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Methods to sample air borne propagules of *Aspergillus flavus*

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Abstract

Three techniques (cyclone samplers, filter samplers and rotorods) were evaluated for sampling airborne propagules of *Aspergillus flavus* in a cultivated region of southwest Arizona. Analysis of variance indicated no significant difference between cyclone and filter samplers in quantity of colony forming units caught, but there was a positive correlation between catches of the three separate impaction samplers ($r = 0.84$–0.99, $P > 0.05$). There was no detectable correlation between impaction sampler catches and cyclone catches ($r = 0.12$–0.33; $P > 0.05$). Cyclone samplers collected a dry sample that was easy to process for quantification of fungal propagules. Size of conidia of *A. flavus* (combined with filter retention studies) suggests that the predominant propagules caught by the cyclone sampler were conidia, rather than sclerotia or infected vegetative matter. Using a water-soluble coating, rotorods collected viable conidia of *A. flavus* under controlled environment conditions, but not in the field, although viable propagules of other fungi were caught, including other *Aspergilli*. In the desert environment the rotorods became overloaded with particles of dust if operated for more than 2 h. Where isolate culture is required, cyclone samplers are ideal for collecting airborne *A. flavus* propagules in dry climates.

Air-dispersed propagules of *Aspergillus flavus* can infect and colonize many agricultural products, including cottonseed and corn (Diener et al., 1987), which can lead to contamination with potent carcinogenic aflatoxins. To help reduce the risk of toxin entering the food chain, aflatoxin content is strictly regulated by law (van Egmond, 2002). Appropriate methods to sample bioaerosols in field conditions (McCartney et al., 1997) is necessary for pathogen detection and quantification. Some sampling of airborne propagules of *A. flavus* has been done (Holtmeyer and Wallin 1981; Lee et al., 1986; Silas et al., 1986; Olanya et al., 1997; Bock et al., 2005). These studies used a range of samplers (Andersen samplers, all glass impingers, Millipore aerosol samplers, rotorods and Hirst-type 7-day samplers and Burkard cyclone samplers), and sampling periods ranged from a few hours to one week or more. The size and shape of conidia of *A. flavus* (spherical, < 10 μm in diameter) makes them difficult to differentiate from similar fungal spores based on morphology. Identification is based on species- and strain-specific colony morphology, colour and other characteristics (Wallin and Loonan, 1976) and Vegetative Compatibility Group testing (VCG) (Cotty, 1989). We evaluated three methods (cyclone, filter and rotorod samplers) that allowed culture-based quantification. The advantages and disadvantages of these methods are discussed and the results compared to previously published methods.

All samples were suspended in sterile distilled water (SDW), serially diluted and plated onto media in 0.1 ml aliquots. Colony forming units (CFUs) of *A. flavus* were counted on modified rose bengal (MRB) agar (Cotty, 1994), and CFUs of *A. niger* and other fungi were counted on 5/2 agar (Cotty, 1989). The 5/2 agar was amended with streptomycin (0.05 g l^{-1}) and chloramphenicol (0.05 g l^{-1}), both added after autoclaving. All plates were incubated at 31 °C for 5 days. Propagules m^{-3} air was calculated from the CFU data and volume sampled.

In the first set of experiments a Burkard cyclone sampler (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK) was operated alongside filter samplers (pore size 0.8 μm, Millipore Corporation, Bedford, Massachusetts, USA) to compare efficiency of sample catches (all samplers were within 3 m of each other). Air pumps (12 V DC, P/N MTR1002, Gast Manufacturing Corporation, Benton Harbor, Michigan, USA) were used to draw air through the filter samplers and were operated at 16.5 l min^{-1}. Sampling orifices were horizontal and 1.2 m above ground level and placed approximately 0.5 m apart. Cyclone samples were suspended in 0.5 ml SDW. Filters were removed from housings and washed three times serially, each in 2 ml SDW. Propagules were quantified by dilution plating (washed filters were also placed face-up on agar). Comparisons were made on three dates. For each date, three repeat samples of 2 h each were taken consecutively and treated as replicate samples for each day. One cyclone sampler and three impaction samples were operated simultaneously for each experiment. Samplers were centred on four 16 ha fields planted to cotton and irrigated at Roll, in the Mohawk Valley, a cultivated desert region of southwest Arizona, unless stated otherwise. This location has less than 5 cm of rain per annum.

