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ORIGINAL PAPER

Isopathic treatment effects of *Arsenicum album* 45x on wheat seedling growth – further reproduction trials

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Background: **Two experimental studies on wheat preintoxicated with Arsenic trioxide yielded a significant shoot growth increase after an isopathic application of *Ars-alb* 45x. One independent reproduction trial however, yielded an effect inversion: wheat shoot growth was significantly decreased after application of *Ars-alb* 45x.**
Aims: **In this study we investigated the role of three potential confounding factors on the experimental outcome: geographical location of the experiments, influence of the main experimenter, and seed sensitivity to Arsenic poisoning. Laboratory-internal reproducibility was assessed by meta-analysis.**
Material and methods: **Wheat poisoned with Arsenic trioxide was cultivated *in vitro* in either *Ars-alb* 45x, water 45x, or unpotentised water. Treatments were blinded and randomised. Shoot length was measured after 7 days. The stability of the experimental set-up was assessed by systematic negative control (SNC) experiments.**
Results: **The SNC experiments did not yield significant differences between the three groups treated with unpotentised water. Thus the experimental set-up seemed to be stable. We did not observe any shoot growth increase after a treatment with *Ars-alb* 45x in any of the newly performed experiments. In contrast, the meta-analysis of all 17 experiments performed (including earlier experiments already published) yielded a statistically significant shoot growth decrease (-3.2%, $p = 0.017$) with isopathic *Ars-alb* 45x treatment. This effect was quantitatively similar across all five series of experiments.**
Conclusions: **Ultramolecular *Ars-alb* 45x led to statistically significant specific effects in arsenic poisoned wheat when investigated by two independent working groups. Effect size and effect direction differ, however. The investigated factors (geographical location, experimenter, seed sensitivity to Arsenic poisoning) did not seem to be responsible for the effect inversion. Laboratory external reproducibility of basic research into homeopathic potentisation remains a difficult issue.** *Homeopathy* (2009) **98**, 198–207.

Keywords: Homeopathy; Isopathy; Ultra high dilution; Arsenic trioxide

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Introduction

The existence of specific effects of homeopathic remedies in ultramolecular dilution is controversial,[1] although nearly all quantitative meta-analyses of randomised controlled trials are in favour of specific homeopathic remedy effects when studying defined medical conditions.[2–7] Experimental studies in basic research may help to clarify this situation, for instance by providing empirical evidence for specific homeopathic drug effects, and by eventually elucidating the mode of action of homeopathic preparations.

There is a considerable number of experimental studies in homeopathic basic research.[8] There are several models that consistently yield consistent results within the same laboratory,[9–12] and some models resulted in at least similar results in multi-centre trials[13,14] or independent replications.[15,16] However, we do not know any single model that yielded exactly the same result (regarding effect size and direction for a given specific potency level) when independently reproduced in another laboratory.[17] Independent reproduction trials often either yielded no significant effects,[18–22] inverted effects,[23] or effects at different potency levels.[16,24–26] Whether these reproducibility problems are due to unknown and consequently uncontrolled parameters relevant for the experimental system or whether they reflect the very nature of homeopathic effects in preclinical systems, is an unresolved issue.[17]

An Italian team developed an isopathic model to study homeopathic treatment effects. Wheat seedlings were poisoned with a sublethal dose of Arsenic (1‰), and were subsequently treated with an ultramolecular homeopathic preparation of arsenic trioxide, *Arsenicum album* 45x (*Ars-alb* 45x). This treatment led to an increase in shoot growth of 24% after 7 days cultivation *in vitro*, compared to an unpotentised water control.[27] This finding could be reproduced in an independent trial eight years later.[28]

A Swiss-German research team adapted this model to study reproducibility in another laboratory. Interestingly, this investigation also revealed statistically significant effects of *Ars-alb* 45x, however, inverted in direction compared to the original trial: shoot length was reduced by −3% after 7 days.[23] This effect was laboratory internally reproducible over 8 independent experiments, working with two different lots of wheat cultivars in two experimental series.

These surprising results prompted us to determine possible reasons for this effect inversion. We set out to investigate three parameters which could have played a decisive role in effect inversion:[23] 1. the geographical location (or an unknown factor associated with the original laboratory); 2. the main experimenter; 3. seed sensitivity towards arsenic.

We therefore designed three further series of independent reproduction trials to empirically test these hypotheses. First, the experiment was reassessed by a member of the Swiss-German research team (MB) in the Italian laboratory. Second, another experimenter (LL) of the Swiss-German group reproduced the experiment in the Swiss laboratory. Third, we screened several different wheat cultivars to determine a seedling lot exhibiting maximal shoot growth reduction through arsenic poisoning whilst maintaining germination rate at a maximal level. With the corresponding lot, another experimental series was conducted.

The size of these three further experimental series (number of seedlings per parameter) was determined according to statistical power calculations: a shoot growth increase of 20% (corresponding to an effect size of 0.3) as observed in the Italian experiments[27,28] would be detected with a power of at least 0.99 for $\alpha = 0.01$.

For economic reasons, experiments were *not* designed as reproduction of the first Swiss-German reproduction trial[23] with effects of about 3%. In order to reveal such small effects (effect size ≈ 0.04) with a high statistical power (0.99), a considerable number of treated seedlings would have been necessary ($n > 10{,}000$).

