzen auf zwei Hefearten, *Saccharomyces cerevisiae* und *Schizosaccharomyces pombe*, in einem stabilen und zuverlässigen Testsystem mit systematischen Negativkontrollen. **Material und Methoden:** Hefezellen wurden in Anwesenheit von potenzierten Substanzen und Wasserkontrollen in Mikrotiterplatten kultiviert und ihre Wachstumskinetik wurde photometrisch gemessen. Wasser-Kontrollläufe wurden wiederholt durchgeführt, um die Stabilität des experimentellen Aufbaus zu untersuchen (systematische Negativkontrollen). **Ergebnisse:** In einem Screening scheinen 4 von 14 Substanzen die Wachstumskurven-Parameter Steigung und Ertrag beeinflusst zu haben. Von diesen Substanzen wurden Azoxystrobin und Phosphor für 8 weitere Wiederholungsexperimente ausgewählt, die die Resultate aus dem Screening teilweise bestätigten. Im Durchschnitt aller Experimente beeinflusste Azoxystrobin die Steigung der Wachstumskurve von *Saccharomyces cerevisiae* ($p < 0{,}05$), und Phosphor beeinflusste die Steigung der Wachstumskurve von *Schizosaccharomyces pombe* ($p < 0{,}05$). Keine Effekte wurden in den Wasser-Kontrollläufen beobachtet. Zusätzlich wurden in allen Experimenten mit potenzierten Substanzen signifikante Wechselwirkungen zwischen der Behandlung mit potenzierten Substanzen und der Experimentnummer beobachtet, nicht jedoch in den Wasser-Kontrollläufen. **Schlussfolgerungen:** Beide Hefearten reagierten auf bestimmte potenzierte Substanzen mit Änderung ihrer Wachstumskinetik. Die gefundenen Wechselwirkungen deuten jedoch auf zusätzliche, noch unbekannte Faktoren hin, welche die Effekte von potenzierten Substanzen modulieren. Das vorgestellte stabile Hefe-Testsystem könnte für weitere Studien zur Wirksamkeit homöopathischer Potenzen geeignet sein.

KARGER
Fax +49 761 4 52 07 14
E-mail Information@Karger.de
www.karger.com

Accessible online at:
www.karger.com/fok

Claudia Scherr
Verein für Krebsforschung, Institut Hiscia
Kirschweg 9, CH-4144 Arlesheim, Schweiz
Tel. +41 61 706-7225, Fax -7200
E-mail scherr@vfk.ch

Introduction

In both human and veterinary care homeopathic dilutions (potencies) are widely used in complementary medical therapies, e.g. homeopathy and anthroposophical medicine. These remedies consist of highly diluted substances, such as plant or animal extracts or minerals, which are stepwise diluted and vigorously succussed. This procedure is called (homeopathic) potentisation. Above a certain dilution step, there are virtually no molecules of the original substance left (potency levels higher than 24x respectively 12c). Due to this fact the possibility of a remedy-specific therapeutic effect is often questioned [1–3], even though there are some meta-analyses of clinical trials which indicate corresponding effects [4, 5]. Moreover, there is currently no generally accepted theory explaining a mode of action. Some hypotheses have been set up, but they still await experimental verification [6–9].

Besides well performed controlled clinical trials there is a great need for preclinical research with high methodological standards [10]. Simple physical, chemical and biological laboratory models are required to investigate effects of high dilutions as well as quality aspects of the remedies, and finally, to get an idea of their mode of action. Additionally, it is important that the results gained with different models are replicated by independent researchers [11–13]. In vivo as well as in vitro, several promising preclinical test systems with higher organisms, like plants, animals or human cell cultures are currently being investigated [14–25].

Microorganisms have also been used as test organisms for potentised substances. They have many advantages, like fast growth and easy handling and one would expect good reproducibility [26–32]. However, the methodology of some of the studies was rarely described sufficiently for exact reproduction. In studies with the same test organism and similar test substances, the results are often divergent, like the studies with Chlorella [33, 34]. Also for yeasts some authors reported effects of potentised substances on selected metabolic or growth parameters [35–39], while others were not able to detect any [40–43]. Given the principal advantages of yeasts as test organisms, it seemed important to us to thoroughly investigate the sensitivity of yeast to highly diluted and succussed substances. Both species examined here are well studied and often used in industrial and medical biotechnology as well as biological models and sensitive test organisms in environmental studies [44–46].

The aim of this study was thus to investigate if potentised substances show any effects on growth kinetics of two different yeast species in a stable, reliable test system with systematic negative controls. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were grown in the presence of the succussed test substances or corresponding water controls in microplates under strictly controlled environmental conditions. Among these test substances there were unspecifically and specifically toxic substances, a growth regulator and different homeopathic remedies with a relation to human growth processes. Growth kinetics were measured photometrically for 40 hours and statistically analysed using a standard parametrisation of the growth curve.

Materials and Methods

Yeast Media

Universal medium for yeast 186 (YM) (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was used to grow yeasts on petri dishes and contained: 3 g l^{-1} yeast extract (Oxoid, Basingstoke, Great Britain), 5 g l^{-1} proteose peptone (Oxoid), 3 g l^{-1} malt extract (Oxoid), 10 g l^{-1} Bacto® dextrose (Difco, Detroit, USA), 15 g l^{-1} agar bacteriological No. 1 (Oxoid). The pH was adjusted with HCl (Fluka, Buchs, Switzerland) to 5.9 ± 0.1.

Liquid yeast cultures were grown in Yeast N base medium as defined nutrient source, pH 5.4 ± 0.1, which contained: 6.7 g l^{-1} Difco™ yeast nitrogen base (Becton, Dickinson and Company, Sparks, USA) and 5 g l^{-1} Bacto® dextrose (Difco).

The growth kinetic measurements were performed on Yeast N base medium agar containing 6 g l^{-1} agar bacteriological No. 1 (Oxoid) in addition.

