Complementary Therapies in Medicine (2014) 22, 333—340

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Droplet evaporation method as a new potential approach for highlighting the effectiveness of ultra high dilutions

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Available online 24 February 2014

KEYWORDS
Droplet evaporation method;
Fractal dimension;
Fluctuating asymmetry;
Wheat seedling growth;
Arsenic trioxide;
Ultra high dilutions;
Polycrystalline structures

Summary
Objective: This study sought to verify whether the droplet evaporation method (DEM) can be applied to assess the effectiveness of ultra-high dilutions (UHDs). We studied the shape characteristics of the polycrystalline structures formed during droplet evaporation of wheat seed leakages.
Methods: The experimental protocol tested both unstressed seeds and seeds stressed with arsenic trioxide 5 mM, treated with either ultra-high dilutions of the same stressor substance, or with water as a control. The experimental groups were analyzed by DEM and *in vitro* growth tests. DEM patterns were evaluated for their local connected fractal dimension (measure of complexity) and fluctuating asymmetry (measure of symmetry exactness).
Results: Treatment with arsenic at UHD of both stressed and non-stressed seeds increased the local connected fractal dimension levels and bilateral symmetry exactness values in the polycrystalline structures, as compared to the water treatment. The results of *in vitro* growth tests revealed a stimulating effect of arsenic at UHD *vs.* control, and a correlation between the changes in growth rate and the crystallographic values of the polycrystalline structures was observed.
Conclusions: The results indicate that polycrystalline structures are sensitive to UHDs, and so for the first time provide grounds for the use of DEM as a new tool for testing UHD effectiveness. DEM could find application as a treatment pre-selection tool, or to monitor sample conditions during treatment. Moreover, when applied to biological liquids (such as saliva, blood, blood serum, *etc.*), DEM might provide information about UHD effectiveness on human and animal health.

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http://dx.doi.org/10.1016/j.ctim.2014.02.005
0965-2299/

Introduction

The droplet evaporation method (DEM) is based on the phenomenon by which particles suspended in fluids self-organize to form nano- and micro-structures during droplet evaporation,[1] and has been studied by researchers working in a variety of fields. The DEM has found applications in DNA/RNA[2–4] and protein microarray deposition,[5] DNA molecule stretching,[6] drug discovery,[7] inkjet printing,[8] and the manufacture of novel electronic and optical materials,[8,9] including thin films and coatings.[10] There have also been studies investigating possible applications of DEM in medical diagnostics.[11–18] As we have previously reported in detail,[19] pattern formation during droplet evaporation is a complex process, dependent on the phase transitions and different flow dynamics which occur during evaporation.[1,20–22] Those phenomena are in their turn dependent on conditions external to the droplet (*e.g.*, temperature, relative humidity, pressure),[7,20,23,24] as well as on conditions internal to the droplet (*e.g.*, liquid composition, viscosity).[23,25] However, for droplets that evaporate under the same external conditions, any variations in the phase transitions — and the resultant variations in patterns — will depend exclusively on differences in the droplets' internal conditions.[23] This sensitivity to liquid composition suggests a wide range of potential applications for DEM, for example as a tool for qualitative analysis of agricultural products and foods. Recently, our research team investigated using DEM for the quality analysis of wheat, and found that different wheat cultivars,[19] or different specimens of the same cultivar, some untreated and some treated with a chemical stressor,[25] show significant differences in their evaporation patterns. We also found that those differences correlated with the vigor of the analyzed seeds. In that study, the quality indicators which we used to objectively evaluate the patterns were: (i) the pattern complexity, measured as the local connected fractal dimension (LCFD) characterizing local variations in image complexity, and (ii) the bilateral symmetry exactness of the polycrystalline structures (PCS) expressed as fluctuating asymmetry (FA). Both those parameters have been previously shown[19,25] to correlate with seed vigor expressed as germination rate.