Particle sizes collected by the cyclone sampler were measured (Olympus X100 S Plan, dry objective, ocular micrometer) for seven independent samples. The sample was suspended in SDW and an aliquot was mounted under a cover slip. The first 100 particles observed during an 80 μm wide transect across the slide were measured (length×width). In a similar manner, conidia from 7 day old cultures of *A. flavus* (AF36) grown on 5/2 agar were measured to compare to the size range of the particles being collected by the cyclone sampler. The length and width of both fresh conidia suspended in SDW, and conidia dried in an oven at 50 °C for 4 h were measured. Dried conidia were dusted directly onto slides (conidia were dried in an attempt to simulate dehydration of conidia in desert environments).

Filter membranes were used to further characterize the size of cyclone-collected propagules of *A. flavus* and to investigate whether they had dimensions of conidia (~10 μm diameter), sclerotia (~50–1000 μm diameter), or fragments of infected organic matter with viable mycelium (probably >20 μm diameter). Two additional samples were collected at the same location by the cyclone sampler and suspended in 5 ml SDW and repeatedly filtered through polycarbonate membranes (Poretics Corporation, Livermore, CA) with decreasing pore size (20, 12, 5, and 3 μm). These filters are perforated with precisely sized pores and have sharp cut-off retention characteristics (Ballew, 1997). Proportions of *A. flavus* propagules passing through each filter were determined by dilution plating. This test was performed twice with independent samples.

The ability of rotorod samplers (Perkins, 1957) to collect *A. flavus* propagules was tested. Rotorods were coated by hand with a water-soluble formulation composed of carboxy-methyl cellulose (CMC, 14 g), glycerol (20 ml) and water (40 ml), which formed a stiff, tacky grease that spread evenly on 0.1 cm diam quartz glass collecting rods (Friedrich and Dimmock Glassworks, Millville, New Jersey). Glass rods (2.5 cm) were used as the coating coalesced on rods composed of plastic or metal. *In vitro* tests at room temperature indicated no loss of viability by conidia of *A. flavus* in CMC over 14 days (data not shown), and sampling efficiency of the CMC–glycerol coating was compared with silicone grease by operating the rotorods in biological safety cabinets while gently tapping an inverted agar culture to dislodge conidia of an atoxigenic strain of *A. flavus*. On each rotorod, one rod was coated with CMC, the other with silicone grease. The glass rods (16 cm apart) were placed in 1 mm holes drilled in the ends of the plastic tube of a ballpoint pen. Locking nuts were glued on the end and used to hold the rods in place and a lock nut attached the middle of the rigid plastic tube to the motor spindle (12 V DC electric motor, #2M197, Dayton Electric Manufacturing Company, Niles, IL). The motor rotated at 3000 rpm. The number of

conidia sampled was counted on 15 replicate transects across each rod using a microscope (300×).

For field sampling the rotorod motors were supported within 10 cm (internal diam) polyvinylchloride pipe joints. Three motors were positioned by using a four-way pipe split with 50 cm sections of tube placed in the horizontal outlets and motors placed in the open ends of the pipes. Thus each experiment had three replicates. The base of the piping was buried in the ground for support. Sampling period was investigated by operating the motors for 2 and 4 h periods and was performed twice. Four samples (two replicates each) were also taken at four different locations within a 4 km area over a 2-day period (sampling period 2 h). The glass rods were removed and both placed in a single microfuge tube, 0.5 ml SDW was added and the sample vortexed to redissolve the CMC coating and suspend any fungal propagules. The sample was subject to dilution plating on MRB agar and amended 5/2 agar. CFUs were counted and the number of CFUs m^{-3} air was calculated.

