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ORIGINAL PAPER

Polycrystalline structures formed in evaporating droplets as a parameter to test the action of *Zincum metallicum* 30c in a wheat seed model

Maria Olga Kokornaczyk[1,*], Stephan Baumgartner[2,3] and Lucietta Betti[1]

[1]*Department of Agricultural Sciences, University of Bologna, Viale Fanin 44, Bologna 40127, Italy*
[2]*Society for Cancer Research, Kirschweg 9, 4144 Arlesheim, Switzerland*
[3]*Institute of Integrative Medicine, University of Witten-Herdecke, Gerhard-Kienle-Weg 4, 58313 Herdecke, Germany*

Background: **Polycrystalline structures formed inside evaporating droplets of different biological fluids have been shown sensitive towards various influences, including ultra high dilutions (UHDs), representing so a new approach potentially useful for basic research in homeopathy. In the present study we tested on a wheat seed model *Zincum metallicum* 30c efficacy versus lactose 30c and water.**
Materials and methods: **Stressed and non-stressed wheat seeds were watered with the three treatments. Seed-leakage droplets were evaporated and the polycrystalline structures formed inside the droplet residues were analyzed for their local connected fractal dimensions (LCFDs) (measure of complexity) using the software *ImageJ*.**
Results: **We have found significant differences in LCFD values of polycrystalline structures obtained from stressed seeds following the treatments ($p < 0.0001$); *Zincum metallicum* 30c lowered the structures' complexity compared to lactose 30c and water. In non-stressed seeds no significant differences were found.**
Conclusions: **The droplet evaporation method (DEM) might represent a potentially useful tool in basic research in homeopathy. Furthermore our results suggest a sensitization of the stressed model towards the treatment action, which is conforming to previous findings.** *Homeopathy* (2016) **105**, 173–179.

Keywords: Ultra high dilutions (UHDs); Droplet evaporation method (DEM); Crystallization; *Zincum metallicum*; Wheat seeds

Introduction

Generally, experimental models applied in basic research regarding the efficacy of ultra high dilutions (UHDs) can be divided into physical and biological models. Physical models mainly concentrate on studies regarding water structure and how this structure gets modified following the subsequent dilution and succussion steps applied during the UHD preparation process.[1–4] Complementary, biological models[5–14] study the influence of UHDs on living organisms, and include *in-vitro* models involving e.g. cell-cultures[6,7] or plants[8–12] and *in-vivo* models involving animals.[13,14] In biological models, as an efficacy measure of the tested UHD different parameters get measured, serving for the comparison of the treated experimental conditions vs. control groups. In most cases the choice of such parameters depends on the experimental model and kind of organism it involves, as also on the tested UHD and its expected action on the organism. For instance, in *in-vitro* studies on basophils treated with UHD of

*Correspondence: Maria Olga Kokornaczyk, Department of Agricultural Sciences, University of Bologna, Viale Fanin 44, Bologna 40127, Italy.
E-mail: maria.kokornaczyk@unibo.it, Stephan.Baumgartner@uni-wh.de, lucietta.betti@unibo.it
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histamine, basophil activation has been found to respond to the UHD's action[7]; whereas in studies on a plant model involving duckweed treated with UHD of a plants growth hormone (gibberellic acid), growth parameters (e.g. the foliar surface) represented the proper parameter.[8]

Recently some studies appeared proposing polycrystalline structures formed during evaporation induced crystallization processes as a new parameter able to assess UHDs efficacy. It has been shown in two different plant models that UHD treatments have modified the polycrystalline structures obtained from evaporating solutions prepared from treated plants vs. controls.[15–17] In particular, in[15,16] wheat seeds, previously stressed with a ponderal concentration of arsenic trioxide (As_2O_3), were watered with arsenic trioxide 45th decimal dilution (As45x) or water as control; subsequently, the wheat seed leakage were analyzed by means of the droplet evaporation method (DEM).[18,19] During evaporation polycrystalline structures are formed, which complexity degree, measured by means of the local connected fractal dimension (LCFD), differed significantly between the As45x treated seeds and controls.