Growth Conditions for Yeast Starter Cultures

Two different yeast species, *Saccharomyces cerevisiae* X2180 (MATa/α *SUC2/SUC2 mal/mal mel/mel gal2/gal2 CUP1*/CUP1), and *Schizosaccharomyces pombe* (972 h^{-}), were used in the experiments. For long time storage the cultures were harvested and resuspended in 15% (w/v) glycerol (Appli Chem, Darmstadt, Germany). Then the cultures were dispensed into 1 ml aliquots and frozen at –80 °C. Yeasts from these storage cultures were taken approximately every 4 months, were grown on YM 186 for 2 days at 30 °C and were stored at 4 °C. Every 2 weeks they were transferred to new YM 186. Before an experiment was carried out one colony was added to 20 ml liquid Yeast N base medium and was grown at 30 °C for 24 h. Turbidity was measured in order to control that the log phase was reached (Turbidity Meter, ESD Engineered Systems and Designs, Newark, USA). As additional criterion for the metabolic state of the culture it was checked, whether there was still enough dextrose in the medium left (Keto-Diastix, Bayer AG, Zurich, Switzerland; without dextrose the yeast cells change their metabolism from fermentation to respiration). Then the test organisms were used as inoculum in the growth kinetic measurements.

Stability of the Test System

In order to test the stability of the test system, to determine the coefficient of variance and to test for false positive results 9 full experiments were carried out with (unsuccussed) water as the only test substance for both yeast species (systematic negative controls). In these water control runs the same number of samples, an analogous randomisation scheme for the statistical analysis and the same technical procedure were used as in the experiments with potentised substances. The water used was taken from the same glass bottle that served as reservoir for the potentisation in the experiments with test substances.

Design of the Experimental Investigations

All experiments were carried out by the corresponding author between May 2002 and March 2004. There were two different phases of the experimental investigations. During the first phase 14 different test substances were screened for their potential to influence growth kinetics of yeasts. In the second phase of the experimental investigations replication of the effects found in the screening was examined.

Screening: Among the substances chosen were some exerting a known effect on yeast growth in substantial concentrations and different modes of

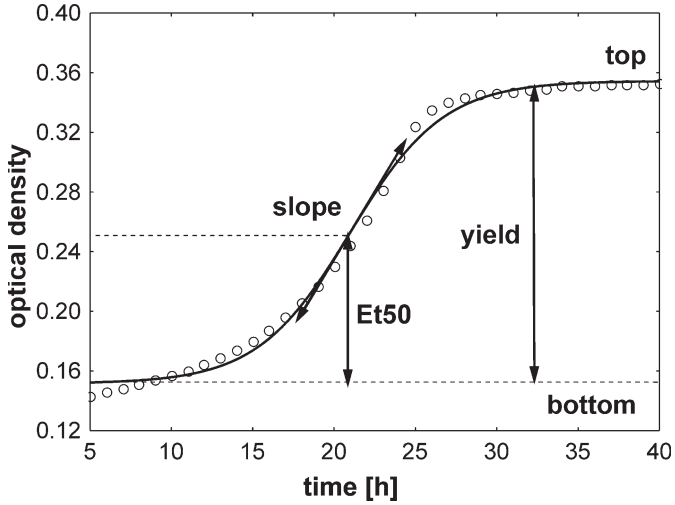

Fig. 1. Example of a growth curve and its parametrisation. A four parameter regression (top, bottom, slope, Et50; straight line) was individually fitted to the measured yeast growth data of each well of the microplate (empty circles).

action, like unspecifically toxic substances (copper(II)sulfate-5-hydrate [$CuSO_4$ * 5 H_2O] (E.Merck, Darmstadt, Germany) and copper(II)chloride-2-hydrate [$CuCl_2$ * 2 H_2O] (Riedel-de Haën, Seelze, Germany), fungicides (azoxystrobin PESTANAL® [methyl(E)-[2-(6-(2-cyanophenoxy) pyrimidine-4-yloxy) phenyl]-3-methoxyacrylate] [$C_{22}H_{17}N_3O_5$], benomyl PESTANAL® [methyl 1-(butylcarbamoyl)benzimidazole-2-yl carbamate] [$C_{14}H_{18}N_4O_3$], folpet PESTANAL® [n-(trichloromethylthio)phthalimide] [$C_9H_4Cl_3NO_2S$], triadimefon PESTANAL® [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazole-1-yl)butanone] [$C_{14}H_{16}ClN_3O_2$] (all Riedel-de Haën) and a growth regulator (3-indolylacetic acid PESTANAL® [$C_{10}H_9NO_2$]; Riedel-de Haën). Also lactose [$C_{12}H_{22}O_{11}$], (medium of some of the remedies bought) and homeopathic remedies (arsenicum album 6x Trituratio [arsenic trioxide] [As_2O_3], apatit 6x Trit. [natural calciumfluorophosphate] [$Ca_5F(PO_4)_3$], phosphorus 4x Dilutio [white modification of phosphorus] [P], quartz 6x Trit. [natural silicon dioxide] [SiO_2], sulfur 6x Trit. [natural sulphur] [S] and witherit 6x Trit. [natural barium carbonate] [$BaCO_3$] (all Weleda, Arlesheim, Switzerland) were included in the screening. The list of these homeopathic remedies (with a relation to human growth processes) was chosen in cooperation with physicians from KIKOM (Institute of Complementary Medicine, University of Berne, Switzerland) and veterinarians from FiBL (Research Institute of Organic Agriculture, Frick, Switzerland). All water-soluble substances were potentised according to homeopathic standard procedures, i.e. diluted and succussed, in sterile distilled water (Büchi, Fontavapor 250, Flavil, Switzerland) from 1x to 30x (details see below), fungicides as acetone-soluble substances were potentised in acetone (AppliChem Darmstadt, Germany) to 1x, then further in a acetone-water mixture with 80, 60, 40, 20% acetone, respectively, until 5x and from 6x on in pure distilled water. The homeopathic remedies were obtained in the lowest potency available, as 4x or 6x, and further potentised in pure distilled water.

As controls untreated pure solvent (sterile distilled water) as well as sterile distilled water being succussed once were used in every experiment with potentised substances, based on the considerations of Baumgartner et al. [10].