Data were analyzed using Statistica V3.0 (Statsoft, Inc., Tulsa, OK) and SAS V8.0 (SAS Systems, Cary, NC). An analysis of variance (SAS PROC GLM) was used to investigate the relationship between the samples collected by the Burkard and the filter-impaction samplers with main effects of sampler and day and their interaction. Correlation analysis was used to further investigate the relationship between the catches by the cyclone and impaction samplers (independent variables of sample date and sampler). Prior to analysis the data were log transformed to reduce the heterogeneity of variance among catches. The total numbers of conidia and numbers of conidia encounters sampled by rotorods coated with silicone grease or CMC was analyzed with a *t*-test assuming independent samples. Correlation analysis was also used to compare the counts of conidia sampled in various replicates using the two coatings.

In the first experiments both the cyclone and the filter-impaction samplers consistently collected propagules of *A. flavus* (Table 1). There was no

Table 1. The quantity of *Aspergillus flavus* propagules in the air sampled either by [a]filter impaction samplers or by a Burkard cyclone sampler in the Mohawk Valley, Arizona, 1998

[b]Date	Rep	CFU m^{-3} (LOG CFUs m^{-3})				
		[c]Cyclone	Filter samplers			Date means
			1	2	3	
June 17	1	67.6	67.7	17.3	15.8	34.0 (1.24)
	2	16.5	1.6	2.0	3.1	
	3	67.6	12.7	64.5	71.8	
	Mean	50.6 (1.63)	27.3 (1.05)	27.9 (1.12)	30.2 (1.18)	
November 18	1	7.6	857.6	187.5	173.6	429.8 (2.40)
	2	135.5	607.8	750.2	1414.2	
	3	165.8	224.3)	327.6	305.7	
	Mean	103.0 (1.74)	563.2 (2.69)	421.8 (2.56)	631.2 (2.63)	
November 19	1	713.8	144.4	213.0	–	170.0 (2.03)
	2	194.4	70.0	60.2	64.9	
	3	258.4	56.7	47.2	47.0	
	Mean	388.9 (2.52)	90.4 (1.92)	106.8 (1.93)	56.0 (1.74)	
	Sampler means	180.8 (1.96)	227.0 (1.89)	185.5 (1.87)	262.0 (1.85)	
[d]SED sampler means	0.172					
SED date means	0.149					
SED sampler*date means	0.298					

[a]ANOVA results for samplers type $F = 0.08$, $P = 0.97$ and for sample day $F = 15.75$, $P < 0.001$.
[b]Each replicate of each sampler is a single measurement taken over 2 h.
[c]Values in parentheses are log transformations.
[d]SED = standard error of the difference.

significant difference between the sampler catches (df 3, $F=0.08$, $P=0.97$), although significantly different numbers of CFUs were collected between tests on different days (df 2, $F=15.75$, $P<0.001$). The quantity of conidia sampled varied substantially with both replicate and day of sampling with both the cyclone and filter impaction samplers. Correlation analysis showed that the numbers of *A. flavus* propagules collected by the filter samplers were positively correlated with each other (0.84–0.99, df3, 33 $P<0.05$). Samples collected by the Burkard were not significantly correlated to samples trapped by any of the filter-impaction traps (0.12–0.33, df3, 33 $P>0.05$).

Particles most commonly sampled by the cyclones ranged in size from 1 to 2.5 μm (Figure 1). Particles >100 μm were occasionally observed under microscopic examination (plant and insect debris, dust and sand). Suspensions of the cyclone samples passed through Poretics filters indicated that most propagules of *A. flavus* were within the range expected for conidia (Figure 2). Few propagules passed through 5 μm pores, but the number of propagules passing through 12 μm pores was not significantly different from the number in unfiltered controls. Fresh conidia of *A. flavus* were spherical (3.2–5.2 μm diam), while oven-dried conidia were ovoid and crumpled in shape (2.4–5.6 μm$\times$1.76–3.6 μm).