As reported in,[16] the LCFD of the polycrystalline structures showed to be also sensitive towards the number of strokes applied following each dilution step during the As45x preparation: As_2O_3 pretreated seeds watered with As45x treatments prepared with growing stroke numbers created polycrystalline structures characterized by significantly higher LCFD values. Furthermore, positive correlations found between the LCFD values and germination tests indicate that LCFD might be sensitive to the seeds vitality. In[17] instead, four days old cress seedlings watered with *Stannum metallicum* 30c or water as control were analyzed by means of the copper chloride crystallization method.[17,20–22] The experimentation was performed in two independent laboratories. The results showed that 15 second-order texture variables of the resulting crystalline patterns differentiated significantly the two experimental conditions.

In addition to plants, the copper chloride crystallization method may be also applied on blood. There are some older studies indicating that this method may revel differences in blood from a cancer patient with addition of low doses of plant extracts vs. the homeopathically untreated controls.[22] Theoretically, crystallization based methods, being applicable to a very wide range of biological substances and fluids[23–27] might be a useful tool in investigations of all kinds of biological models applied in basic research on homeopathy.

Other studies indicate that polycrystalline structures obtained by means of DEM can be used to study the water's structure[28] and the structure of UHD's,[29] and thus might also serve as an useful parameter for investigations performed on physical models. Since the choice to use polycrystalline structures formed during evaporation-induced crystallization as an outcome parameter does neither depend on the UHD's expected action nor on the specific model in use, polycrystalline structures might constitute a 'first line' parameter able to show if, generally, the UHD treatment has induced some kind of modification into the model.

All studies concerning the evaporation induced crystallization applied to test UHDs[15–17,27] and also studies with the copper chloride crystallization method applied to test food quality[19,20] reported on a strong day-factor influence upon the crystallization results. However, until now no plausible explanation of such a phenomenon is known.

Efficacy tests of *Zincum metallicum* UHDs applied on different models are the topic of many recent studies[30–35] performed within the International Research Group on Very Low Dose and High Dilution Effects (GIRI)[35] indicating on a wide application area of this preparation. Two studies performed on animal models showed that treatments with *Zincum metallicum* UHDs: (i) increased the survival rate and improved the clinical state of mice infected by *Trypanosoma cruzi*[31] and (ii) in a multigeneration study improved reproducibility and behavioral parameters in F1 mice generation in case of *Zincum metallicum* 200c, whereas *Zincum metallicum* 5c reduced the number of births.[32]

In a study performed with a physical model,[34] aiming at nanoparticle tracking during first dilution steps, the authors analyzed *Zincum metallicum* 2c and 3c in respect to lactose 2c and 3c and unsuccussed, undiluted water. The findings suggest that the modification in 100–300 nm particle concentrations caused by the dilution step from 2c to 3c was different in *Zincum metallicum* dilutions in respect to the lactose controls: in *Zincum metallicum* the average count of nanoparticles increased only slightly (from 4.7×10^8 in 2c to 5.1×10^8 in 3c particles/ml), whereas in lactose this difference was much bigger (1.3×10^8 in 2c to 7.5×10^8 in 3c particles/ml). However, a count of 4×10^7 particles/ml was also obtained in laboratory ultrapure water suggesting that careful controls must be applied in research on nanoparticle content of solutions.

In the present study we analyzed wheat seeds treated with *Zincum metallicum* 30c (Zm), lactose 30c (L), and undiluted unsuccussed water (C) by means of DEM. Since in our previous study[15] it was observed that there is a significant increase in the UHD action in the stressed model (compared to the non-stressed model), also in the present experiment we tested the treatments on both stressed (s-seeds) and non-stressed wheat seeds (ns-seeds). Moreover, the here reported experimentation inclined us to state a hypothesis on possible lunar influences on the crystallization process, which might contribute to explain the high significance of the day factor in this and similar studies.