Replication series: Two test substances, azoxystrobin and phosphorus, exhibited the most pronounced effects on yeast growth kinetics in the screening. Therefore these two substances were chosen in order to repeat the experiments 8 times with both yeast species during one and a half years. The results were compared with those of the water control runs (see above), performed during the same period of time (systematic negative controls).

Preparation of the Test Solutions

For every experiment the potencies of the test substances and the controls were freshly prepared according to the German Homeopathic Pharmacopoeia [47] under sterile conditions under a laminar flow (Skan AG, Basel, Switzerland) before 11 a.m. on the day of the experiment. The stock solutions were prepared in either distilled water or in aceton (see above). Copper(II)sulphate-5-hydrate and copper(II)chloride-2-hydrate were dissolved in distilled water in concentrations of 0.4 M. In order to dissolve 1 mg lactose or trituratio in 9 ml distilled water, these solutions were gently heated to approximately 38 °C. Substances which are usually not used as homeopathic dilutions were prepared in concentrations according to their application in agriculture (azoxystrobin 0.62 mM, benomyl 1.72 mM, folpet 2.7 mM, triadimefon 0.85 mM, 3-indolylacetic acid 0.057 mM) [48]. 8 ml of the stock solution were carefully sterile filtered (0.2 µm, Macherey-Nagel, Düren, Germany) and filled in the first 14 ml polypropylene tube (always brand-new and sterile from Greiner Bio-One, Frickenhausen, Germany). After vigorously shaking by hand in a vertical line for 1 min at a rate of approximately 2 Hz at room temperature, 0.8 ml of this solution were added to the second test tube containing 7.2 ml sterile distilled water and shaken in the same manner. This process of successive 10-fold dilution steps and vigorously shaking proceeded until the 30th dilution step was reached. All prepared test solutions were stored at room temperature in the dark until they were used. For the water controls, the first tube containing 8 ml of sterile distilled water remained unsuccussed and was only gently inverted (C0). From this tube 0.8 ml were added to another tube with 7.2 ml sterile distilled water and succussed like described before (C1). In every experiment the potency levels 9x, 12x, 15x, 18x, 21x, 24x, 27x and 30x of one test substance as well as unsuccussed (C0) and succussed (C1) water controls were examined. After preparation of the test solutions, they were coded (blinded) and randomised by another person not being involved in the experiments. The code remained unbroken until statistical analysis was accomplished.

Microplate Bioassay

100 µl of sample was added to the wells of a new, sterile 96-well microplate (flat bottom with lid, Sarstedt, Newton, NC, USA) in a block randomisation scheme for all experiments. Both columns at the edge were filled with water, but were not included in the analysis because of measured edge effects in pre-investigations with pure water as test substance. The remaining 80 wells were filled with 10 coded samples, either potentised substances or controls, in 8 replicates each, in two blocks of four adjacent wells. Samples were pipetted with a multiple channel pipette (Socorex, Ecublens/Lausanne, Switzerland) as two times 4 × 100 µl. This was followed by 150 µl of Yeast N base medium agar containing about 60,000 cells ml^{-1} yeast cells (PP-reagent reservoir, Socorex; Exp8 channel impact pipettor, Matrix Technologies, Hudson, NH, USA) resulting in a final concentration of about 36,000 cells ml^{-1} in each well.

For growth kinetic measurements the microplate was put in a temperature controlled microplate reader (MRX II TC, Dynex Technologies, Chantilly, VA, USA) at 30 °C, which in turn was situated in a plant growth chamber (Heraeus, Geneva, Switzerland) with 25 °C and relative humidity (rH) of 50–60% in order to improve temperature stability (according to pre-investigations). The optical density (I_0 / I; intensity of incident light / intensity of transmitted light) was measured at a wavelength of 600 nm every hour for a period of 40 h. After the experiment the microplate was inspected for any visible changes of the medium. For data reduction a regression with four free parameters was fitted to the yeast growth data (5^{th} hour until 40^{th} hour) individually for each well of the microplate (fig. 1):

$$\text{optical density (OD)} = \text{bottom} + ((\text{top} - \text{bottom}) / (1 + 10 \exp((\text{Et50-time}) * \text{slope}))) \quad (1)$$

Table 1. Statistical analysis of the screening experiments with two yeast species for all potentised substances tested and three growth curve parameters (p-values of F-tests of the corresponding ANOVA)

	Saccharomyces cerevisiae			*Schizosaccharomyces pombe*		
	Et50	slope	yield	Et50	slope	yield
Apatit	0.1198	0.2911	0.6281	0.9530	0.5154	0.2541
Arsenicum album	0.8650	0.2928	0.1480	0.4482	0.0948	0.4492
Azoxystrobin	0.1269	0.0095*	0.0017*	0.5970	0.1379	0.1135
Benomyl	0.3849	0.0193*	0.8336	0.0773	0.1541	0.1176
$CuSo_4$	0.0543	0.4188	0.7454	0.6289	0.6627	0.6167
$CuCl_2$	–	–	–	0.5576	0.4819	0.8629
Folpet	0.7962	0.1404	0.3178	0.8699	0.3867	0.2586
3-Indolylacetic acid	0.2852	0.5104	0.7529	0.9031	0.3141	0.6235
Lactose	0.1420	0.4869	0.2296	0.1652	0.0881	0.4235
Phosphorus	0.9063	0.6174	0.3682	0.8770	0.0026*	0.1985
Quartz	0.2504	0.8238	0.9283	0.7904	0.7263	0.1135
Sulfur	0.6566	0.1430	0.0758	0.9743	0.4403	0.5685
Triadimefon	0.1746	0.9089	0.4896	0.1735	0.2007	0.6942
Witherit	0.4556	0.0344*	0.0984	0.3434	0.3378	0.3076

*$p < 0.05$.

Three parameters were finally used for statistical analysis: slope [h^{-1}], yield (top – bottom) and Et50 (point in time when the yield was half maximum) [h].

From all experiments a total of 6,160 growth curves was obtained. In 4% of all cases air bubbles or cracks in the agar medium falsified the measurements, therefore these data had to be excluded. 6% of the data were eliminated due to other reasons (e.g., regression fit < 99%, negative slope or standard error not calculated in the regression). Finally, 90% of all data were integrated in the statistical analysis.