Material and methods

General experimental design

For experiments with homeopathic preparations, wheat seed were pre-treated (poisoned) with As_2O_3 (see below). Each experiment typically consisted of 450 seeds: 150 seeds treated with *Ars-alb* 45x, 150 seeds with water 45x, and 150 seeds with unpotentised water (due to time and space constraints, group size had to be reduced in series III to 200 (=66 + 67 + 67) seeds in total). Wheat shoot length was measured after 7 days (see below). Within the experimental series, we conducted 2, 3 or 4 independent experiments (see Table 1, column 'Potency experiments', series III–V). The number of independent experiments was determined based on a power calculation, on previous experiences, and on existing financial possibilities.

Table 1 Overview of all experiments performed. Arsenic = concentration of As_2O_3 poisoning. SNC = systematic negative control experiment. Data of experimental series I and II have been published earlier;[23] experimental series III, IV and V are new data sets and have not been reported before. Wheat cultivar includes year of harvest when known. Abbreviations for countries and nationalities: DE = Germany, CH = Switzerland, IT = Italy

Exp. Series	*Experimenter/ Nationality*	*Date of experiments*	*Location/ Country*	*Wheat cultivar*	*Arsenic*	*Experiment number*				
						SNC	*Potency experiments*			
I	MB/DE	2001	Arlesheim/CH	Pandas 2000	1.0‰	E11	E12	E13	E14	E15
II	MB/DE	2002/2003	Arlesheim/CH	Mec	1.0‰ & 1.2‰	E21	E22	E23	E24	E25
III	MB/DE	2002	Milano/IT	Pandas 2001	1.0‰	E31	E32	E33		
IV	LL/DE	2004	Arlesheim/CH	Pandas 2000	1.0‰	E41	E42	E43	E44	
V	LL/DE	2004	Arlesheim/CH	Pandas 2001	1.6‰	E51	E52	E53	E54	E55

In addition, we performed one full-scale systematic negative control (SNC) experiment per series, consisting of three groups with 150 seedlings each (66, 67, 67 seedlings in series III), which were all treated with unpotentised water. Shoot length data were measured as usual (see below) and were analysed for differences between the three identically treated groups in order to test the stability of the experimental set-up.

In series I, experiments were performed by a member of the Swiss-German research team (MB) in the laboratory at Arlesheim (Switzerland). Four independent experiments were carried out to replicate the original trial.[27] In these experiments, wheat of the cultivar 'Pandas' was used since seed of the cultivar 'Mec' that had been used in the original trial[27] was no longer officially available. 'Pandas' wheat has successfully been used in germination rate experiments of the Italian team (unpublished results) and was therefore adopted for the replication trial. Methods were described in detail in an earlier publication,[23] and were essentially identical to the experimental series III–V reported in this publication.

In series II, four further independent experiments with homeopathic preparations were performed by a member of the Swiss-German research team (MB) in the laboratory at Arlesheim (Switzerland). The only difference to the first series was the wheat cultivar. After being unable to reproduce the results of the original trial[27] with 'Pandas' wheat the Swiss-German team tried to get seeds of the wheat cultivar 'Mec' in order to test the hypothesis that the wheat cultivar was decisive for experimental outcome. After finally acquiring a small lot of 'Mec' wheat this hypothesis could be tested. Also for this series of experiments, methods were described in detail in an earlier publication,[23] and were essentially identical to the experimental series III–V reported in this publication.

In series III, experiments were performed by a member of the Swiss-German research team (Mascha Binder, MB) in the laboratory at Milan (Italy), where the original trial had been carried out.[27] This approach was chosen to test the hypothesis, that the geographical location of the experiments (or an unknown factor associated with the original laboratory) was crucial for successful reproduction. In contrast to the original trial, seed of wheat cultivar 'Pandas' had to be used because 'Mec' was no longer available in the amounts needed.

In series IV, experiments were performed by a member of the Swiss-German research team (Lisa Lahnstein, LL) in the laboratory at Arlesheim (Switzerland), where the first two experimental series of the independent reproduction trials (I and II) had been carried out before by MB.[23] We used the same wheat cultivar lot ('Pandas' harvest 2000 poisoned by MB for the experimental series I) to test the hypothesis, that the experimenter responsible for potentisation and plant treatment somehow influences the results.

Prior to series V (see Table 1) we conducted a series of screening experiments to determine a seedling lot exhibiting maximal shoot growth reduction after arsenic poisoning whilst maintaining germination rate at a maximal level. This approach was chosen since in experimental series I and II, we had not been able to induce a shoot length reduction of about 50% as observed in the original trial[27] after application of 1‰ or 1.2‰ arsenic.[23] One might therefore argue that the poisoning effect in experimental series I and II was too weak (−15% compared to −50% in the original trial) to allow a clear positive effect of *Ars-alb* 45x. We therefore treated 20 different wheat seed lots (see below) with various concentrations of arsenic (0–1.6‰) to determine the lot that causes maximal shoot growth reduction whilst maintaining a reasonable germination rate for a given arsenic concentration. Each of these screening experiments was performed with 200 seeds per parameter. The main experiments of series V (see Table 1) were finally conducted by LL in the laboratory at Arlesheim (Switzerland), with the wheat cultivar 'Pandas' from harvest 2001 poisoned with 1.6‰ arsenic.