There were no significant differences in the quantity of spores collected by the rotorods coated with CMC–glycerol and those coated with silicone (Figure 3a, means of 108 and 122 conidia/slide transect, respectively; t-value $=-0.36$, df 14, $P=0.38$) or conidial clusters between the two coatings (Figure 3b, both had a mean of 12 encounters of conidia or clusters of conidia). The number of conidia sampled by CMC–glycerol or silicone was positively correlated ($r=0.95$, df 14, $P<0.001$ and $r=0.91$, df 14, $P=0.002$ respectively) with the number of encounters. Cluster size of conidia ranged from one conidium to clusters of more than 10 on both surfaces (Figure 3c).

In field tests the 4 h sampling period collected fewer fungal propagules m^{-3} than the sum of the consecutive 2 h sampling periods, suggesting that overloading of the rotorods occurred after 2 h sampling resulting in reduced sampling efficiency, and microscopic observation of the rods after 2 h operation showed a dense cover of particles. Although propagules of various fungi (*Alternaria*, *Cladosporium* and *Aspergillus* spp.) were consistently sampled in the field, *A. flavus* was not detected. *Aspergillus niger* was consistently sampled.

Burkard cyclone samplers, filter-samplers and rotorods all sampled propagules of fungi in the cultivated desert regions of southwest Arizona. The cyclone sampler, when operated for periods of 2 h, consistently sampled viable propagules of *A. flavus*, and Bock et al. (2005) showed that they could operate continuously for at least 1 week. The cyclone can handle large quantities of dust, limited only by catch bin size, while filters could

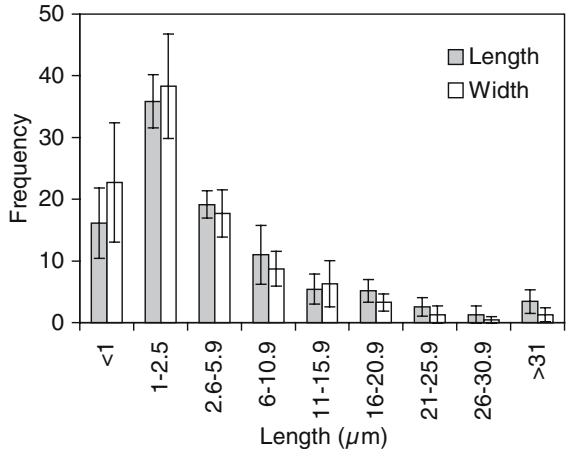

Figure 1. Frequency of different size particles sampled by the Burkard cyclone sampler. Standard deviations of the means are indicated.

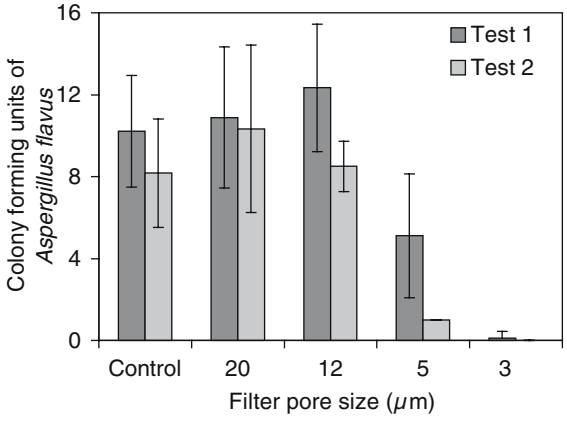

Figure 2. The number of propagules of *Aspergillus flavus* m^{-3} of air sampled by the Burkard cyclone sampler that pass through membrane filters with different size-retention cut-off characteristics. Control is unfiltered sample. Standard deviations of the means are indicated.

