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C. R. Krause
United States Department of Agriculture

C. C. Powell
Ohio State University

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MEASUREMENT OF FUNGICIDE SMOKE APPLICATION BY ELECTRON BEAM ANALYSIS

C.R. Krause[1,*] and C.C. Powell[2]

[1]United States Department of Agriculture, Agricultural Research Service, Nursery Crops Research Laboratory, Delaware, OH 43015

[2]Ohio State University, Department of Plant Pathology, Columbus, OH 43210

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Abstract

Low volume fungicide smoke particle deposits were studied with scanning electron microscopy and energy dispersive X-ray analysis. Under greenhouse conditions, particles deposited on *Rhododendron* leaf surfaces and onto inert, carbon planchets adjacent to *Rhododendron* leaves were characterized by electron beam analysis. Angular particles containing chlorine, identified as vinclozolin deposition, were quantified on a per unit area basis. Leaf samples collected from plants immediately adjacent to the smoke applicator had more particles than leaf samples collected elsewhere. More deposition was measured on leaf surfaces than on inert planchet surfaces. This study increased our knowledge about the effectiveness of using less fungicide to control plant disease and could increase consumer safety and reduce work hazard.

Key words: Particle deposition, *Rhododendron* spp., scanning electron microscopy, energy dispersive X-ray microanalysis, inert surfaces.

*Address for correspondence:
Charles R. Krause,
USDA-ARS, Nursery Crops Research Laboratory,
359 Main Road
Delaware, OH 43015
Phone number: (614) 363-1129

Introduction

Within the greenhouse industry, the need to reduce the cost of disease control measures has led to new fungicide application methods with greater overall effectiveness (8). Among these are: low volume hydraulic spray equipment which produces smaller droplet size (10), thermal pulse jet foggers (12), and self-dispersing smoke generators (8). All of the above seek to reduce the amount of active ingredient applied to target surfaces, thereby reducing the cost, worker exposure, product waste and environmental hazard. Little is known, however, of the behavior and fate of particles produced from these newer application techniques (8).

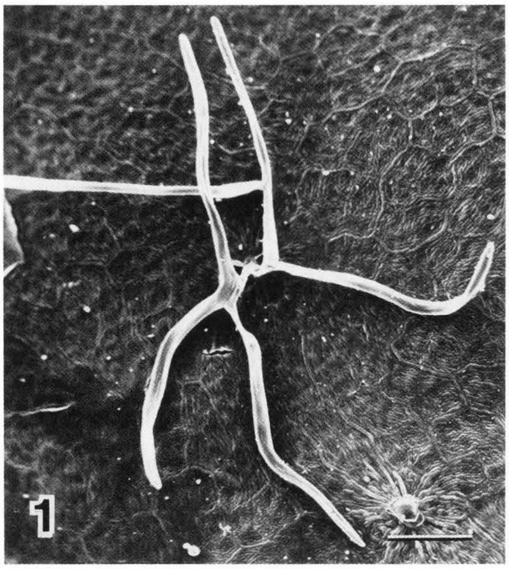
Electron beam analysis (EBA), a combination of scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDXA), has been used to characterize various particulate air pollutants such as fly ash (1), road deicing salt (4) and other atmospheric particles (5). Copper hydroxide particles were traced by Krause (6) on red maple (*Acer rubrum*) leaves using EBA. Krause and Powell (7,11) have used EBA to characterize vinclozolin particles from self-dispersing smoke generators. Vinclozolin is widely used to control gray mold, a serious disease of greenhouse and nursery crops caused by the fungus, *Botrytis cinerea*.

The present study was conducted to develop a basic understanding of the airborne transport and distribution of vinclozolin particle produced by a self-dispersing smoke generator onto foliage and inert surfaces in a greenhouse environment. The other purpose of the current study was to determine if inert surfaces could be used as an adjunct substrate for studies of particle deposition in place of plant surfaces. Since fungicide deposition studies are best done through the examination of hydrated plant surfaces, problems can be introduced due to vacuum and electron beam damage (3,7).