The materials used repeatedly, such as glass vessels to prepare the stock solutions or reagent reservoirs, were cleaned with tap water, thoroughly rinsed twice in hot demineralised water, twice in cold demineralised water and once in cold distilled water and then dried at 80 °C. If necessary, they were autoclaved at 121 °C for 15 min (GE 406; GETINGE AB, Getinge, Sweden).

Statistical Analysis

In general, statistical analysis was based on analysis of variance (ANOVA) F tests, after checking the data for normal distribution with Shapiro-Wilk W Test.

For the screening experiments the independent variable was treatment (potentised substances or controls) and the dependent variables were slope, Et50 and yield, respectively ($\alpha = 5\%$). To detect any effects of the succussion procedure itself, the differences between the unsuccussed (C0) and succussed (C1) water controls in all experiments with potentised substances were determined using a 2-way ANOVA with the independent variables experiment number and succussion (yes/no) and the dependent variables slope, Et50 and yield, respectively ($\alpha = 5\%$).

For the replication experiments and the water control runs the independent variables were experiment number and treatment (either 8 potencies and 1 control C, or 9 'pseudo-treatments' with water only) and the dependent variables were slope, Et50 and yield, respectively ($\alpha = 5\%$). In order to compare the 9 experiments of one test substance, the data of each experiment had been normalised to its mean value. Besides the main effect of treatment the statistical model included the interaction of treatment with experiment number. Any such significant interaction would indicate a real treatment effect, but being modulated by external factors varying with time.

Only if the global F test for treatment was significant ($p < 0.05$) planned comparisons were evaluated with the LSD test. This protected Fisher's LSD gives a good safeguard against type I errors as well as type II errors [49]. All analyses were made using the software STATISTICA version 5, (Stat Soft, Inc., Tulsa, OK, USA).

Results

Screening Experiments

In the screening 14 different potentised substances were investigated for their effect on growth of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* compared to the corresponding water controls (table 1). Growth kinetics were assessed over 40 hours by hourly measurements of the optical density and were described with three parameters: Et50, slope and yield. Table 1 shows that the two yeast species performed differently in the presence of the potentised substances tested. For *Saccharomyces cerevisiae*, benomyl and witherit affected slope while azoxystrobin affected slope and yield of the growth curves. In experiments with *Schizosaccharomyces pombe*, only slope of the growth curves was influenced by phosphorus. All other substances tested showed no significant effect on the growth kinetics of the yeasts.

Succussion Effect

In order to detect any effects of the succussion procedure itself, unsuccussed (C0) and succussed (C1) water controls from all experiments with potentised test substances (no water control runs) were compared. In a 2-way ANOVA of growth curve parameters (raw data) both water controls differ significantly for the experiment number ($p = 0.0000$ for Et50, slope, yield and both yeast species, respectively), but no significant succussion effect (*Saccharomyces cerevisiae*: Et50 $p = 0.2239$, slope $p = 0.6196$, yield $p = 0.7977$; *Schizosaccharomyces pombe*: Et50 $p = 0.7886$, slope $p = 0.6289$, yield $p = 0.2372$) and no significant interaction (*Saccharomyces cerevisiae*: Et50 $p = 0.3157$, slope $p = 0.6639$, yield $p = 0.7219$; *Schizosaccha-*

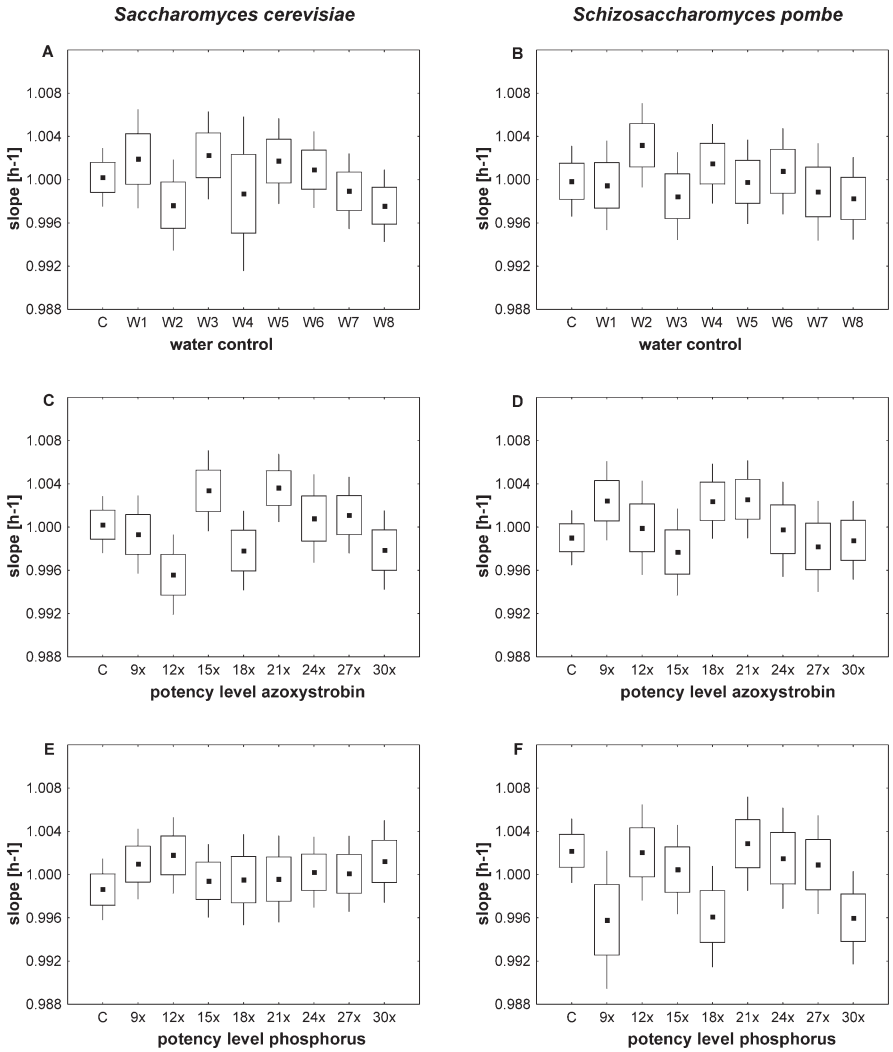

Fig. 2. Slope of the normalised growth curves of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* for eight potency levels of azoxystrobin (C, D) and phosphorus (E, F) in comparison to their corresponding water control C. Figure 2 A and B show corresponding graphs for the pure water control runs (systematic negative controls) with samples of identical origin (unsuccussed water = dilution medium used). Mean values (dots) (± 1.00 (boxes) and ± 1.96 standard error (whiskers), respectively) for nine independent experiments each.