Materials

Wheat seed (*Triticum aestivum* L.) was obtained from different sources: cultivars 'Asita', 'Ataro' and 'REG.100' from Getreidezüchtung Peter Kunz (Hombrechtikon, Switzerland); cultivars 'Titlis' and 'Tamaro' grown in five farming systems each (2 organic, 2 conventional and 1 unfertilised control; samples drawn from the DOK trial[29]) from Paul Mäder (Research Institute of Organic Agriculture, Frick, Switzerland); cultivar 'Pandas' from three different harvests (2000, 2001, 2003) from Maurizio Peruzzi (Association for Sensitive Crystallization, Milan, Italy); cultivars 'Janak', 'Madraji', 'Sonalika', and 'Jharna' from Anirban Sukul (Department of Zoology, Visva-Bharati University, Santiniketan, India).

Arsenicum album (As_2O_3, BDH Chemicals Ltd., Poole, England, no. 27274) and pure water (pro analysis (p.a.) Merck, no. 1.16754.9010, VWR, Dietikon, Switzerland) was used from the same source as for the original trial.[27] Clay to fix the wheat seeds on the chromatography paper (2043 A, Schleicher & Schuell, Dassel, Germany) was obtained from Maurizio Peruzzi (Milan, Italy) and was from the same source as for the original trial.[27]

Polyethylene bags (15 × 30 cm, N° 9978, Plastik-Haus, Arlesheim, Switzerland) for *in vitro* cultivation of the wheat seedlings were cut to a size of 15 × 20 cm. Potentisation was performed in 100 ml polyethylene vessels (N° 1654, Plastik-Haus, Arlesheim, Switzerland) for potency levels up to 44x. The last potentisation step (45x) was done in 500 ml polyethylene vessels (N° 7605, Plastik-Haus, Arlesheim, Switzerland).

Seed pre-treatment

Wheat seed was pre-treated (poisoned) by immersion for 30 min in a 1‰, 1.2‰ or 1.6‰ As_2O_3 solution (see Table 1) in water p.a. and by subsequent rinsing under running tap water for another 60 min. Seeds were allowed to dry in ambient air and stored in darkness until used in the experiments.

Preparation of homeopathic dilutions and controls

For each experiment, aqueous decimal potencies were freshly prepared on the day of the experiment using the multiple-glass method, starting from the mother tincture (As_2O_3

1‰ solution). For each potentisation step, 10 ml of the preceding potency level was diluted in 90 ml water p.a., except for the 45x potency where 50 ml 44x were diluted in 450 ml water. Succussion was done by hand: 70 strong beats directed downwards against a firm base (this method was adopted from the Italian team). Exactly the same procedure was adopted for the potentised water control (water 45x); the only difference to *Ars-alb* 45x was that the 'mother tincture' was pure water (instead of a 1‰ As_2O_3 solution). The same batch of water p.a. was used to prepare *Ars-alb* 45x, water 45x and unpotentised water being used in the same experiment.

Conduct of experiments

After potentisation, a person not otherwise involved in conduct of the experiment (Stephan Baumgartner, SB) coded (blinded) the three treatment solutions with a random letter code. The code was kept secret in a sealed envelope until all measurements and basic data processing were finished.

Each seed was fixed by a piece of clay (0.20 ± 0.05 g) on chromatography paper (14×17 cm). The paper was inserted in a polyethylene bag (15×20 cm), which in turn was placed into a half-open paper envelope covering the roots, but not the shoots.[27] This technique ensured that shoots and roots would grow in natural light and darkness, respectively. Each chromatography paper was wetted with 3.3 ml fluid (*Ars-alb* 45x, water 45x, or unpotentised water) using a pipette (Eppendorf Research 5000 μl, Schönenbuch, Switzerland).

The paper envelopes comprising the differently treated seeds were placed in cardboard boxes. Envelopes were pierced with a hole puncher. Two metal rods were put through the two holes, and envelopes were suspended by the metal rods abutting on the edges of the cardboard box. One cardboard box accommodated 90 envelopes with 1 seedling each. A total of 5 cardboard boxes were used for a total of 450 seedlings in one experiment. Treatments were allocated in randomised block sequences. The 90 envelopes of each cardboard box were grouped in 9 groups of ten seeds each. The 9 groups of each box were randomly allocated to 3×3 treatment groups with the aid of a computer generated randomization list. The list was prepared newly for each box and each experiment. Thus, in one box, ten seeds of the first group were followed by ten seeds of the second group, and so on (corresponding to the order in which they were planted).

The cardboard boxes were placed without cover on a laboratory bench. Windows were shielded by curtains to protect plants from direct sunlight. Thus, seedlings germinated and grew for 7 days in diffuse natural light assuring a natural day-night-rhythm.

Measurements

Plants were photocopied at the 7th day of growth (C187, UTAX, Embrach, Switzerland). True curve length of the plant shoots was measured by means of the software 'Tracking 0.2.6' (A. Fritschy, Informatik-Lösungen, Zürich, Switzerland), using a Summagraphics Summa-Sketch III digitizing tablet (GTCO CalComp, Columbia, USA) connected to an Apple ibook G3 computer (Apple, Cupertino, USA).

Data evaluation and statistical analysis

Primary measurement parameter was the mean shoot length of all germinated plants. Secondary outcome parameters were germination rate and shoot length of all plants (including non-germinated seedlings). Tertiary (descriptive) outcome parameters were variability (standard deviation(SD)) within experiments, and data distribution shape.

A total of 4650 seeds were used in the experiments of series III–V. Data from 16 seeds (0.34%) were excluded from statistical analysis, in all cases due to mistakes in experimental handling (missing cultivation fluid).