Our purpose in the present work was to evaluate whether DEM is a suitable tool for studying the effectiveness of ultra high dilutions (UHDs), a form of treatment commonly used in homeopathy and, in recent years, also in agriculture.[26] UHDs are prepared through a process of repeated dilution and rhythmic shaking (succussion), starting from a mother tincture. Dilutions are usually performed on a decimal (1:10) or centesimal (1:100) scale, designated with the letters x and c, respectively. The biological effectiveness of UHDs remains controversial: since their dilution levels are beyond the Avogadro limit, the probability that such ''solutions'' contain molecules of the original substance is close to zero.[27] Therefore, according to the conventional scientific paradigm of the ''molecule/receptor'' model, any biological activity is highly unlikely. To tackle this problem, experimental studies of high methodological quality have been carried out in different fields of basic research on UHDs, providing empirical evidence of specific UHD activity *in vitro*[28–30] and *in vivo*[31,32] and suggesting a possible mechanism of action.[33–36] These contributions hypothesize, based on physicochemical measurements, that the technique used to prepare UHDs (*i.e.*, repeated dilution and succussion steps) may cause structural alterations in the aqueous solvent which in their turn trigger the formation of molecular aggregates of water molecules.[37] In previous works[38–41] our group has demonstrated stimulating effects of arsenic trioxide (As_2O_3) UHDs on wheat seeds previously stressed (poisoned) with As_2O_3 5 mM. In these types of models, referred to as ''isopathic'', a biological subject is first poisoned with some agent in molecular concentration, and then an attempt is made to counteract the toxicity by applying a UHD of the same agent.[42,43] We adopted this approach because our previous research has always detected an ''isopathic sensitization'', meaning a notable increase in UHD effectiveness when working with seeds previously stressed with the same substance.

Recently, a crystallographic approach (biocrystallization method) has been successfully integrated into a seedling test system for UHDs, yielding results which show crystallographic structures to be treatment-specific.[44] The biocrystallization method, developed around a hundred years ago[45] and mostly used for quality analysis of foods and agricultural products,[46,47] involves the formation of evaporation-induced patterns from a watery extract prepared by mixing the analyzed sample with dihydrate copper chloride.

The aim of the present study was to investigate whether DEM might serve as a test to evaluate the effectiveness of As_2O_3 UHDs applied to both not-stressed and stressed wheat seeds (ns- and s-seeds, respectively); the stress consisted in pre-treating the seeds with As_2O_3 5 mM. In particular, we focused on the following research questions: (i) whether DEM patterns produced out of ns- and s-seeds show significant differences; (ii) whether the UHD treatment applied to both ns- and s-seeds may induce changes in DEM patterns and, if so, (iii) whether these changes reflect the seed viability revealed by the *in vitro* growth tests. For what concerns the pattern shapes, a further aim of this study was to investigate whether the same parameters, LCFD and bilateral symmetry exactness, already applied in our previous works,[19,25] are sensitive to seed viability changes due to UHD treatments.

Finally, we would like to underline that this study was carried out taking into account the present guidelines for basic research investigations with homeopathic remedies.[48]

Materials and methods

Plant material, classes of treatments and experimental protocol

Seeds of the common wheat (*Triticum aestivum* L.) cultivar ''Pandas'', from organic farming, were used after being selected for integrity and uniformity of size, shape and color. A portion of the seeds was stressed (s-seeds) by immersion for 30 min in an As_2O_3 5 mM water solution (As_2O_3, Sigma-Aldrich, Milan, Italy; H_2O p.a. Merck, Darmstadt, Germany), and then rinsed in tap water for 60 min, dried in ambient air until the seeds reached 12% moisture content, and stored in the dark at room temperature until use. This sub-lethal poisoning protocol was selected on the basis of previous