romyces pombe: Et50 p = 0.9962, slope p = 0.7472, yield p = 0.0690) were observed. Hence a succussion effect could be excluded. It had been defined a priori, that if no significant differences between C0 and C1 were found, the controls of one experiment were pooled to one control value C.

Stability of the Test System

Nine experiments with (unsuccussed) water as the only test substance were carried out to assess the stability of the experimental set-up during the course of the investigations. These systematic negative controls showed a good stability of the test system with small coefficients of variation for both yeast species depending on the growth curve parameter (Et50 0.006, slope 0.018, yield 0.050 for *Saccharomyces cerevisiae* and Et50 0.011, slope 0.017, yield 0.066 for *Schizosaccharomyces pombe*). In a 2-way ANOVA neither treatment (here 9 'pseudo-treatments', distilled water only) nor the interaction of treatment with experiment number yielded significant effects (table 2a, fig. 2A, B). Thus, this showed that the test system was stable and did not generate false positive results.

Replication Experiments

According to the results of the screening we selected azoxystrobin and phosphorus for independent replication experiments in order to test if the screening results could be confirmed and to determine if or if not the corresponding null hypothesis ('no differences in the effects of treatment with potentised substances and water controls on yeast growth') had to be dismissed.

For the azoxystrobin experiments, the results of the 2-way ANOVA of the growth curve parameters are listed in table 2b; for slope they are graphically shown in figure 2C, D. The analysis yielded for *Saccharomyces cerevisiae* a significant effect of the treatment with potencies of azoxystrobin on slope. In the pooled data set of all nine experiments the LSD test for the main effect treatment on slope yielded no significant difference for any potency level when compared to the corresponding water control C. However, the LSD test yielded significant ($p < 0.05$) differences for the potency levels 15x and 21x when compared to the potency levels 12x, 18x and 30x. In addition, a significant interaction was found for the growth

Table 2. Results of ANOVA F-tests for the normalised data of the growth curve parameters Et50, slope and yield for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Effect of experiment number, treatment and their interaction for 9 independent experiments each: water control runs (2a), azoxystrobin (2b) and phosphorus (2c)

	Saccharomyces cerevisiae			*Schizosaccharomyces pombe*		
Main effects	Et50	slope	yield	Et50	slope	yield
2a. Water control runs						
Exp. no.	1.0000	0.9998	0.9998	0.9999	1.0000	0.9998
Treatment	0.4813	0.7682	0.2618	0.9727	0.8268	0.9980
Interaction	0.5399	0.8719	0.1243	0.9994	0.2412	0.9176
2b. Azoxystrobin experiments						
Exp. no.	1.0000	0.9988	0.9994	1.0000	0,9988	1.0000
Treatment	0.8134	0.0472*	0.6308	0.8995	0.3934	0.8964
Interaction	0.0773	0.0006*	0.0024*	0.9966	0.0001*	0.7805
2c. Phosphorus experiments						
Exp. no.	1.0000	0.9980	0.9998	1.0000	1.0000	0.9970
Treatment	0.6370	0.9428	0.1782	0.9458	0.0436*	0.3187
Interaction	0.9905	0.0065*	0.0787	0.9997	0.0167*	0.0690

*$p < 0.05$.

parameters slope and yield. For *Schizosaccharomyces pombe*, for slope also a highly significant interaction of the experiment number and the treatment was detected.

In the experiments with potencies of phosphorus (table 2c, fig. 2E, F) the 2-way ANOVA of the growth curve parameters yielded a significant effect of the treatment on slope of the growth curves of *Schizosaccharomyces pombe*. In the following LSD test for the main effect treatment on the growth parameter slope the potency levels 9x, 18x and 30x differ significantly from the water control C (fig. 2F) when pooling all nine experiments. Again, significant interactions of experiment number and treatment were detected for the growth parameter slope of both *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (table 2c).

For the growth parameter Et50 no significant main effect of treatment and no significant interaction could be detected in the azoxystrobin experiments as well as in the phosphorus experiments for any yeast species (table 2b, c).

Discussion

Stability of the Test System

Profound knowledge of the test system chosen is a prerequisite for a correct interpretation of any results obtained. One has to know how the organisms grow under controlled conditions over time without any further influences and to what extent natural variations occur. In this study the experimental set-up to examine the growth kinetics of two different yeast species was assessed in many pre-investigations as well as in the nine water control runs during the time of investigations with potentised substances. The results of these systematic negative controls showed that with both yeast species the experimental system was stable and had small coefficients of variation for all growth curve parameters. Therefore it was possible to measure even small effects induced by the substances tested in the screening as well as in the replication experiments. Furthermore, in our experiments false positive results can be excluded with high certainty: neither treatment nor interaction between experiment number and treatment yielded significant effects for any growth curve parameter or any yeast species.

Succussion Effect

During preparation of high dilutions physico-chemical alterations occur by the succussion of water within the polypropylene tubes, e.g. dissolved and suspended gases (oxygen, carbon dioxide), dissolution of inorganic and organic substances (plasticiser) or other unspecific effects [50, 51]. Both yeast species do not seem to be influenced by these alterations since no significant effect of succussed water (C1) on any growth parameter was observed, compared to unsuccussed water (C0). Consequently, any detected effect of potentised substances compared to controls cannot be caused by these physico-chemical alterations since the succussed water controls were prepared analogously to the high dilutions. As it had been defined a priori, for further analysis the succussed and unsuccussed water controls were pooled to one single control (C) due to the lack of any difference.