Statistical analysis of mean wheat shoot length of germinated seedlings was based upon parametrical analysis of variance (ANOVA). We adopted the two-step procedure of a preceding F-test for global significance (at the level $p < 0.05$) and following planned pairwise comparisons by the least significant difference test (LSD-test) , which yields optimal protection against type 1 and type 2 errors.[30] This type of analysis is also empirically quite robust against minor violations of its theoretical assumptions (normality, homogeneity of variances).

Differences in germination rate were assessed with the chi-square test. Wheat shoot length of all (germinated and not germinated) seedlings was assessed by Kruskal–Wallis-ANOVA followed by Mann–Whitney-U-test for pairwise comparisons, since these data sets showed bimodal distributions due to the non-germinated seeds with length 0 mm.

Descriptive statistics, parametric and non-parametric ANOVA, chi-square tests, LSD- and U-tests were calculated with the software ''Statistica 4.1'' (Statsoft, Inc., Tulsa, USA). Power calculations were performed with 'G*Power 3.0.10' (Institut für Psychologie, Universität Düsseldorf, Germany).[31] Data distribution shape was examined with ''Kaleidagraph 3.6.4'' (Synergy Software, Reading, USA).

Results

SNC experiments

For each series of experiments, we conducted one full scale SNC experiment (see Table 1). All seedlings were poisoned as in experiments with homeopathic preparations, but treated with unsuccussed water only. Statistical evaluation investigated whether there were any differences between three seedling groups ($n = 3 \times 150 = 450$, except for series III with $n = 200$ in total), which corresponded in spatial set-up and experimental progress in time to those groups that were allocated to the three different treatments in the 'true' experiments.

Statistics for the earlier SNC experiments of series I and II have been reported in detail in an earlier publication.[23] None of the experiments yielded significant differences regarding shoot length for the three identically treated groups.[23] Germination rate also did not yield significant differences.[23]

Table 2 Descriptive statistics for the newly performed experiments in series III, IV and V: shoot length of all germinated seeds [mm], SD [mm], CV = coefficient of variation [%], number *n*, and germination rate [%]. Experiments N° 31, 41, and 51 were systematic negative control experiments: after poisoning with arsenic, seeds were all treated with unpotentised water (parameters water (1), (2), (3))

Experimental series	*N°*	*Parameter*	*Length germinated seedlings*			*Number*	*Germination*
			Mean [mm]	*SD [mm]*	*CV [%]*	n	*Rate [%]*
III	31 (SNC)	Water (1)	58.3	20.6	35.2	65	97.0%
		Water (2)	59.9	22.9	38.3	58	87.9%
		Water (3)	62.0	21.9	35.4	64	95.5%
III	32	Water	63.4	27.2	42.9	64	95.5%
		Water 45x	63.8	26.5	41.5	64	97.0%
		Ars-alb 45x	59.0	29.1	49.3	65	97.0%
III	33	Water	65.3	17.9	27.5	66	98.5%
		Water 45x	62.8	19.3	30.7	61	91.0%
		Ars-alb 45x	65.2	19.7	30.2	64	97.0%
IV	41 (SNC)	Water (1)	40.8	16.9	41.4	121	82.3%
		Water (2)	44.0	17.7	40.3	128	85.3%
		Water (3)	41.7	17.9	43.0	122	81.3%
IV	42	Water	44.1	16.7	37.8	125	85.0%
		Water 45x	40.8	19.3	47.2	130	86.7%
		Ars-alb 45x	41.7	17.5	42.0	121	80.7%
IV	43	Water	38.0	16.4	43.2	130	86.7%
		Water 45x	41.3	17.7	42.9	109	74.2%
		Ars-alb 45x	38.0	19.2	50.6	128	87.1%
IV	44	Water	40.8	16.8	41.1	126	84.0%
		Water 45x	38.7	16.7	43.1	113	75.8%
		Ars-alb 45x	39.8	13.8	34.7	115	77.7%
V	51 (SNC)	Water (1)	41.2	28.1	68.2	104	69.3%
		Water (2)	41.4	25.7	62.0	102	68.0%
		Water (3)	36.5	28.1	77.1	94	62.7%
V	52	Water	34.7	23.8	68.7	91	60.7%
		Water 45x	33.7	23.0	68.3	92	61.3%
		Ars-alb 45x	29.0	21.5	74.0	83	55.3%
V	53	Water	35.8	26.4	73.8	97	64.7%
		Water 45x	39.2	28.7	73.3	104	69.3%
		Ars-alb 45x	37.1	30.8	83.0	104	69.3%
V	54	Water	38.8	29.7	76.5	93	61.6%
		Water 45x	40.5	28.4	70.3	104	69.3%
		Ars-alb 45x	39.9	30.3	76.0	89	60.1%
V	55	Water	49.0	27.6	56.4	68	45.3%
		Water 45x	51.5	32.2	62.6	76	50.7%
		Ars-alb 45x	46.8	30.3	64.8	76	50.7%

Descriptive statistics for the newly performed SNC experiments (series III–V) are given in Table 2 (experiments 31, 41, 51). None of the three individual experiments yielded significant differences regarding shoot length for the three identically treated groups (one-way ANOVA *F*-test, analysing all germinated seedlings: $p = 0.63$ for E31, $p = 0.32$ for E41, $p = 0.37$ for E51; Kruskal–Wallis-test, analysing all seedlings, including non-germinated seed with length 0 mm: $p = 0.48$ for E31, $p = 0.26$ for E41, $p = 0.13$ for E51). Germination rate also did not yield significant differences (Chi-square-test: $p = 0.07$ for E31, $p = 0.63$ for E41, $p = 0.43$ for E51).