Materials and methods

Wheat seeds

Whole, undamaged wheat seeds (*Triticum aestivum* L. cv. Inallettabile, harvesting year 2010) were used for the experiment. The distinction between s- and ns-seeds was based upon different storing conditions from the harvest until the analysis day (4 years): the ns-seeds were kept in controlled conditions at 5°C, under vacuum, and in the dark, whereas the s-seeds were kept in lab at varying temperature, humidity, and light conditions.

Treatments

In total three replicates of diluted and succussed treatments Zm (Zm1−3) and L (L1−3) were prepared. Zm1 and L1 were prepared starting from Zm and L 3c triturations (received from the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil; courtesy of Carla Holandino) by vertical mechanical succussions (DYNA HV1 by Debofar N.V.S.A Belgium) and following centesimal dilutions. Whereas, Zm2,3 and L2,3 were prepared departing from the Zm 28c and L 28c potencies remained from the preparation of the first treatment replicates: Zm1 and L1, respectively. For the preparation process polypropylene, cylindrical vessels (VWR international, Milan, Italy) of 150 ml capacity were used; the filling level was 100 ml (99 ml ultrapure water and 1 ml potency). Negative control (C) was undiluted, unsuccussed ultra-pure water. For the experimentation two C replicates (C1,2) were used: C1 coming from the water batch used for dilutions during preparation of Zm1 and L1, and C2 coming from the water batch used for dilutions during preparation of Zm2,3 and L2,3.

DEM

DEM experimental protocol is described in detail elsewhere.[15] In short: 5 undamaged wheat seeds were placed in an experimental tube and watered with 5 ml of treatment. The seed imbibition lasted 1 h; during this time the tubes were slightly mixed by hand twice, at the beginning of the imbibition and after 30 min. 3 μl droplets of the 1-h seed leakages were collected by a micropipette and placed on microscope slides (5 drops from one tube placed in one row). The slides were put into a thermostat and left for ca. 1 h for evaporation at controlled conditions at 25°C and UV light. The droplet residues were photographed by means of a CMOS microscope camera at dark field and in 100× magnification. One picture per droplet residue was taken; the area to be photographed was specified in order to embrace all polycrystalline structures formed within one droplet or their main part.

Experimental design

The experimental design is depicted in Figure 1. During one experimentation day all treatments (Zm1−3, L1−3 and C1,2) were tested on one seed lot (s- or ns-seeds) in three replicates (three test tubes filled with seeds per each treatment). The 24 test-tubes were letter-coded by a person not involved into the experimentation and treated as blind samples. From each test-tube 5 droplets were placed in one row on a 100 × 90 mm microscope slide. The different rows followed a completely randomized design. The experiment was repeated on 6 days within one month (s-seeds were analyzed on 10th, 11th and 24th February 2014 and ns-seeds on 12th, 26th February and 3rd March 2014). In total 720 patterns were obtained. The samples were decoded after pattern evaluation.

Pattern evaluation

The images of the polycrystalline structures obtained from the evaporating droplets were evaluated by means of the ImageJ software.[36] All images were adjusted to