Screening

In the screening it was observed that both yeast species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, seem to react to some of the potentised substances tested. Among the latter, there were substances which are commonly used as remedies for human beings and animals [47, 52], as well as succussed highly diluted growth regulators [53], toxic and non-toxic substances [54, 55]. It is essential to choose appropriate

test substances when assessing possible effects of highly diluted substances on any organism in a new experimental set-up since in our study only 4 out of 14 different substances tested seem to affect yeast growth in the screening. This does not exclude the possibility that a substance showing no effect in the screening might have an effect in further experiments, but with this procedure one can easily get a hint which test substance might be useful to test. Therefore, screening tests are recommended to select appropriate test substances prior to longer series of experiments which are necessary to yield reliable information about the effects of homeopathic potencies.

Replication Experiments

In the replication experiments the results of the screening were confirmed for phosphorus and partly confirmed for azoxystrobin. In this experimental set-up the slope of the growth curve was the only parameter to detect any reproducible changes in yeast growth kinetics induced by potentised substances. In the global F-test azoxystrobin affected the slope of the growth curves of *Saccharomyces cerevisiae* and phosphorus that of *Schizosaccharomyces pombe*, respectively. Comparison of the effects of the single potency levels of azoxystrobin on the growth of *Saccharomyces cerevisiae* yielded no significant differences to the water control (fig. 2C). However, the slope of the growth curve was significantly higher for the potency levels 15x or 21x when compared to 12x, 18x or 30x, respectively. Since the corresponding LSD test was not predefined the results have to be viewed with some caution. However, they do not contradict the hypothesis that some potency levels of azoxystrobin stimulate and others retard the growth of *Saccharomyces cerevisiae*.

For *Schizosaccahromyces pombe* the potency levels 9x, 18x and 30x of phosphorus decreased the slope of the growth curves significantly compared to the corresponding water control (fig. 2F). Seemingly no stimulating effect of phosphorus potencies could be observed.

Changes between effective and ineffective levels of homeopathic dilutions can hardly be traced back to substantial remnants of the test substances, since the Hahnemanns' method (different vessel for every dilution step) was used to prepare the test substances. Moreover, traces of phosphorus are ubiquitously present in the environment. Also such changes have repeatedly been reported from different test systems [15, 16, 18, 35, 38, 56].

However, the effects found are very small. This may probably be due to the use of 'healthy' test organisms which may react less to homeopathic preparations than 'unhealthy' ones. Larger effects have been described with the so called intoxication model, where the test organisms are first poisoned with high concentrations of the test substance and subsequently treated with homeopathic dilutions of the same substance [12, 57–59]. Thus, we propose to test the effects of homeopathic remedies on yeasts using an intoxication model.

Even more striking than the significant overall effect of the treatment are the highly significant interactions between treatment and experiment number. These interactions occurred with both yeast species in all replication experiments with potentised test substances, but not in the water control runs. In a study with the marine bacterium *Vibrio fischeri* using similar statistical methods the same kind of interactions between treatment with potentised substances and the experiment number was observed (Brack A, personal communication). These findings indicate that there are still unknown external or internal influences which modulated the effects caused by the homeopathic dilutions. To investigate these influences, statistical correlations were calculated between the experimental results and possible modulating factors during the preparation process of the potencies, like mood of the person potentising, weather, season or astronomical constellations. For every experiment the results were parametrised by three methods: (1) standard deviation of mean values, (2) sum of successive differences between means of all potency levels, and (3) maximal difference between mean values of the highest and lowest potency level. The Pearson product moment correlation yielded no definitive statements about the influences, since with only nine replication experiments per yeast and test substance there were not enough data to detect any other than very strong correlations. To get a hint regarding the nature of these unknown influences further independent well controlled replications as well as experiments with potentised water are proposed.

The reproduction of published effects by independent researchers has been and still is one of the main challenges of basic research with homeopathic dilutions. Strüh suggested a possible influence of the investigator when measuring the CO_2-production of yeast in the presence of different potency levels of plumbum aceticum [60]. In reproduction experiments with a wheat-arsenic-intoxication model Binder et al. observed significant effects of highly diluted arsenic, however, the direction of the effects found was inverted: instead of a stimulating effect as described by Betti et al. [59, 61] an internally reproducible inhibiting effect was seen [62]. One might raise the hypothesis that such problems of reproduction by other researchers may be related to the variability of the effects of homeopathic potencies observed in this study. The highly significant interactions between treatment and number of the experiments indicate that there are still unknown factors which modulate the effects of the potentised substances.

In summary, in this study it was observed that the two yeast species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, are able to react to potencies of some test substances by changing their growth kinetics. The effects on the healthy microorganisms are statistically significant, though they are only small and modulated by additional internal or external factors. Due to the inherent use of systematic negative controls, false positive results can be excluded with high certainty. In this study we observed severe violations of the classical

dose-response-curve [63]. Similar observations have previously been reported by different authors [15, 19, 20, 24, 59]. Our findings support the notion of homeopathy, that homeopathic potencies may exert specific biological effects. Thus, in future, further research efforts with methodological well defined studies and various test organisms are needed to correctly examine the efficacy of homeopathic potencies on different organism levels, identify possible effect modulating factors, and eventually enlighten their mode of action.

Acknowledgements

Thanks are due to Peter Klocke and Ingo Schroeder for valuable discussion. We thank C. de Virgilio for providing both yeast cultures as well as the physicians from KIKOM and veterinarians from FiBL for discussing the list of homeopathic potencies tested. This work was financed by the official budget of the Research Institute of Organic Agriculture. Additional financial support by the Sampo Foundation, Dornach, Switzerland and the Asta Blumfeld Foundation, Dornach, Switzerland is gratefully acknowledged.