In a meta-analysis, we analysed the length data of all germinated seedlings recorded in all five SNC experiments of all experimental series (I–V, see Table 1) in a two-way ANOVA with the independent variables experiment number (E11, E21, E31, E41, E51) and allocation group (1, 2, 3). Neither the *F*-test for the main allocation group effect ($p = 0.48$) nor its interaction with experiment number ($p = 0.71$) were significant.

Thus, there was no indication for any systematic differences associated with the experimental set-up that could lead to false-positive results.

Results of the first two experimental series (I and II) with homeopathic preparations

As reported in detail in an earlier publication,[23] the analysis of series I and II yielded an effect inversion compared to the original trial.[27] Shoot length was reduced by −3% after application of *ars-alb* 45x, compared to both water ($p = 0.011$, LSD-test) and water 45x ($p = 0.037$, LSD) used as controls (see also Figure 1A (series I and II) for the 2×4 individual experiments, and Figure 1B (experimental series I and II) for mean values of the experimental series). Results did not differ for the two wheat cultivars used ('Pandas' and 'Mec'). Analogously, there was a non-significant tendency for germination rate to be reduced after application of *Ars-alb* 45x. In other experiments, the Italian team also had observed enhancement of germination rate

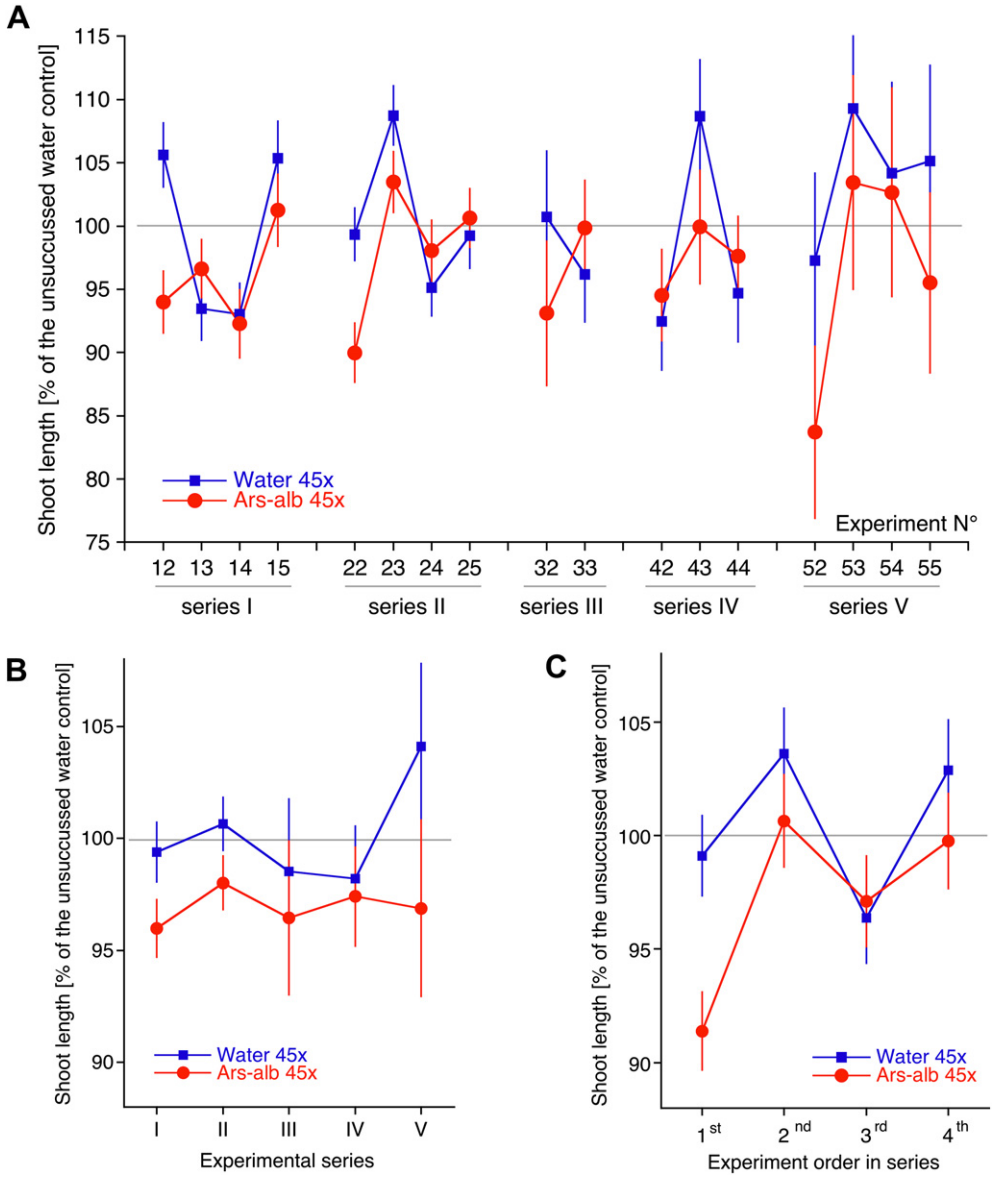

Figure 1 Shoot length of wheat seedlings, treated with water 45x or *Ars-alb* 45x (mean ± standard error). Data are plotted relative to the unsuccussed water control group [%]. A: results of individual experiments (see Table 1). B: mean results of the five experimental series I–V (see Table 1). C: mean results as a function of the experimental ordering (1st, 2nd, 3rd, 4th experiment) within each series. Data of experimental series I and II (experiments 12–15 & 22–25) have already been published[23] and are included here for comparison. Connecting lines were inserted to guide the eye; they are not meant to suggest interpolations.