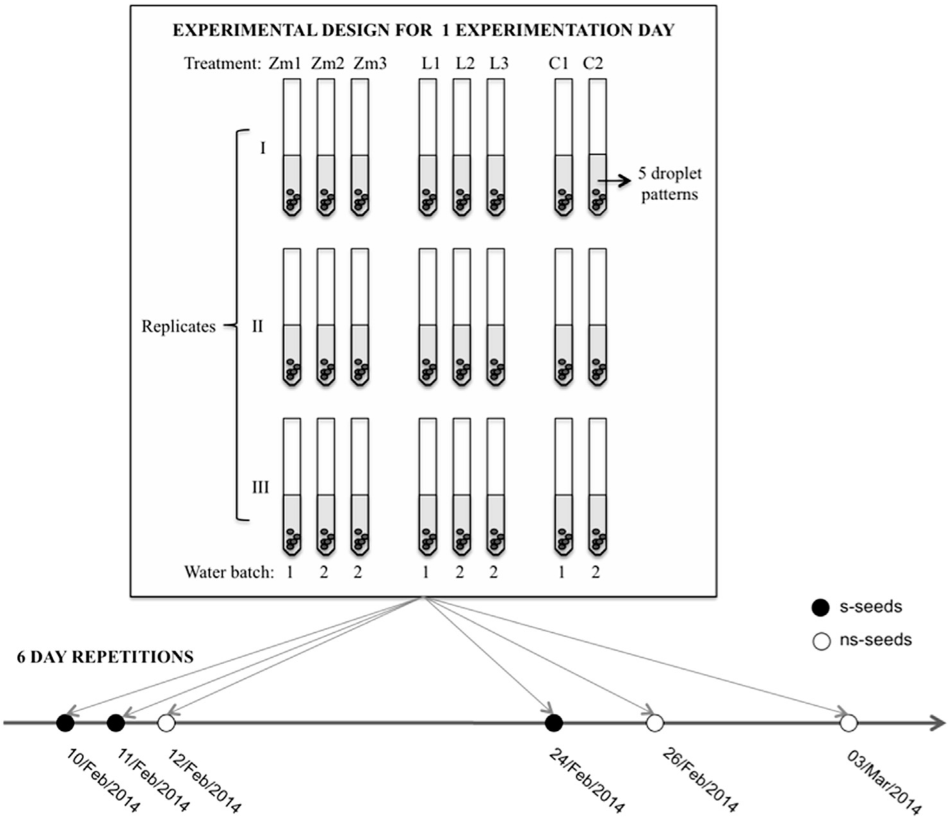

Figure 1 Experimental design. Graphical representation of the experimental design.

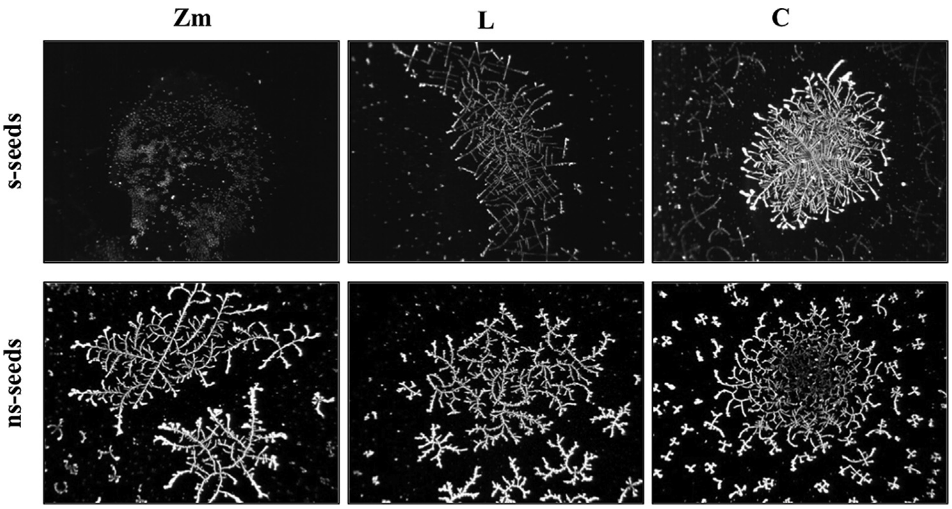

Figure 2 Polycrystalline structures. Examples of structures formed in droplets of wheat seed leakages prepared from stressed seeds (s-seeds) and non-stressed seeds (ns-seeds) treated with *Zincum metallicum* 30c (Zm), lactose 30c (L), and water control (C); magnification 100×.