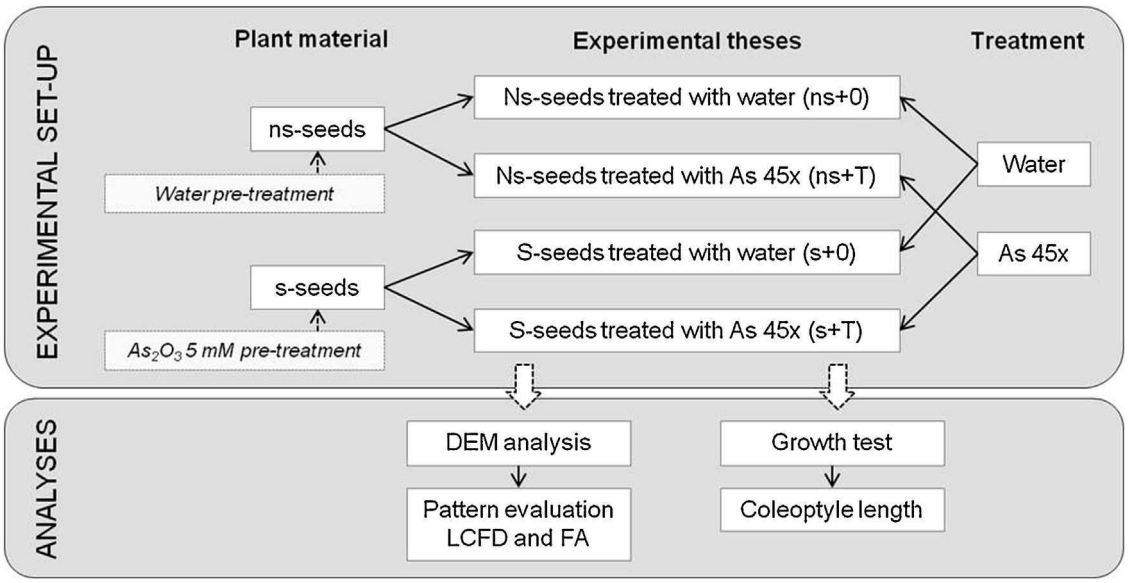

Figure 1 General experimental design.

experiments.[40,49] Non-stressed seeds (ns-seeds), used as a control, were pre-treated in the same way, but using distilled water instead of As_2O_3 5 mM.

The treatment classes for testing were:

- Distilled water (control, 0)
- As_2O_3 at the 45th decimal dilution/succussion level (As 45×, T).

Both test substances were freshly prepared from the same batch of water and supplied by Laboratoires Boiron, Sainte-Foy-lès-Lyon, France. The As 45× solution was prepared following the Hahnemann multi-vial-method in accordance with the European Pharmacopoeia, by a process of repeated serial dilutions at decimal scale (starting from a mother tincture of As_2O_3 0.01 M) followed by mechanical shaking (dynamization) at each dilution step. This procedure was continued until the 45× dilution/dynamization level was reached. To reduce microbial growth, Pyrex glass bottles were stored at a cool temperature (4 °C) until use. All treatments were letter-coded (blinded) by a person not involved in the experiments, and the codes were kept by independent people until the time of disclosure.

As shown in Fig. 1, the following four experimental groups were formed:

(1) ns-seeds treated with distilled water (ns + 0)
(2) ns-seeds treated with As 45× (ns + T)
(3) s-seeds treated with distilled water (s + 0)
(4) s-seeds treated with As 45× (s + T)

These four experimental groups were analyzed by both DEM and *in vitro* growth tests.

Droplet evaporation method and pattern evaluation

The experimental procedure, drawn from Kokornaczyk et al.,[19] is briefly described here. From each set of seed samples (s-seeds and ns-seeds) we prepared two sub-samples, each consisting of five entire kernels selected for integrity and uniformity of size, shape and color. The kernels were weighed, cleaned by rinsing them in distilled water, and placed in four test tubes. Two of the four sub-samples (one from s-seeds and one from ns-seeds) were watered with distilled water and the other two with As 45×, in w/v proportion 1:20. The test tubes were slightly shaken by hand and left at room temperature. After 1 h, leakage drops were collected using a micropipette, placed on a clean microscope slide, and allowed to dry in a thermostat at 25 °C and under UV light PHILIPS TL-D 18W BLB 1SL, Monza, Italy. The residues were then photographed under a dark field microscope MT4300H, MEIJI Techno, Saitama, Japan, with a connected CMOS Camera UK1175-C, EHD imaging GmbH, Damme, Germany, in QXGA (2048 × 1536) resolution, and magnifications 25× and 100×. The experiment was repeated four times to obtain a total of 240 droplet residues (2 samples, 2 treatments, 3 replicates, 5 droplet residues per replicate, 4 experimentation days).

The PCS were evaluated for their LCFD and FA using the software *Image J for microscopy 1.43 m*,[50] as described in detail in Kokornaczyk et al.[19,25] The evaluation was performed on pattern images in 100× magnification. The LCFD measurement was done on images converted to binary using the installed fractal analysis plug-in *FRAC-LAC 2.5*.[51] The analysis was designed to avoid any experimenter bias. FA, a parameter inversely correlated to bilateral symmetry (BS), was measured only on images containing structures that exhibited BS (BS structures; an example is depicted in Fig. 2). BS structures were preselected on the basis of clearly defined end-points of the first-order ramifications. Subsequently, in up to three randomly chosen BS structures per