through *Ars-alb* 45x.[32] Thus, results of the Swiss-German reproduction trials were inverted compared to the results of the Italian group.[27,32]

Influence of the geographical location (experimental series III)

Descriptive statistics for the two experiments performed by MB in Milan (Italy) are given in Table 2 (exp. N° 32, 33). The main outcome parameter 'length of all germinated seedlings' was analysed in a two-way ANOVA with the independent variables experiment number (E32, E33) and treatment group (water, water 45x, and *Ars-alb* 45x). Neither the F-test for the main treatment effect ($p = 0.75$, achieved power = 0.998 for an estimated effect of +20% and $\alpha = 0.01$) nor its interaction with experiment number ($p = 0.48$) were significant. Numerically, seedlings treated with *Ars-alb* 45x were smaller than those of the two control groups (−1.9% or −3.5% compared to water 45x or water, respectively). None of the secondary outcome parameters yielded significant effects between treatment groups. Numerically, results were similar to those of the first two series[23] performed by the Swiss-German team (Figure 1B).

Influence of the main experimenter (experimental series IV)

Descriptive statistics for the three experiments performed in Arlesheim by LL are given in Table 2 (exp. N° 42–44). The main outcome parameter 'length of all germinated seedlings' was analysed in a two-way ANOVA with the independent variables experiment number (E42, E43, E44)

and treatment group (water, water 45x, and *Ars-alb* 45x). Neither the *F*-test for the main treatment effect ($p = 0.66$, achieved power > 0.999 for an estimated effect of +20% and $\alpha = 0.01$) nor its interaction with experiment number ($p = 0.25$) were significant. Numerically, seedlings treated with *Ars-alb* 45x were smaller than those of the two control groups (−1.0% or −2.8% compared to water 45x or water, respectively). Again, results were numerically similar to those of the first two series[23] performed by the Swiss-German team (Figure 1B).

Among the secondary outcome parameters, only germination rate yielded significant effects between treatment groups ($p = 0.049$, chi-square-test); subsequent pairwise comparisons yielded significant differences only for water 45x (78.9%) compared to water (85.2%) ($p = 0.014$, chi-square test).

Optimization of seed lot properties (experimental series V)

In several screening experiments we tested 20 different wheat seed cultivar lots regarding their sensitivity towards poisoning with arsenic (data not shown). Harvest 2001 of cultivar 'Pandas' exhibited the most pronounced shoot depression (≈50%) after treatment with arsenic 1.6‰ whilst maintaining a reasonable level germination rate (≈70%). We thus used this lot for experimental series V.

Descriptive statistics for the four experiments performed by LL in Arlesheim (Switzerland) are given in Table 2 (exp. N° 52–55). The main outcome parameter 'length of all germinated seedlings' was analysed in a two-way ANOVA with the independent variables experiment number (E52, E53, E54, E55) and treatment group (water, water 45x, and *Ars-alb* 45x). Neither the *F*-test for the main treatment effect ($p = 0.35$, achieved power > 0.999 for an estimated effect of +20% and $\alpha = 0.01$) nor its interaction with experiment number ($p = 0.91$) were significant. Numerically, seedlings treated with *Ars-alb* 45x were smaller than those of the two control groups (−7.3% or −3.5% compared to water 45x or water, respectively). None of the secondary outcome parameters yielded significant effects between treatment groups. Thus, results for *Ars-alb* 45x treated seedlings were again similar to those of the first two series[23] performed by the Swiss-German team (Figure 1B).

Meta-analysis of all experimental series (I–V)

The main outcome parameter 'length of all germinated seedlings' was analysed in a two-way ANOVA with the independent variables experiment number (17 independent experiments, E12–E55, Table 1) and treatment group (water, water 45x, and *Ars-alb* 45x). The *F*-test for the main treatment effect ($p = 0.017$) yielded evidence for statistically significant differences between treatment groups. In the pairwise comparison, the effects of *Ars-alb* 45x could be differentiated from water 45x (−3.2%, $p = 0.01$, LSD-test) and from unsuccussed water (−3.2%, $p = 0.01$, LSD-test). The two water controls did not differ in their effects ($p = 0.99$, LSD). ANOVA did not yield any evidence for an interaction between experiment number and treatment effect ($p = 0.31$, *F*-test). Thus, from a statistical point of view, the effects of *Ars-alb* 45x were reproducible over all 17 experiments. In all five experimental series (I–V), the effect of *Ars-alb* 45x was comparable (Figure 1B). In 12 out of 17 experiments, seedlings treated with *Ars-alb* 45x were on the average smaller than the control samples treated with unsuccussed water (Figure 1A).

Interestingly, in 4 out of 5 experimental series, the first experiment of the series seemed to have the largest effect (Figure 1A). When combining all experiments according to the ordering within the experimental series (Figure 1C), only the 1st experiments within all series yielded significant differences between *Ars-alb* 45x and water (−8.0%, $p = 0.003$, LSD-test) or water 45x (−7.3%, $p = 0.007$, LSD), respectively. The 2nd, 3rd, and 4th experiments did not yield significant effects of *Ars-alb* 45x compared to the water controls ($p > 0.32$, LSD). Also, there were no significant differences between the two water controls ($p > 0.19$, LSD) for any of the four experimental order groups.