500 × 375 pixels sizes, converted to binary and analyzed by means of the Frac-Lac plug-in[37] for their LCFD.

Data on moon position

Data on moon height with respect to the horizon (measured as angle horizon-position on Earth—moon), moon distance from Earth, and moon phase (percentage of illumination of the moon disc) for each analysis day was collected from the online tool Virtuelles Telescope[38] by introducing into the tool the longitude and latitude of experimental position (Bologna/Italy: 44°29′ N/ 11°20′ E), experimentation date, and time (on the beginning of the evaporation processes).

Statistical analysis

The dataset was analyzed by means of three-way analysis of variance (ANOVA) with independent factors (treatment, treatment repetition, experimental day) followed by the *post-hoc* multiple mean comparison with LSD test using the CoStat statistical software (v. 6.4, CoHort Software). In addition, the Bravais—Pearson linear coefficient of correlation r 1⁄4 (Cov(X,Y))/(SD(X)SD(Y)) was computed to determine the degree of association between the DEM data (LCFD) coming from the 6 experimental days and corresponding data on the moon position at the beginning of each evaporation process.

Results

Figure 2 depicts examples of polycrystalline structures formed inside the evaporated droplet residues obtained from s- and ns-seeds following treatments with Zm, L, and C (s- and ns-seeds were analyzed on different days). Within the s-seed row (Figure 2, upper row) it can be observed that: (i) the C pattern contains well formed and good visible polycrystalline structures, which create one connected structure placed in the middle, surrounded by cross-like, single structures; (ii) the polycrystalline structures visible in the L pattern appear darker and thinner, when compared to C, and the connected structure is surrounded only by single spots, and (iii) the Zm pattern example shows hardly any structures, it contains amorphous deposits, which do not form dendrites. Whereas within the ns-seed row (Figure 2 lower row), all three patterns examples appear very similar: all contain well-formed, complex, connected polycrystalline structures surrounded by cross-like, single dendrites.

Table 1 presents the p- and F-values of three-way ANOVA with independent factors treatment, treatment repetition, and experimentation day. As can be noted the factor treatment was highly significant only in the s-seed lot.

The results of the treatment influence on the overall LCFD mean values of both seed lots are depicted in Table 2. The LCFD means for the s-seed lot differed significantly ($p = 0.0000$) between each other and mounted to 1.33 (a) for C, 1.26 (b) for L, and 1.19 (c) for Zm, whereas in the ns-seed lot no significant differences were found ($p = 0.4124$).

As further shown in Table 1, regarding the treatment repetitions (Zm1—3, L1—3, and C1,2), Zm2 differed

Table 1 Results of F-tests of analysis of variance of the local connected fractal dimension (LCFD) values. Effects of treatment, treatment repetition, and experimentation day are given for the stressed seeds (s-seeds) and non-stressed seeds (ns-seeds). Significant values ($p < 0.05$) are bold

		s-seeds	*ns-seeds*
1. Treatment	*F-Value*	**12.86**	0.89
	p-Value	**0.0000**	0.4124
2. Treatment replicate	*F-Value*	**2.59**	1.95
	p-Value	**0.0254**	0.0842
3. Day	*F-Value*	**30.67**	**173.41**
	p-Value	**0.0000**	**0.0000**
1/3 Interaction	*F-Value*	**7.32**	0.87
	p-Value	**0.0000**	0.4807
2/3 Interaction	*F-Value*	**6.09**	1.08
	p-Value	**0.0000**	0.3746

Table 2 Mean values of the local connected fractal dimension (LCFD) of polycrystalline structures obtained from droplets of stressed and non-stressed seeds watered with *Zincum metallicum* 30c (Zm), lactose 30c (L) and water (C). Different letters indicate significant differences at p = 0.0000 (LSD)

	N	*s-seeds*		*ns-seeds*	
		LCFD	*SE*	*LCFD*	*SE*
C	90	1.33 (a)	0.02	1.28 (a)	0.02
L	135	1.26 (b)	0.02	1.25 (a)	0.02
Zm	135	1.19 (c)	0.02	1.28 (a)	0.02

Legend: N – number of patterns; SE – standard error.

significantly from Zm1 and Zm3 ($p = 0.025$) within the data concerning the s-seeds, whereas in the ns-seed lot no significant differences were found.