References

1 Falus A: Homeopathy and high dilutions – is there a real effect? Inflamm Res 2004;53(5):179–180.
2 Maddox J: Waves caused by extreme dilution. Nature 1988;335(27):760–763.
3 Shang A, Huwiler-Müntener K, Nartey L, Jüni P, Dörig S, Sterne JAC, Pewsner D, Egger M: Are the clinical effects of homoeopathy placebo effects? Comparative study of placebo-controlled trials of homoeopathy and allopathy. Lancet 2005;366: 726–732.
4 Kleijnen J, Knipschild P, terRiet G: Clinical trials of homoeopathy. BMJ 1991;302:316–323.
5 Linde K, Clausius N, Ramirez G, Melchart D, Eitel F, Hedges LV, Jonas WB: Are the clinical effects of homeopathy placebo effects? A meta-analysis of placebo-controlled trials. Lancet 1997;350:834–843.
6 Bastide M, Lagache A: A communication process: a new paradigm applied to high-dilution effects on the living body. Altern Ther Health Med 1997;3(4): 35–39.
7 Walach H: Entanglement model of homeopathy as an example of generalized entanglement predicted by weak quantum theory. Forsch Komplementärmed Klass Naturheilkd 2003;10(4):192–200.
8 Weingärtner O: What is therapeutically active ingredient of homeopathic potencies? Homeopathy 2003;92(3):145–151.
9 Milgrom L: Patient-practitioner-remedy (PPR) entanglement part 4. Towards classification and unification of the different entanglement models for homeopathy. Homeopathy 2004;93(1):34–42.
10 Baumgartner S, Heusser P, Thurneysen A: Methodological standards and problems in preclinical homoeopathic potency research. Forsch Komplementärmed 1998;5:27–32.
11 Kleijnen J: What research is needed to show the effectiveness of homeopathy? Br Hom J 2000;89 (suppl 1):1–2.
12 Linde K, Jonas WB, Melchart D, Worku F, Wagner H, Eitel F: Critical review and meta-analysis of serial agitated dilutions in experimental toxicology. Hum Exp Toxicol 1994;13:481–492.
13 Scofield AM: Homoeopathy and its potential role in agriculture – a critical review. Biol Agric Hortic 1984;2:1–50.
14 Jones RL, Jenkins MD: Plant responses to homoeopathic remedies. Br Hom J 1981;70(3):120–128.
15 Pongratz W, Endler PC: Reappraisal of a classical botanical experiment in ultra high dilution research. Energetic coupling in a wheat model; in Endler PC, Schulte J (eds): Ultra High Dilutions Physiology and Physics. Dordrecht, Kluwer Academic, 1994, pp 19–26.
16 Brizzi M, Nani D, Peruzzi M, Betti L: Statistical analysis of the effect of high dilutions of arsenic in a large dataset from a wheat germination model. Br Hom J 2000;89:63–67.
17 Betti L, Lazzarato L, Trebbi G, Brizzi M, Calzoni GL, Borghini F, Nadi D: Effects of homeopathic arsenic on tobacco plant resistance to tobacco mosaic virus. Theoretical suggestions about system variability, based on a large experimental data set. Homeopathy 2003;92:195–202.
18 Baumgartner S, Thurneysen A, Heusser P: Growth stimulation of dwarf peas (*Pisum sativum* L.) through homeopathic potencies of plant growth substances. Forsch Komplementärmed Klass Naturheilkd 2004;11:281–292.
19 Youbicier-Simo BJ, Boudard F, Guelati M, Mekaouche M, Baylé JD, Bastide M: The role of the bursa of fabricius and highly dilute bursin in immunoendocrine interactions in the chickens; in Bastide M (ed): Signals and Images. Dordrecht, Kluwer Academic, 1997, pp 121–147.
20 Zausner C, Lassnig H, Endler PC, Scherer-Pongratz W, Haidvogl M, Frass M, Kastberger G, Lüdtke R: Die Wirkung von 'homöopathisch' zubereitetem Thyroxin auf die Metamorphose von Hochlandamphibien – Ergebnisse einer multizentrischen Kontrollstudie. Perfusion 2002;15:268–276.
21 Endler PC, Lüdtke R, Heckmann C, Zausner C, Lassnig H, Scherer-Pongratz W, Haidvogl M, Frass M: Pretreatment with thyroxine (10–8 parts by weight) enhances a 'curative' effect of homeopathically prepared thyroxine (10–13) on lowland frogs. Forsch Komplementärmed Klass Naturheilkd 2003; 10:137–142.
22 Pedalino CMV, Perazzo FF, Carvalho JCT, Martinho KS, de O Massoco C, Bonamin LV: Effect of *Atropa belladonna* and *Echinaceae angustifolia* in homeopathic dilution on experimental peritonitis. Homeopathy 2004;93(4):193–198.
23 Schmidt F, Süss WG, Nieber K: In-vitro Testung von homöopathischen Verdünnungen. Biol Med 2004;33(1):32–37.
24 Belon P, Cumps J, Ennis M, Mannaioni PF, Roberfroid M, Sainte-Laudy J, Wiegant FAC: Histamine dilutions modulate basophil activation. Inflamm Res 2004;53:181–188.
25 Lorenz I, Schneider E, Stolz P, Brack A, Strube J: Sensitive flow cytometric method to test basophil activation influenced by homeopathic histamine dilutions. Forsch Komplementärmed Klass Naturheilkd 2003;10(6):316–324.
26 Noiret R, Glaude M: Activité de diverses dilutions homéopathiques de Cuprum sulfuricum sur quelques souches microbiennes. Ann Hom Fr 1977;19: 91–109.
27 Brack A, Strube J, Stolz P, Decker H: Effects of ultrahigh dilutions of 3.5-dichlorophenol on the luminescence of the bacterium *Vibrio fischeri*. Biochim Biophys Acta 2003;1621:253–260.
28 Junker H: Die Wirkung extremer Potenzverdünnungen auf Organismen. Pflügers Arch ges Physiol 1928;219 B(5/6):647–672.
29 Singh RMM, Badamchian M: Reversal of inhibitory effect of 4-Nitrophenol by its own infinitesimal concentrations in *Escherichia coli*. J Am Inst Hom 1978;71:166–172.
30 Glatthaar-Saalmüller B, Fallier-Becker P: Antiviral action of Euphorbium compositum and its components. Forsch Komplementärmed Klass Naturheilkd 2001;8:207–212.
31 Kumar R, Kumar S: Effect of certain homoeopathic medicines on fungal growth and conidial germination. Indian Phytopath 1980;33:620–621.
32 Khanna KK, Chandra S: Effect of homoeopathic drugs on respiration of germinating fungal spores. Indian Phytopath 1992;45(3):348–353.
33 Graviou E, Biron MA: Action d'une 15 CH de sulfate de cuivre sur la culture de *Chlorella vulgaris*. Ann Hom Fr 1971;13(7):539–548.
34 Moss VA, Roberts JA, Simpson HKL: The effect of copper sulphate on the growth of the alga Chlorella. Br Hom J 1977;66:169–177.
35 Jones RL, Jenkins MD: Comparison of wheat and yeast as in vitro models for investigating homoeopathic medicines. Br Hom J 1983;72(3):143–147.
36 Jones RL, Jenkins MD: Effects of hand and machine succession on in vitro activity of potencies of Pulsatilla. Br Hom J 1983;72(4):217–223.
37 Nebel: Offener Brief an Dr. Friedrich Gisevius, Berlin. AHZ 1905;150:132–134.
38 Hauschka R: Hohe Verdünnungen und ihre Wirksamkeit: Substanzlehre. Frankfurt/M., Vittorio Klostermann, 1981, pp 140–152.
39 Baker RD, Smith CW: Comment on the paper, 'Growth of yeast cultures as in vitro model for investigating homoeopathic remedies'. Br Hom J 1985;74(2):93–95.
40 Steffen WA: Growth of yeast cultures as in vitro model for investigating homoeopathic medicines – a critical assessment. Br Hom J 1984;73(4):198–210.
41 Steffen WA: Growth of yeast cultures as in vitro model for investigating homoeopathic medicines. Some further studies. Br Hom J 1985;74(3): 132–140.
42 Koch HP, Partilla A: Experimentelle Untersuchungen zur Wirksamkeit homöopathischer Potenzen im 'Hefe-Test'. Pharmazie 1995;50(1):73.
43 Hagelberg E: The effect of homoeopathic medicines on yeast growth. Br Hom J 1987;76(3): 126–129.
44 Schmitt M, Gellert G, Ludwig J, Lichtenberg-Fraté H: Phenotypic yeast growth analysis for chronic toxicity testing. Ecotoxicol Environ Saf 2004;59(2): 142–150.
45 Walker GM: Yeast physiology and biotechnology. Chichester, Wiley, 1998.
46 Grundler W: Sharp resonances in yeast growth prove nonthermal sensitivity to microwaves. Phys Rev Lett 1983;51(13):1214–1216.