Materials and Methods

Plant Material

Rooted cuttings of *Rhododendron* spp. were grown in two adjacent greenhouse chambers of 20 x 20 x 10 m. The plants were watered as needed, and fertilized biweekly with nitrogen/phosphorus/potassium in a ratio of 20.8/8.6/16.8. Natural light intensity was supplemented with 26,000 lux from a high pressure sodium vapor source on a 16 hour photoperiod. Temperature was maintained at 24 ± 1 C (day)



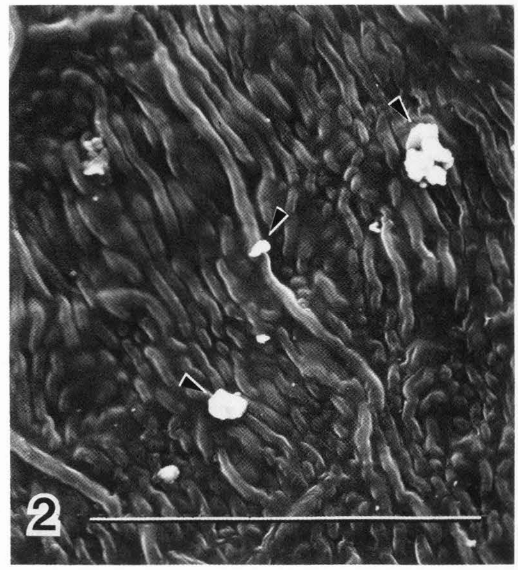


Fig. 1. Upper leaf surface of Rhododendron not exposed to vinclozolin smoke (control). Note multicellular trichomes and striated surface. Bar = 50 μm.

Fig. 2. Background particles (arrows) on control leaf. Bar = 5 μm.

and 21 ± 1 C (night). Prior to fungicide applications, test plants were located in the center (C) at each corner (northeast, NE; northwest NW; southeast, SE; southwest, SW) of the respective greenhouse rooms for a total of five plants per room. Next to each fully expanded leaf to be sampled (1/plant) an inert collection surface was positioned. The inert surface used in this study was a carbon planchet coated with graphite specimen adhesive attached to an aluminum stub (Ted Pella, Redding, CA). Graphite adhesive coating was used to provide a smooth substrate for particle deposition analysis. Stubs were placed in specimen boxes (Ted Pella, Redding, CA), supported on ring stands adjacent to the exposed leaves.

Fungicide Treatment

One of the greenhouse chambers was designated as the control chamber with no vinclozolin application. The other chamber was used as the treatment chamber into which vinclozolin was applied as a self-dispensing smoke at the rate recommended by the fungicide manufacturer. The self-dispensing generator used in this study consisted of a canister with a fuse that was ignited, releasing the vinclozolin smoke. Application was made at 4:00 pm with both fungicide treatment and control chambers having been sealed from air movement until the next morning (8:00 am). After 18 hours, leaf samples of plants that were not exposed to vinclozolin smoke were mounted on specimen stubs along with the corresponding inert surface (stubs with planchets). Samples from smoke-fumigated leaves were identically mounted on the corresponding inert surfaces.

Microscopy and Microanalysis

All specimens were carbon-coated in a vacuum-evaporator and examined with a Hitachi S-500 SEM at 20 kV. A TN-2000 (Tracor Northern, Middleton, WI) X-ray analyzer system was used to detect and characterize the presence of chlorine (Cl) in the vinclozolin molecule [3-(3,5-dichlorophenyl-5-ethenyl-5-methyl-2,4-oxazolidinedione)]. The plant wall and stubs were not exposed to any other chlorine source. Particles were examined in the raster mode with a raster size of 50 μm. The beam current was 100 mA and the dead time was 32-35 seconds. Each 50 μm^2 field required 20 to 25 minutes for EDXA.

The experiment was repeated four weeks later with previously unexposed Rhododendron spp. plants in the same greenhouse chambers. Data were collected on the numbers of vinclozolin particles per 50 μm^2 of area either directly dispersed on leaves or planchet surface placed adjacent to the corresponding leaves. Three 50 μm^2 areas of each sample, either stub or leaf, were analyzed.