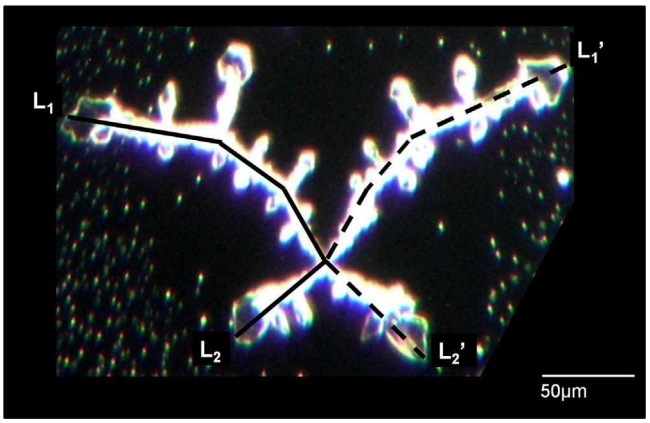

Figure 2 Example of a BS structure from an evaporated droplet of wheat seed leakage and the FA measurements: letters indicate the measured lengths of both symmetric branch pairs (L_1 and L_1', L_2 and L_2') (Kokornaczyk et al.[25]).

image, the first-order ramification lengths were measured with the segmented line tool: L_1, length of the upper left branch; L_2, length of the lower left branch; L_1', length of the upper right branch; and L_2', length of the lower right branch (Fig. 2). The FA value was calculated for each structure, as follows:

$$FA = \frac{|L_1 - L_1'|}{L_1 + L_1'} + \frac{|L_2 - L_2'|}{|L_2 + L_2'|}.$$

In vitro growth test

Following a procedure described in previous papers,[38,41] each of the seeds (280 s- and 280 ns-seeds) was attached to the top of a piece of filter paper (Perfecte 2-extrarapid, Cordenons, Pordenone, Italy) with clay (0.20 ± 0.05 g). The filter paper was inserted into a transparent polyethylene envelope (12 cm × 20 cm) which was in its turn placed in a larger black cardboard envelope, in such a way as to allow the shoots to develop in natural light and the roots in the dark. Each seed was treated by wetting the filter paper with 3.2 ml of distilled water or As_2O_3 45×. The cardboard envelopes (80 envelopes, 20 for each experimental group) were fixed, following a completely randomized design, to a wooden support (85 cm × 100 cm), positioned one beside the other, hanging vertically. The experiment was repeated 7 times and carried out in a glasshouse at a temperature of 20 ± 1 °C, in diffuse natural light, following a natural day—night rhythm. A total of 140 seedlings for each treatment was analyzed. The main variable considered was the shoot length on day 7 of growth, determined by scanning the whole plants (Epson Perfection 2480 Photo) and measuring the length of coleoptiles using the Assess 2.0 software.[52]

Statistical analysis

All data were processed by two-way analysis of variance; the separation of means was performed using Fisher's least significant difference test at a significance level of $p \leq 0.05$. Correlations between average shoot growth data and LCFD or FA were evaluated by *r* Pearson coefficient. To the statistical analysis the CoStat software (version 6.002, Cohort Software, Monterey, CA, USA) was used.

Results

All DEM patterns consisted of a border line (BL), a rather structure-free peripheral zone (PZ), and polycrystalline structures (PCS) placed in the middle zone (MZ) of the residue (Fig. 3). The centrally placed PCS were the only elements that varied, and so our analysis focused only on these structures. As can be seen in Fig. 4, the patterns obtained from ns-seeds treated with water (ns + 0) consist of ramified PCS surrounded by single spots. These PCS are all centered, and their complexity ranges from simple ramified structures to well-structured and complex ramification networks (Fig. 4a). For ns-seeds treated with As 45× (ns + T) we see that the complexity and regularity of the PCS structures is increased compared to those for ns + 0 (Fig. 4b). The s-seeds treated with water (s + 0) created only non-centered patterns with no PCS and a large number of separate spots; the patterns were partially dissolved and contained amorphous agglomerates in their MZ and PZ (Fig. 4c). For s-seeds treated with As 45× (s + T) we instead notice a partial recovery of the structured patterns, characterized by an increase in the amount, size and complexity of PCS with respect to s + 0 (Fig. 4d).