Finally, the day factor influence was strongly significant ($p = 0.0000$) in both seed lots. Moreover, in the s-seed lot, there were strong interactions between the day factor and treatment, as also between the day factor and treatment repetition.

When comparing the results obtained on the six experimentation days separately (Figure 3), in s-seed model Zm lowered the structures LCFD vs. C on all three analysis days, whereas difference between Zm and L (significance at $p < 0.05$) could be observed only on first and third analysis day (Figure 3a). For the ns-seeds no significant differences between the three tested treatments were observed in any of the three experimentation days.

As shown in Table 3 the mean LCFD values of polycrystalline structures obtained on the six experimental days from seeds treated with Zm, L, and C correlated significantly with the data describing the moon position and phase. In particular all correlations between the LCFD values and moon height in respect to horizon were positive, whereas those between the LCFD values and moon distance from Earth and moon phase were negative. In average strongest correlations were noted between LCFD values and moon height ($r = 0.91$) followed by those with moon phase and moon distance ($r = -0.86$ and -0.9, respectively). The data describing the moon position and phase created strong correlations also between each other (moon height and moon distance $r = -0.92$; moon height and moon phase $r = -0.98$; moon distance and moon phase $r = 0.97$).

Table 3 Values of the r Pearson coefficient of correlations between the local connected fractal dimension and foreground pixel values of polycrystalline structures obtained on six experimentation days from wheat seeds treated with *Zincum metallicum* 30c (Zm), lactose 30c (L) and control water (C) and data on moon height, distance and phase ($p < 0.05$)

	Zm	*L*	*C*
Moon height	0.97	0.91	0.86
Moon distance	−0.89	−0.76	−0.72
Moon phase	−0.97	−0.82	−0.80

Discussion

In this study we have found that polycrystalline structures formed by means of DEM applied to wheat seeds treated with *Zincum metallicum* 30c (Zm) revel differences when compared to controls (lactose 30c (L) and unpotentized water (C)) and thus may be considered a parameter able to verify treatment efficacy. In so far this result conforms to our previous findings where polycrystalline structures obtained by means of DEM showed to be sensitive towards As45x applied on a wheat seed model.[15,16] In the present study we did not compare the DEM results with any other analysis e.g. germination test, and so we can only state that Zm induced a change to the model, which lowered the LCFD and the area of the polycrystalline structures. In our previous studies,[15,16,18,19] when compared with the results of a growth- and germination-test, LCFD correlated positively with the seeds viability; in case of the present study the relation between LCFD and seed viability should be reconfirmed in follow-up studies.

Furthermore, as shown here, the Zm inhibiting action could be observed only in the stressed model. The increased response of stressed models towards UHD action has been also reported by other authors,[12] who concluded that the stress induces a pre-sensitizing effect on the model. Also in the case of our previous study[15,16] seeds pretreated with ponderal concentrations of As_2O_3 responded better to the As45x treatment than ns-seeds.