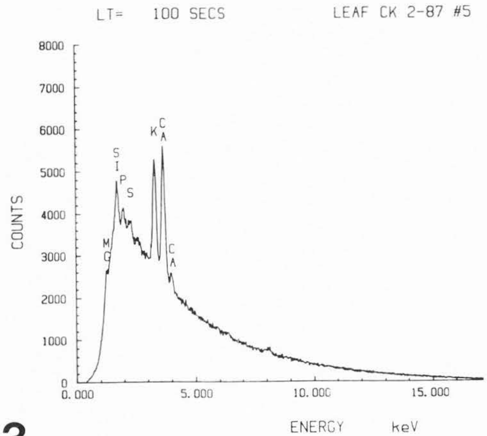
Statistical Analysis

Due to the highly non-normal nature of the experimental design, the standard analysis of variance was not appropriate. The odds ratio was the most accurate estimate of actual difference (2).

Variables of interest that were recorded included the number of particles detected per 50 μm^2 of surface area for both leaf and stub surfaces, location of specimen in chamber (C, NE, NW, SE, SW), and month of treatment (February or March). The logistic formula (9) was also reduced to check for the effect of location. Maximum likelihood estimates (expressed as the odds ratio) and comparison of these three models were made. The ability of vinclozolin smoke applicators to control disease was not tested in this study.

Results and Discussion

EBA of upper leaf surfaces on controls revealed multicellular trichomes and striated cuticular surfaces (Fig. 1). Background particles of various shapes and sizes (arrows) were observed on control samples (Fig. 2), and were identified as primarily containing silicon and calcium with EBA (Fig. 3). Stubs from the control chamber also yielded background particles of



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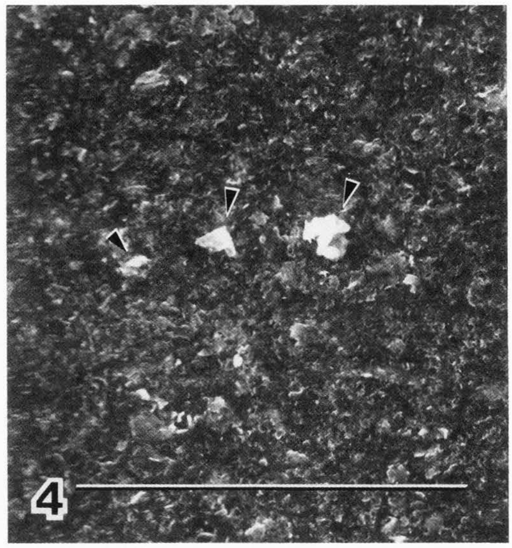

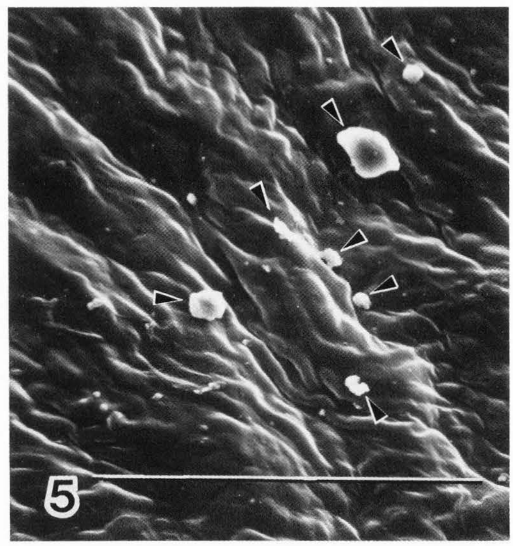

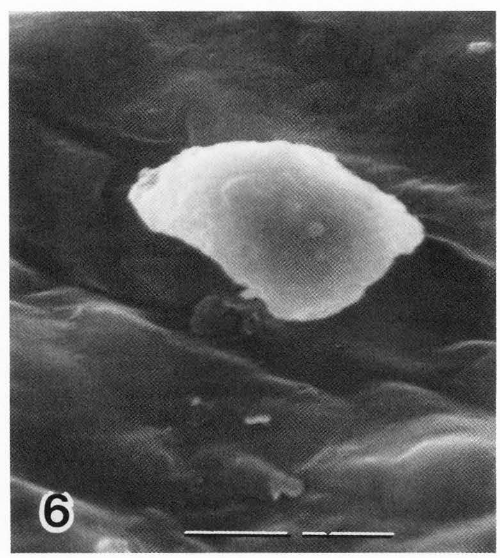

Fig. 3 (above). X-ray spectrum from leaf sample after control treatment; particles primarily contain Si or Ca.