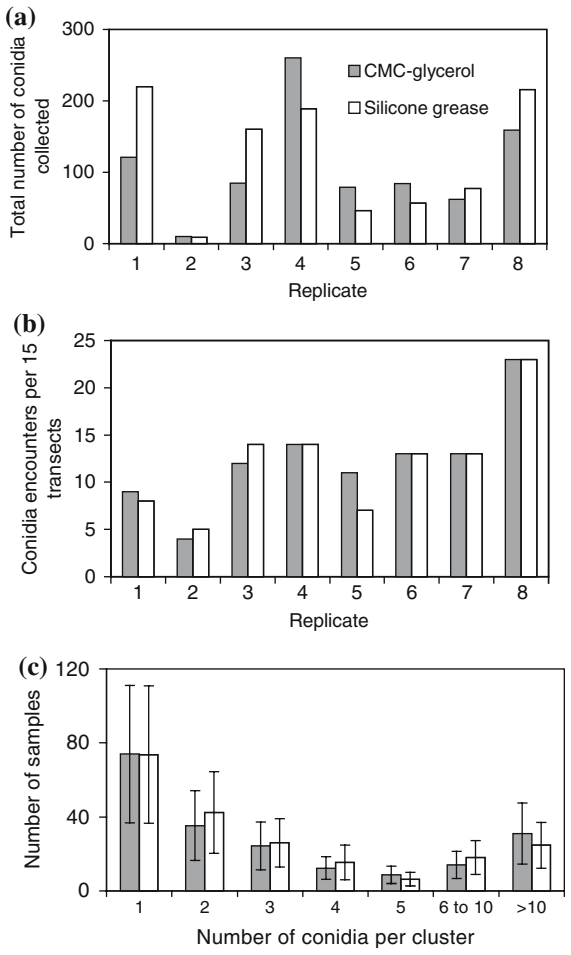

Figure 3. The results of laboratory tests using rotorods coated with either silicone grease or carboxy-methyl cellulose/water/glycerol to sample conidia of *Aspergillus flavus* (a) numbers of conidia sampled, (b) the frequency of encounters with conidia, and (c) the range of cluster sizes of conidia sampled. Standard deviations of the means are indicated.

clog after prolonged sampling. The lack of correlation between the Burkard and filter-samplers remains unclear, but might be related to spatial heterogeneity in propagule quantities at the scale of the sampler collection orifices. The particle filtration tests suggested the propagules of *A. flavus* were primarily conidia (< 12 μm diam), although small sclerotia of the S-strain that form on colonized locules (Garber and Cotty, 1997) may be dispersed by strong winds, but these were not evident in the sample size-sorted by filtration.

Rotorods collected viable conidia of *A. flavus* in laboratory tests, but not in the field (although they caught other fungal propagules), despite previous studies having shown that rotorods can collect propagules of *A. flavus* in the field (Holtmeyer and Wallin, 1981). The rapid loading of rotorods with particles suggested they would be best suited for short-term sampling. To the best of our knowledge rotorods have not been operated previously with a water-soluble coating like CMC–glycerol, which allows culture and thus reliable quantification of viable propagules.

Cyclone samplers have several advantages. They are low maintenance; they collect dry samples that are stable, and they operate continuously depending on the desired resolution of data and the capacity of the collection vessel (Bock et al., 2005). The sample can be microscopically examined and cultured for purposes of identification. Long sampling periods can even out transient fluctuations in propagule numbers. Lee et al. (1986) used the Andersen sampler for short-term sampling, but longer-term studies led to media overload or desiccation, and storage or subdivision of the sample is difficult and premature spore germination may occur. Rotorods, glass impingers and filter samplers are also suited to short-term sampling, up to a few hours. Cyclone samplers have not been widely used in plant pathological studies (Emberlin and Babbonian, 1995; Williams et al., 2001; Bock et al., 2005), yet they have practical value for the study of airborne plant pathogens in some environments (rain could wet the sample resulting in microbial degradation).

The final choice of sampling apparatus should be taken after consideration of the data desired, particularly taking into account sampling periods, resolution of data (hourly, daily, weekly etc), the need to culture propagules for quantification and identification, the environment and the cost of the sampler.

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