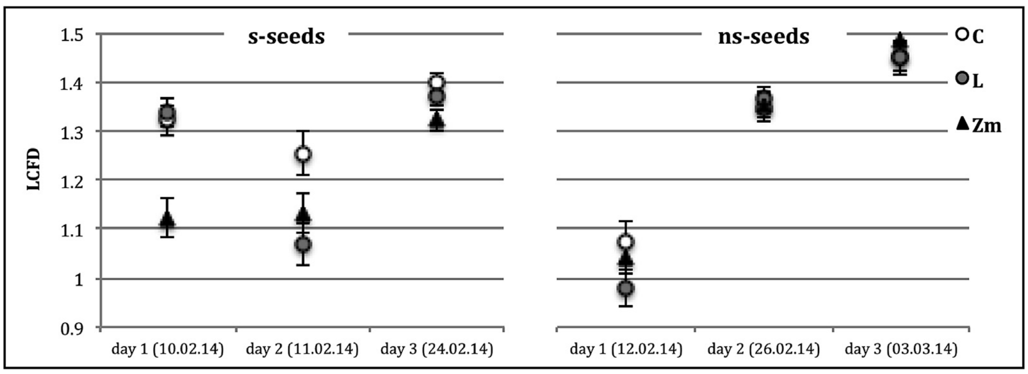

Figure 3 Results of the local connected fractal dimension (LCFD) analysis. Graphical representation of LCFD values of polycrystalline structures obtained on three experimentation days from stressed (s-seeds) vs. non-stressed (ns-seeds) wheat seeds treated with *Zincum metallicum* 30c (Zm), lactose 30c (L), and control water (C).

Concerning model robustness, as revealed by three-way ANOVA with independent factors (treatment, treatment repetition, experimentation day) the factor treatment repetition was significant only in the stressed model (Zm2 differed from Zm1,3) and on a significance level, which was much lower than the significance level of the treatment influence ($p = 0.0254$ vs. $p = 0.0000$). This result suggests that the treatment preparation process did not induce major differences to the treatments, as also that the formation of the polycrystalline structures from the treatment replicates was repetitive within the same experimentation day. The day factor influence instead proved to be highly significant ($p = 0.0000$) for both seed lots; moreover, as can be seen in Figure 3, the difference between the LCFD mean values from two different days could be even bigger than the difference between the differently treated conditions from same day.

We have previously reported on the significance of day factor on DEM outcome.[15,18,19] In other crystallization-based methods also, like the copper chloride crystallization, the day factor showed a highly significant influence on the results[17] also when applied to test the quality of foods and agricultural products.[20,21] We cannot explain why in controlled conditions the crystallization process shows such big variations between different experimentation days. In the present study, we observed strong correlations between the LCFD mean values of polycrystalline structures formed on different days and data on the moon height, moon distance from Earth, and moon phase collected at the beginning of each evaporation process; and were strongest for the moon height above horizon. As shown in Table 3 the data describing the moon position (moon height and moon distance from Earth) and phase created also strong correlations between each other; therefore further experimentation is needed to disentangle these data sets. Since DEM applied to a wheat seed model is not a standardized method, these correlations can be considered simultaneous, and not causal, however their strength gives good reasons for further studies.

The stress treatment applied in the present experimentation consisted in varying storing conditions; in case of ns-seeds (stored in controlled conditions) these conditions were known, whereas the s-seeds were exposed to varying, accidental conditions. Further studies on the identification of precise environmental stress-factors on seeds (e.g. exposure to varying temperature and/or relative humidity) would be interesting.

Other studies performed within the GIRI-Group[35] on *Zincum metallicum* UHDs report on the use of both, animal[30–32] and physical models.[33,34] To the best of our knowledge the present study is the first on *Zincum metallicum* UHDs applied on plant model.

Conclusions

The results of the present study seem to encourage further DEM experiments on s-seeds following UHD treatments. For further confirmation of the inhibiting effect of Zm on s-seeds, germination tests should be added. The correlations between LCFD values of the polycrystalline structures from DEM patterns and moon position should be studied in depth and monitored over a longer period of time. Such studies might have importance for the research on biorhythms.

DEM, being a time-saving and fairly economic approach with a wide range of possible applications (biological and physical models), seems to represent a promising tool for basic research in homeopathy. It might find useful applications as a screening method, for instance for the specification of the proper treatment in a given experimental model. The applicability of crystallization processes to studies on UHDs efficacy on different models will be addressed in further studies of our research group.

Conflict of interest statement

None.

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