Fig. 4 (right top). Background particles of Si or Ca (arrows) on control stub. Bar = 5 μm.

Fig. 5 (right center). Sharp-edged particles (arrows) identified as vinclozolin smoke deposition on an upper leaf surface. Bar = 5 μm.

Fig. 6 (right bottom). Vinclozolin smoke particle with agglomerated texture on leaf surface. Bar = 5 μm.

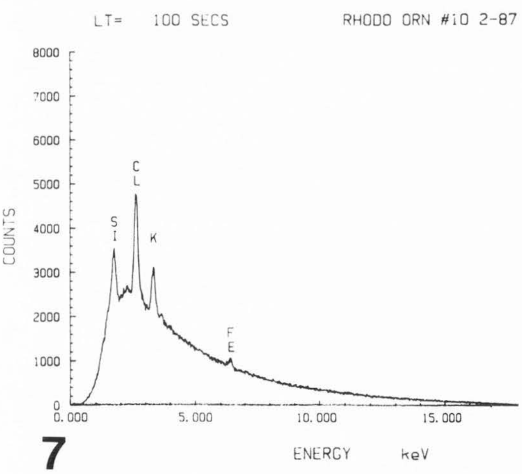

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Fig. 7. Representative EDXA spectrum of a vinclozolin smoke particle detected on a Rhododendron leaf.

various configurations (Fig. 4). Vinclozolin particles were not found on control surfaces.

Fungicide smoke particles were not macroscopically visible on leaves of treated plants. Sharp-edged particles, 1 to 15 μm in diameter, containing chlorine were identified as vinclozolin smoke deposition (Fig. 5, arrows) on treated leaves. Higher magnification revealed an agglomerated texture (Fig. 6). Fig. 7 is a representative spectrum of vinclozolin smoke particles, note the chlorine peak. Stubs collected from the center and four corners of the treatment chamber also yielded vinclozolin smoke particles (Fig. 8) of similar agglomerated appearance, size, and Cl composition to those detected on treated leaf surfaces as noted in Fig. 9.

The number of vinclozolin particles identified by EBA (SEM + EDXA), either on specimen stubs or on Rhododendron upper leaf surfaces according to location within the greenhouse chamber appears in Table 1. Since normal distribution was not observed, standard analysis of variance was not appropriate. An odds ratio was performed and yielded the following results. Categories of particle deposition were as follows: low = 5 particles per 50 μm^2; medium = 6 particles per 50 μm^2; and high = 7-8 particles per 50 μm^2. When vinclozolin particle deposition was compared among the five greenhouse locations, a location effect was observed (Table 2). The likelihood of location of stubs or plants having an effect was 100.05 with location (6 degrees of freedom, df) and 115.16 without location (2 df) with a Chi square value of 15.11 ($P < 0.01$). The leaf specimen from the center (C) plant location had more particles than leaf specimens from the perimeter locations NE, NW, SE and SW. The latter was probably due to the close proximity of the center location to the vinclozolin smoke applicator. While the latter observation may seem trivial, it has never been quantified with electron beam analysis or any other method. Uniform peripheral distribution was indicated since there were no differences between the other sites (NE, NW, SE, SW).

Table 1. Number of vinclozolin particles measured by electron beam analysis on various substrates.

Position in Greenhouse Chamber	Surface analyzed	Mean number of vinclozolin particles[a]
Center	Leaf[b]	7.33
	Stub[c]	6.33
Northeast Corner	Leaf	6.66
	Stub	6.00
Northwest Corner	Leaf	6.66
	Stub	5.33
Southeast Corner	Leaf	5.66
	Stub	5.66
Southwest Corner	Leaf	6.00
	Stub	5.66

[a]Each entry represents the average number of particles within three 50 μm^2 areas analyzed per sample.

[b]Rhododendron leaf (upper surface only).

[c]Specimen stub.

Analysis of particles detected on planchets compared to leaf tissue samples showed a difference with more deposition evident on leaf surfaces. Possible explanation of the above variation could relate to the leaf's uneven surface and the adhesive quality of leaf surface wax toward vinclozolin particles. The latter data prohibits the use of inert surfaces such as carbon planchets in lieu of actual plant surfaces for future studies.