For the pattern evaluation (Table 1) we measured the LCFD on 60 images for each experimental group, whereas for the FA analysis we only considered those patterns that contained BS structures (these amounted to 201, or 84%, out of the total of 240 patterns). More specifically, the percentages of patterns analyzed for FA for each experimental group were: 96.7% for ns + 0; 95.0% for ns + T; 70.0% for s + 0; and 73.3% for s + T. As regards the effects induced by As 45× treatment, we found that both ns- and s-seeds (ns + T and s + T, respectively) showed a significant LCFD increase — denoting a significant increase in pattern complexity (Fig. 4b and d) — compared to the same seeds treated with water (ns + 0 and s + 0, respectively). The As 45× treatment also produced a decreasing trend in the FA values of the PCS, corresponding to a recovery of more exact bilateral symmetry, to a significant extent for s-seeds (s + T). The effects of stress with As_2O_3 5 mM, evaluated on the s + 0 group, amounted to a significant decrease in LCFD (reduced PCS complexity) and increase in FA (reduced bilateral symmetry) with respect to the ns + 0 group, as also shown in Fig. 4c. The two-way analysis of variance with independent factors treatment and experiment number (experimentation day) performed on LCFD and FA data showed no significance neither for the day factor nor for the interaction between treatment and experimentation day.

For what concerns the results of the *in vitro* growth test (Table 1), the shoot length of the s-seeds treated with water (s + 0) was significantly less than for the ns + 0 group, evincing the effect of the stress on seedling growth. Following the As 45× treatment, both ns- and s-seeds (ns + T and s + T, respectively) showed a significant shoot length increase compared to ns + 0 and s + 0, respectively. The two-way analysis of variance showed no significance for the day factor, whereas the interaction between treatment and experimentation day

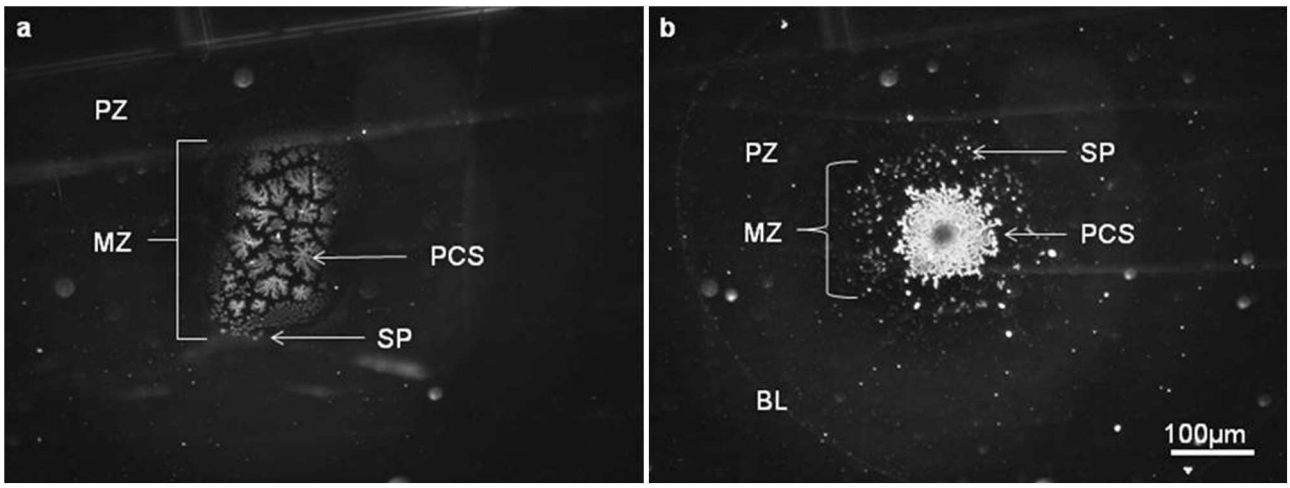

Figure 3 Examples of patterns from the droplet evaporation method: (a) a pattern with a non-centered polycrystalline structure, and (b) a pattern with a centered polycrystalline structure. BL: border line, PZ: peripheral zone, MZ: middle zone, SP: single spots, and PCS: polycrystalline structure.

resulted significant ($p < 0.01$) indicating that the experimentation day influenced the seedling growth. The results of the growth test correlated against the crystallographic values of DEM patterns: we found a positive correlation ($r = 0.91$) with LCFD, and a negative correlation ($r = -0.89$) with FA.