47 Homöopathisches Arzneibuch. Stuttgart, Deutscher Apotheker Verlag, 2003.
48 Pflanzenschutzmittel Verzeichnis 2000. Bern, Eidgenössische Forschungsanstalt für Obst-, Wein- und Gartenbau, 2000.
49 Carmer SG, Swanson MR: An evaluation of ten pairwise multiple comparison procedures by Monte Carlo Methods. J Am Stat Assoc 1973;68(341): 66–74.
50 Houta H, Leupin K: Vergleichende Untersuchungen über die Haltbarkeit von Lösungen in Kunststoff- und Glasbehältern. Pharm Acta Helv 1969; 44:366–384.
51 Jenke DR: Additive model for the evaluation of interactions between aqueous solutes and multi-component container materials. J Parenteral Sci Technol 1991;45(5):233–238.
52 Steingassner HM: Homöopathische Materia Medica für Veterinärmediziner. Wien, Wilhelm Maudrich, 1998.
53 Prusty R, Grisafi P, Fink GR: The plant hormone indolacetic acid induces invasive growth in *Saccharomyces cerevisiae*. PNAS 2004;101(12):4153–4157.
54 Joseph-Horne T, Hollomon DW, Wood PM: Fungal respiration: a fusion of standard and alternative components. Biochim Biophys Acta 2001;1504: 179–195.
55 Kraiczy P, Haase U, Gencic S, Flindt S, Anke T, Brandt U, Von Jagow G: The molecular basis for the natural resistance of the cytochrome bc_1 complex from strobilurin-producing basidiomycetes to center Q_P inhibitors. Eur J Biochem 1996;235: 54–63.
56 Pelikan W, Unger G: Die Wirkung potenzierter Substanzen: Pflanzenwachstums-Versuche mit statistischer Auswertung. Dornach, Philosophisch-Anthroposophischer Verlag am Goetheanum, 1965.
57 Lauppert E, Endler PC: Enhanced inversion effect of thyroxine log 10^{-13} by agitation. Curative effect following hyperstimulation in frogs; in Taddei-Ferretti C, Marotta P (eds): High Dilution Effects on Cells and Integrated Systems. Series on Biophysics and Biocybernetics, vol 3. Singapore, World Scientific, 1998, pp 134–144.
58 Netien G, Boiron J, Marin A: Copper sulphate and plant growth. Br Hom J 1966;55:186–188.
59 Betti L, Brizzi M, Nani D, Peruzzi M: Effects of high dilutions of Arsenicum album on wheat seedlings from seed poisoned with the same substance. Br Hom J 1997;86:86–89.
60 Strüh H-J: Der Einfluss des Experimentators auf das Experiment: Tycho De Brahe-Jahrbuch für Goetheanismus, vol 8. Niefern-Öschelbronn, Tycho Brahe, 1991, pp 68–110.
61 Brizzi M, Lazzarato L, Nani D, Borghini F, Peruzzi M, Betti L: A biostatistical insight into the As_2O_3 high dilution effects on the rate and variability of wheat seedling growth. Forsch Komplementärmed Klass Naturheilkd 2005;12:277–283.
62 Binder M, Baumgartner S, Thurneysen A: The effects of a 45x potency of Arsenicum album on wheat seedling growth – a reproduction trial. Forsch Komplementaämed Klass Naturheilkd 2005; 12:284–291.
63 Lüllmann H, Mohr K, Wehling M: Pharmakologie und Toxicologie. Stuttgart, Thieme, 2003.