The current investigation is a preliminary study. Automated EBA will be used in future studies to obtain more critical insight with exhaustive quantitation. Other areas of interest are the efficacy of smoke applicators related to deposition, the penetration of fungicide smoke particles within the greenhouse plant canopies, and time-course studies that impact on the safety of workers re-entering greenhouse following fumigation. EBA appears to be an extremely useful technique for detecting dispersal and could lead to more effective, safe and prudent use of pesticides.

Table 2. Vinclozolin particle deposition given as an odds ratio with resultant probabilities.

Variable	Relative Deposition (odds ratio)[a]	Probability
Stubs	1.00	NA
Leaf	6.54	< 0.01
February Application	1.00	NA
March Application	0.66	0.43
Center[b]	23.19	< 0.01
Northeast	3.31	0.11
Northwest	2.97	0.18
Southeast	1.68	0.53
Southwest[c]	1.00	NA

[a]Average of three 50 μm^2 areas per sample. An interpretation of the exponentiated beta coefficient as the odds ratio as an estimate of fungicide deposition based on three categories (low = 5 particles per 50 μm^2; medium = 6 particles per 50 μm^2; and high = 7-8 particles per 50 μm^2).

[b]Greenhouse location.

[c]Southwest was the reference location.

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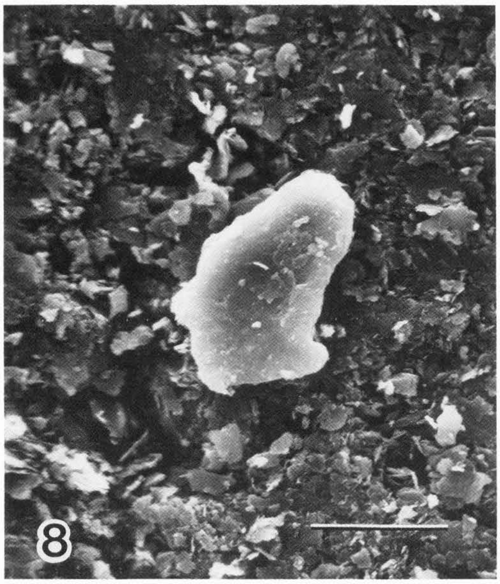

Fig. 8. Sharp-edged, agglomerated particles found on stubs adjacent to Rhododendron leaf when exposed to vinclozolin smoke. Bar = 5 µm.

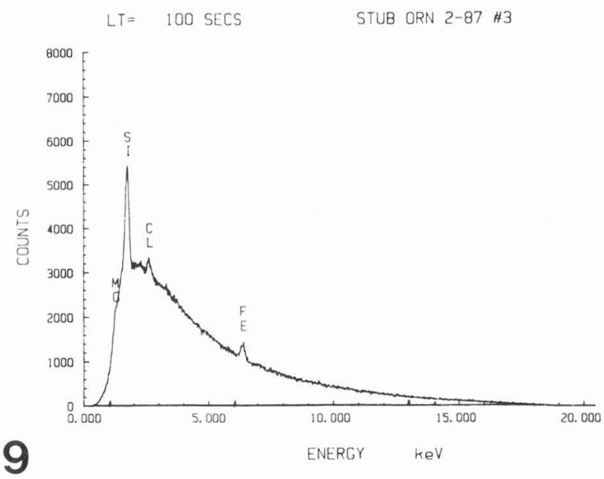

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Fig. 9. Representative spectrum of vinclozolin particles found on stubs. Note presence of chlorine.

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Discussion with Reviewers

Reviewer 4: Vinclozolin is detected by its chlorine signal. Since the leaf surface may also contain chlorine, are the authors sure that vinclozolin can be distinguished from its surroundings?
Authors: Since other chlorine sources were not present in the current study conducted under controlled conditions, we were sure that chlorinated particles present were vinclozolin. In future studies under actual greenhouse operations, where chlorine sources could be present either in particles on plant surfaces or within the leaf tissue, unique vinclozolin crystal characteristics (e.g., shape and size), will be used along with EDXA for unambiguous identification.