Discussion

First of all the conformation of DEM patterns obtained in this experiment coincided with the pattern-type we reported in our previous studies[19,25] and can be considered typical for wheat seed leakages. As far as the PCSs are concerned, partially dissolved patterns without connected structures (Fig. 4c) were obtained from s-seed leakages, reflecting the destructive effect of the arsenic treatment on seedling vigor. Conversely, the growth-stimulating effect of arsenic at UHD positively correlated with an increased complexity and bilateral symmetry exactness of both ns- and s-seeds (Fig. 4b and d, respectively). In our previous studies[19,25] we showed that complexity and bilateral symmetry exactness in DEM patterns prepared from wheat seed leakages reflected the seeds viability differences due to cultivar characteristics and chemical stress influence. The present study is consistent with our previous results and highlights that the same pattern characteristics (LCFD and FA) might be sensitive also to UHD treatment. The UHD effect was higher on s-seeds than on ns-seeds, confirming the ''isopathic sensitization'', *i.e.*, the notable increase in treatment effectiveness when applied to previously stressed seeds.[39,49]

It is worth pointing out that in the present experimentation undiluted/unsuccussed water was used as a control instead of dynamized water, as recommended in the REHBaR guidelines.[48] As demonstrated by Witt et al.,[53] during the succussions applied to the liquid in the dynamization process some trace-elements (*inter alia* Na, Si, Mg, Al, Cu, Rh, and Li) pass from the vessel walls into the liquid changing its composition in confront to the undynamized liquid. In accordance to this study the concentration of trace-elements is expected to be higher in dynamized As 45× than in undynamized water and the differences in DEM patterns could be ascribed to unspecific physicochemical alterations.[54,55] This aspect should be deeply investigated in follow-up trials. Nevertheless, in a pilot study[56] we examined by means of DEM the effects of As 45× and dynamized water 45×, prepared with a different number of strokes between the dilution steps, with respect to undiluted/unsuccussed water. The data showed that LCFD of DEM patterns take higher values for the As 45× in comparison to water 45× for all investigated stroke numbers. This result suggests that

Table 1 Results of the pattern evaluation and shoot length measurements for the four experimental groups.

Experimental thesis	LCFD			FA			Shoot length		
	N	Mean	CI	*N*	Mean	CI	*N*	Mean	CI
ns + 0	60	1.7 (b)	1.64—1.76	58	0.20 (c)	0.18—0.22	140	25.10 (b)	22.67—27.53
ns + T	60	1.8 (a)	1.70—1.90	57	0.15 (c)	0.11—0.19	140	31.40 (a)	27.83—34.97
s + 0	60	1.2 (d)	1.10—1.30	42	0.48 (a)	0.44—0.52	140	19.70 (d)	17.74—21.66
s + T	60	1.5 (c)	1.42—1.58	44	0.30 (b)	0.28—0.32	140	22.40 (c)	20.24—24.56

Different letters indicate significant differences at $p < 0.05$; LCFD local connected fractal dimension, FA fluctuating asymmetry, *N* number of samples, CI confidence interval 95%.