The secondary outcome parameter germination rate was numerically lowest for *Ars-alb* 45x treated seed (82.0%), and somewhat higher for the water control groups (water 45x: 82.5%; water: 83.1%). These differences were not significant ($p = 0.57$, chi-square-test).

Another way to analyse the data set is to analyse the length of *all* seeds, germinated as well as non-germinated seed. In this analysis, non-germinated seed were incorporated in the data set with length 0 mm. Due to the bimodal distribution, data were analysed by means of a non-parametrical Kruskal–Wallis-ANOVA with the independent factor treatment (water, water 45x, and *Ars-alb* 45x). Global analysis was significant ($p = 0.03$). Subsequent *U*-tests differentiated *Ars-alb* 45x from water ($p = 0.014$) and water 45x ($p = 0.038$), but not water from water 45x ($p = 0.72$).

Variability for each of the three treatment parameters over all 17 experiments was assessed by first normalizing all length data of all germinated plants to the experimental mean for each experiment. Then data of all experiments were pooled for each treatment parameter, and the SD calculated. The *Ars-alb* 45x treated group had the highest SD (44.4%), followed by the water 45x group with 43.2%, and the unsuccussed water group with 41.6%. No evident differences in distribution shape of the three differently treated groups could be detected after visual inspection by probability plots (data not shown).

Discussion

Two experimental studies[27,28] by an Italian research group on wheat poisoned with Arsenic trioxide yielded a significant shoot growth *increase* (+24%) after an isopathic application of *Ars-alb* 45x. One independent reproduction trial[23] by a Swiss-German group, however, yielded an inverted effect: wheat shoot growth was significantly *decreased* (−3%) after application of *Ars-alb* 45x in two experimental series using two different wheat cultivars. We therefore designed three further series of experiments to verify or falsify three hypotheses, why the reproduction

trials of the Swiss-German group yielded inverted results compared to ones of the Italian group.

The first hypothesis was that an unknown factor associated with the location of the laboratory of the original trial (Milan, Italy) was crucial for the shoot growth increasing effect. However, our replication trial in the laboratory of the original trial (Milan) did not yield any evidence for a shoot growth increase after application of *Ars-alb* 45x. The power of the experiments performed was larger than 0.998 (for an estimated effect of +20% and $\alpha = 0.01$). Therefore, any increasing effect in the order of +20% should have been detected with very high probability. Since the 2nd trial of the Italian group was performed in another location (Bologna, Italy)[28] and yielded analogous results as the first trial in Milan,[27] location does not seem to be the decisive factor that leads to increased shoot growth in this model.

Secondly we hypothesised that the personality of the main experimenter of the Swiss-German team (MB) had some influence on the results, even though all experiments were strictly blinded. MB had carried out all experimental work of the first three series (I–III, Table 1), i.e. poisoning of the wheat, potentisation of *Ars-alb* 45x and water 45x, selection of wheat grains for the trials, all experimental handling during the experiments, and final measurements by photocopying and subsequent digitizing the seedlings' shoot length. We therefore decided to have the experiments repeated by another person (LL), being responsible for all experimental work with the exception of the poisoning of the wheat seeds, since we wanted to use the same lot as in experimental series I. The results of these series of experiments were also negative, i.e. we did not observe any shoot growth increasing effects after application of *Ars-alb* 45x. The power of these experiments was also very high (>0.999 for an estimated effect of +20% and $\alpha = 0.01$). Therefore, also here, any increasing effect should have been detected with very high probability. Thus, the exchange of experimenter did not explain the results.

The third hypothesis was that the seed responsiveness to arsenic was not optimal in the wheat lots the Swiss-German team had used for series I and II. Due to restrictions in available quantity, we could not use the same seed lot the Italian team used,[27] and had to introduce another seed lot. In experimental series I and II, we were not able to induce a shoot length reduction of about 50% as observed in the original trial[27] after application of 1‰ or 1.2‰ arsenic.[23] One might therefore argue that the poisoning effect in experimental series I and II was too weak (−15% compared to −50% in the original trial) to allow a clear effect of *Ars-alb* 45x to occur (a 'floor' effect). A differing seed lot might produce other results. We therefore treated 20 different seedling lots with various concentrations of arsenic (0–1.6‰) to determine the lot that exhibits maximal shoot growth reduction whilst maintaining a good germination rate for a given arsenic concentration. The seed lot we finally selected was wheat cultivar 'Pandas' from harvest 2001 poisoned with 1.6‰ arsenic, exhibiting a shoot growth depression of about 50% with a germination rate of about 70%. But again in this series of experiments we did not observe any shoot growth increase after application of *Ars-alb* 45x. The power of these experiments was also very high (>0.999 for an estimated effect of +20% and $\alpha = 0.01$). Therefore again, any increasing effect of *Ars-alb* 45x should have been detected with very high probability. Thus, the seed sensitivity hypothesis also had to be dismissed.

Results of all three newly performed experimental series (III–V) were numerically in line with the results of the first two series (I–II) published earlier (Figure 1B).[23] However, one may be troubled by the fact that none of the newly performed experimental series (III, IV and V) yielded statistical evidence for such growth-decreasing effects. However, the experiments were not designed to reveal effects in the order of 3% as observed in the first two series.[23] A posteriori, the power of the three new experimental series (III, IV, V) was calculated to be 0.06, 0.17, and 0.04, respectively (for an effect of −3% and $\alpha = 0.01$). Thus, the power was too low to detect 3%-effects, and no conclusion regarding reproducibility can be drawn from evaluating single experimental series. Only meta-analysis of all experiments can furnish valuable information. The power of the combined analysis[23] of series I and II was 0.60 for an effect of −3% and $\alpha = 0.01$ (a posteriori). The power of series III, IV and V was considerably lower due to the smaller sample size and due to increased SD, especially for series V with stronger poisoning effects using 1.6‰ arsenic.