J.N.A. Lott: The authors mention that chlorine is diagnostic for this fungicide, yet Fig. 9 has very little chlorine!
S.L. Flegler: Why is the chlorine peak so small in Figure 9? If this spectrum is from a vinclozolin particle on a carbon stub, I would expect it to be higher. Are the net counts in the peak greater than three times the square root of the background beneath the peak?
Authors: The chlorine peak was so small because the relative amount of chlorine present in the formulation of vinclozolin was small. Even though the net counts for the chlorine peak in Figure 9 were not three times greater than the square root of the background, we feel that this was an authentic vinclozolin spectrum for this formulation. Semi-quantitative analysis on each particle was not practical.

J.N.A. Lott: What was the source of the large silicon, potassium, and iron peaks shown in Figure 9?
S.L. Flegler: There was an iron peak evident in all three spectra. Is this a system peak? Why do you

have the very large silicon peak in Fig. 9?
Authors: While the information about the formulation of the vinclozolin smoke applicator was of a proprietary nature, we feel that a possible source for Si, K and Fe, that occur in Fig. 9 could be the propellant portion of the formulation. The iron peak that appear in all three spectra could have originated from contamination within the greenhouse chambers even though we attempted to control such background sources. The large silicon peak present in Fig. 9 could be the part of the fungicide formulation.

S. Flegler: No mention is made of cryo-fixation, although the authors have used this method in past studies. Were these specimens carbon coated and examined in the frozen-hydrated state? If the specimens were examined in the wet hydrated state, how were the severe vacuum artifacts avoided? From my experience, it is possible to examine wet hydrated specimens in a SEM. However, the operator usually has only a few minutes of viewing time before severe specimen distortion becomes evident. I am especially curious about how the wet hydrated specimens could withstand the normally lengthy vacuum evaporation process for carbon.
Authors: The samples were neither cryo-fixed nor examined in the frozen hydrated state. We believe that the artifacts are avoided in the specific leaf we selected as a test genera (Rhododendron leaf), because of its thick cuticle, heavy cell walls, and other physiological characteristics not yet studied.

I.B. Sachs: What was the effect of the smoke on the underside of the leaf and stomata?
Authors: We have not studied the underside as yet and plan to investigate fungicide coverage on both sides during future studies.

G.M. Roomans: Were there no areas with less that 5 or more than 8 particles per μm^2?
Authors: No. The actual counts did not have any variation from the data given in Table 1. The current study indicated that self-dispersing fungicide smoke applicator used in this experiment produced a very constant number of particles all over the greenhouse chamber under the above experimental conditions. The chief limiting factor with any space fumigant system, such as self-dispersing smoke applicators, was complete restriction of air movement within the treatment area.

Reviewer 4: What significance do you attach to the results for February and March included in Table 2?
Authors: The analysis showed that no important differences existed due to the month of application. A check was necessary to confirm expectations of uniformity over slight environmental differences.

Reviewer 5: Why would one expect a monthly variation in deposition level? Isn't deposition level a function of the smoke generator and the frequency / duration of its use?
Authors: Temperature and light intensity can vary in greenhouse operations between February and March within the temperate climatic zone that the experiment was conducted. While greenhouses allow culture of plant during extremes of external climatic conditions, chambers do operate with some degree of ambient variation in terms of temperature and light. These variations could alter the dispersal of space fumigants. In addition to the frequency and duration of use you mention, deposition level also depends on temperature, humidity, air movement, formulation of the smoke applicator, etc.

J.N.A. Lott: The results you present (Fig. 5) show great variation in size of the fungicide particles. In terms of delivery of vinclozolin to a leaf, the mass of fungicide transferred could be more important than the number of particles. Is the number of particles per unit area or the mass of fungicide delivered most important in stopping growth of the pathogen? Do you have any data as to the size distribution of particles at your sample locations?
Authors: The total volume of fungicide delivered per unit area of leaf is likely to be an important factor in disease control. We do not have any data addressing this point. We are currently using particle recognition and characterization programs to determine particle size variation and intend to publish that soon.

J.N.A. Lott: I suspect there may have been cases where several particles were clumped together. How were such particles scored?
Authors: Clumped particles were counted as one particle regardless of the size of the clump.