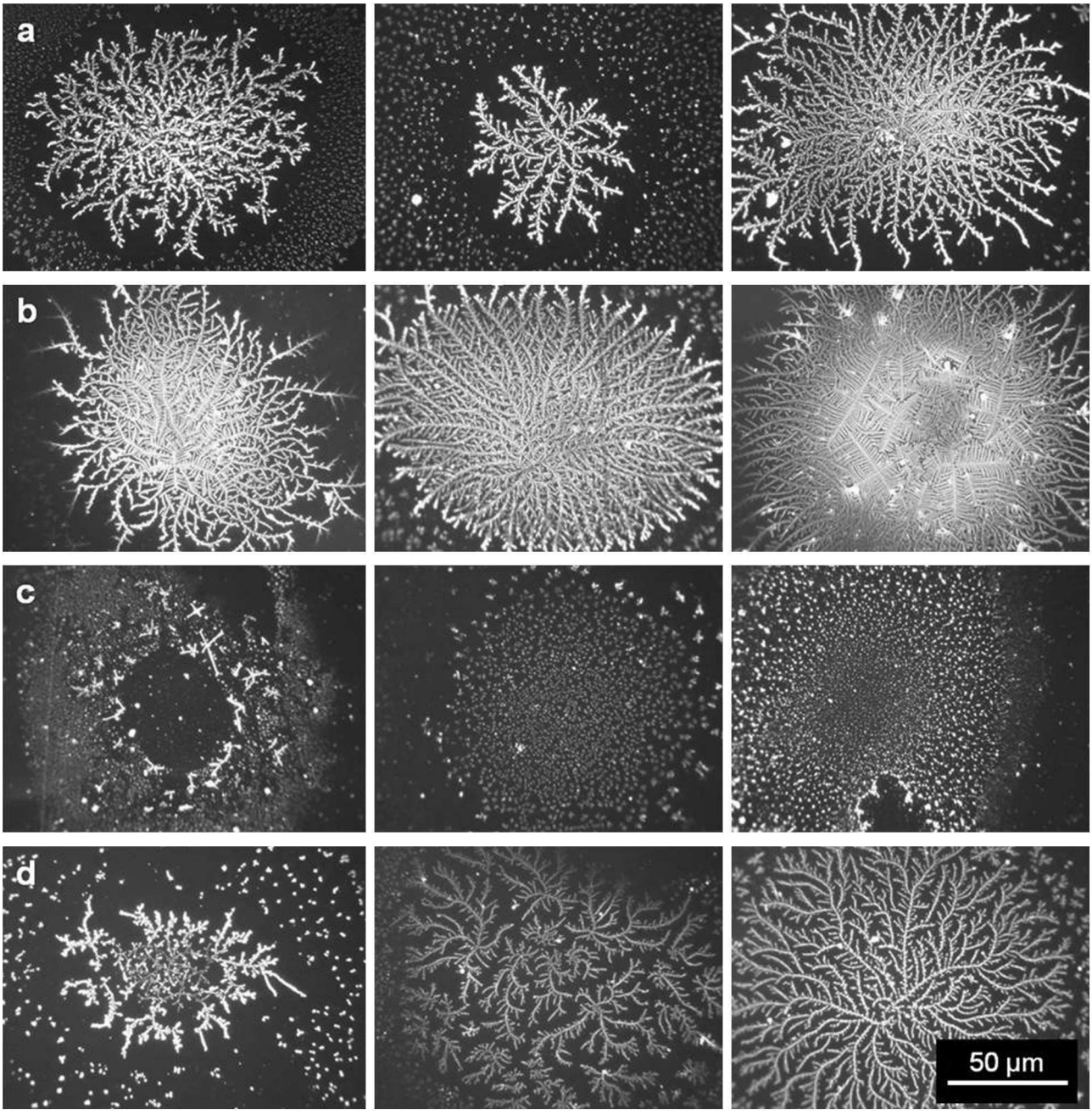

Figure 4 Examples of patterns from evaporated droplets. (a) ns-seeds treated with water, (b) ns-seeds treated with As 45×, (c) s-seeds treated with water, and (d) s-seeds treated with As 45× (magnification 100×).

the differences might be correlated to the action of the dynamized substance and not only to differences in ion content due to succussion.

Furthermore, the here presented method should be tested for its reproducibility, one of the main problems in research with UHD preparations, by repeated investigations with independently prepared homeopathic remedy production lots, different wheat cultivars, several seed harvest lots as well as different dilution/succussion levels. In order to assess the stability of the method, also laboratory external reproduction trials should be performed.

Conclusions

Based on the ability of crystallization methods to reflect sample quality by tracking differences in evaporation patterns, we have here for the first time used DEM as a complementary and rapid tool for evaluating UHD effectiveness. Our findings show that this method can be considered a promising approach to study the viability changes in wheat seeds due to chemical stress and UHD treatments, and we hypothesize that it might also be useful for testing the effects of UHDs on other crops. In fact, since DEM is

a simple and time-saving method, it could be used as a treatment pre-selection tool. Furthermore, because of its noninvasiveness, DEM could also be used to monitor sample conditions even before completing the treatment. Finally, DEM might be applied to biological liquids (such as saliva, blood, blood serum, *etc.*), to also provide information about the effectiveness of UHDs on human and animal health.

Conflict of interest statement

All the authors declare no financial/commercial conflicts of interest.

Acknowledgements

The authors would like to thank Demeter Italy for funding this research, and Dr. Antonello Russo and Dr. Edda Sanesi for their precious support and encouragement. Moreover, the authors wish to thank Laboratoires Boiron, Italy, and in particular Dr. Silvia Nencioni and Dr. Luigi Marrari, for their technical assistance. The sponsors had no influence whatsoever upon the design, conduct, evaluation and manuscript of this investigation.

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