A meta-analysis of all five experimental series performed by the Swiss-German team ($n = 17$ independent experiments in total) yielded evidence for a *decreasing* effect of *Ars-alb* 45x on wheat shoot growth (−3.2%, $p = 0.017$), consistently across all five series of experiments (Figure 1B). There was also a trend that germination rate was *lowest* for seeds treated with *Ars-alb* 45x. Furthermore, the *Ars-alb* 45x treated group had the *highest* intra-experimental SD. Again, this set of results is a complete reversal of the results of the Italian group, which observed that the application *Ars-alb* 45x led to an *increase* in shoot length,[27,28] an *increase* in germination rate[32] and a *decrease* in SD.[33]

The contradiction between the results of the Italian group and the Swiss-German group is puzzling. We think that the probability for all these observations being systematic misinterpretations is very low. After all, the SNC experiments of the Swiss-German group do not yield any evidence for systematic errors. The fact that results *within* working groups seem to be consistent points to 'true' specific effects of homeopathic preparations, which are however influenced by unknown factors leading to a modification of effect size and direction. Thus, the question arises how to identify these unknown factors. For the moment we cannot offer anything but the usual scientific procedure of formulating specific hypotheses and subsequent experimental investigation.

Based on the results accomplished so far, we conclude that neither seed cultivars used ('Pandas' or 'Mec'), nor magnitude of arsenic poisoning (15%–50%), nor geographical location of the original laboratory played a decisive role for the observed effect inversion. Regarding further possible reasons for the observed effect inversion, we currently have two main hypotheses.

The first hypothesis refers to seed 'quality'. In an investigation with dwarf peas[34] we had observed that, of four

different lots of the same dwarf pea cultivar 'Früher Zwerg' (harvests of years 1997, 1998, 1999, 2000), only one (1997) reacted reproducibly to a treatment with gibberellic acid 17x. This specific seed lot differed from the other three by an increased glucose and fructose content, pointing to premature harvest. In another study with barley, extent and type of response to potencies of gibberellic acid (4c–200c) were also dependent on the vigour level of the seed lot.[35] It thus may be hypothesised that the specific seed lot used by the Italian team in the original trial[27] exhibited some special characteristics due to unique cultivation factors such as climate, water supply, soil, fertilization, plant diseases, insect attack, and harvesting date.

The second hypothesis relies on the fact that it has often been observed in biological models of high dilution effects that in a series of succeeding potency levels (e.g. 12x–30x) effective and ineffective potency levels alternate in a seemingly chaotic way.[14,36–38] The effects of adjacent potency levels may differ drastically, in some cases even in a binary mode (effect–no effect).[34,38,39] If for some (still unknown) reason active and inactive potency levels shifted by one or two levels, effects of a single potency level (e.g. 45x) may vanish totally. We therefore recommend testing a series of potency levels (e.g. 40x–50x) with this model to determine any such drifts. Furthermore, testing of a broader base of potency levels (e.g. 30x, 200x, or 6c, 15c, 30c, 200c, 1000c) might yield interesting results.

Duration of storage before use of the poisoned seeds probably had no relevant influence on experimental outcome. This can be concluded from the similarity of the results of series I and IV: in the latter series we had used poisoned seed from series I stored in the meantime for about three years (Table 1). In all other experiments, wheat seed was used within some months after initial poisoning. Thus, length of storage before use of the poisoned seeds most probably had no relevant influence on the results.

In purely physicochemical systems, some investigations observed that effects of homeopathic preparations increased in the course of time.[40,41] Whether such effects may also be relevant in biological systems should be studied more closely in correspondingly designed experiments.

Seasonality as a possible explanation for the differing results has been discussed earlier[23] and can most probably be dismissed. It is well known, however, that biological systems exhibit a broad range of physiological rhythms, with wavelengths of days to months.[42–44] Further exploration might be interesting, provided the number of experiments is large enough.

Reproducibility is one of the major concerns in homeopathic basic research.[17] Though there are several models that yield consistent results within the same working group[9–12] or within multi-center trials,[13,14] we do not know any model that yielded *identical* results when independently reproduced.[17] There are, however, models that yield *similar* results, e.g. significant effects of the same potentized substance, but at differing potency levels.[16,24–26] In our opinion, elucidation of the reasons for the lacking reproducibility constitutes the major present challenge for homeopathic basic research and therefore warrants further investigations.

Conclusion

Ultramolecular treatments with *Ars-alb* 45x led to statistically significant specific effects in arsenic poisoned wheat when investigated by two independent working groups. Effect size and effect direction differ, however: the Italian group observed an *increase* in shoot length and germination rate, and a *decrease* in SD, whilst the Swiss-German group observed a *decrease* in shoot length and germination rate, and an *increase* in SD. The investigated factors (geographical location, experimenter, seed sensitivity to Arsenic poisoning) did not seem to be responsible for the effect inversion. Laboratory external reproducibility of basic research into homeopathic potentisation still seems to be a difficult issue. We want to encourage other research groups picking up this model to contribute identifying the factors responsible for effect size and direction.

Conflict of interest

There are no conflicts of interest.

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