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A novel computerised image analysis method for the measurement of production of conidia from the aphid pathogenic fungus *Erynia neoaphidis*

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Abstract

A semi-automated method has been developed for the quantification and measurement of conidia discharged by the aphid pathogen *Erynia neoaphidis*. This was used to compare conidiation by *E. neoaphidis*-mycosed pea aphid cadavers, mycelial plugs cut from agar plates, mycelial pellets from shake flasks and by mycelial pellets from different phases of liquid batch fermenter culture. Aphid cadavers discharged significantly more and significantly smaller conidia than plugs or pellets. The volume of conidia discharged was stable over the period of discharge (80 h), but more detailed analysis of the size frequency distribution showed that more very small and very large conidia were discharged after 5 h incubation than after 75 h incubation. Biomass harvested at the end of the exponential growth phase in batch fermenter culture produced significantly more conidia than biomass from any other growth phase. The implications of these findings for the development of production and formulation processes for *E. neoaphidis* as a biological control agent are discussed.

Keywords: Image analysis; Production and dimensions of conidia; Biological control; *Erynia neoaphidis*

1. Introduction

Erynia neoaphidis Remaudiere and Hennebert (Zygomycetes: Entomophthorales) (= *Pandora neoaphidis* (Humber)) is an obligate fungal pathogen of aphids (Homoptera: Aphididae) with a wide host range amongst aphids. Hosts include species of economic importance; *Acyrthosiphon pisum* (Harris) (pea aphid), *Aphis fabae* (Scopoli) (black bean aphid), *Myzus persicae* (Sulzer) (peach-potato aphid), *Myzus nicotianae* (Blackman) (tobacco aphid) and *Sitobion avenae* (Fabricus) (English grain aphid). *E. neoaphidis* is responsible for the majority of aphid mycoses reported in the field [1,2] and epizootics which contribute to the regulation of aphid populations are often observed [2–4]. Consequently, *E. neoaphidis* has been studied for many years with a view to its development as a biological control agent. In common with the rest of the Entomophthorales, conidia are the infective propagules of the species. However, there are a number of significant obstacles to the production and formulation of conidia of *E. neoaphidis* in a form suitable for spray application in the field. Firstly, conidia are not readily produced in submerged culture and secondly, conidia have a thick coating of mucus, making them difficult to harvest and suspend uniformly in water. In addition, the mucous coat is essential for adhesion to the aphid and its removal by wetting agents used to achieve suspension could have a significant affect on pathogenicity [5,6]. An alternative approach, therefore, is to develop a system for production of hyphal biomass formulated in such a way that, after application, it will sporulate in situ in the field [7,8].

Although some success in initiating infection in aphid populations by the application of *E. neoaphidis* has been achieved in laboratory and greenhouse studies [9], the results of field trials are inconsistent and have often failed to demonstrate effective population control [10–12]. In order to standardise and optimise production of *E. neoaphidis* mycelial biomass for aphid control it is necessary to quan-

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tify the number of conidia discharged and the time over which discharge persists. It is also advantageous to be able to measure the size and shape of the conidia, as some inferences regarding the physiological status of the conidia can be made on the basis of their size and shape. For example, if a primary conidium of *E. neoaphidis* fails to land on an aphid after discharge, it may germinate to form a short germ tube, then produce and discharge a secondary conidium, which can be distinguished from the primary conidium on the basis of its smaller size and, at temperatures greater than 18°C, more rounded shape [13]. The importance of determining the rate of production of conidia by entomophthoralean fungi has led to the development of a sporulation monitor [14] to collect a time course of conidia produced by a sample of fungal biomass, which are then counted manually under the light microscopic. This is a time-consuming procedure that limits throughput, and manual measurement of the dimensions of a large enough number of conidia to characterise any changes in size distribution over time between treatments is impractical. Image analysis has previously been used to characterise conidia of *Pandora* spp. [15], but not to count them or to investigate changes in conidial dimensions in response to production parameters. This paper describes a semi-automated method for counting and measuring conidia collected by the sporulation monitor using image analysis. It also compares the rate of production, size and shape of conidia discharged by *E. neoaphidis* grown on aphid cadavers and in vitro, and demonstrates differences in the rate of production of conidia by *E. neoaphidis* biomass sampled from different phases of liquid batch fermenter culture.

2. Materials and methods

2.1. Fungal isolate, media and culture conditions

E. neoaphidis strain X4 from the Rothamsted collection was originally collected from *A. pisum* on lucerne (*Medicago sativa*) in the 1970s and has been maintained since then by repeated passage through *A. pisum*. It has also been isolated onto Sabouraud dextrose egg milk agar as isolate reference NW327 in the Rothamsted collection [16].

Plate and shake-flask cultures were grown at 20°C on solid or in liquid semi-defined *Erynia* medium (SDEM) as described previously [17], except that the concentration of oleic acid in the medium was reduced to 0.01% (v/v). The same medium was used for batch fermenter culture, which was initiated by inoculating 200 ml culture taken from 5-day-old shake flasks into 800 ml fresh medium in a 2 l fermenter. Fermenter culture conditions were otherwise as reported previously [18]. The reduction in the oleic acid content of the medium resulted in the formation of regular, 3–5 mm diameter pellets of biomass in both shake-flask and fermenter culture rather than a suspension of discrete hyphal fragments. Dry weight of biomass within the shake-flask and fermenter cultures was determined by vacuum-filtration of triplicate 10 ml samples through pre-weighed, 47 mm diameter 0.2 μm pore size glass fibre filters which were then dried overnight at 105°C and then desiccated to constant mass.

2.2. Preparation of aphid cadavers, plugs from plate cultures and pellets from shake-flask and fermenter cultures for assessment of sporulation

Fresh, *E. neoaphidis*-mycosed cadavers of *A. pisum* were prepared by the method of Wilding [19]. Agar plugs (10 mm diameter) were cut from the colony margin of 3-week-old plate cultures of *E. neoaphidis* using a flame-sterilised cork borer. Shake-flask cultures were harvested after 4 days and the fermenter culture was sampled 48, 96, 144 and 192 h after inoculation. The mycelial pellets were separated from the broth by sieving through an autoclaved 250 μm mesh sieve. The dry weight of biomass in each type of sample was estimated by drying triplicate samples of each type to constant mass as above. In the case of plugs cut from plate cultures, the mycelium was carefully dissected away from the agar prior to dry-weight determination. As there was no way of separating the fungus from the remaining tissue within the cadavers, it was accepted that the method used would generate a slight over-estimate of the fungal biomass present.

2.3. Sporulation monitor and image analysis

Conidia were collected using a sporulation monitor developed for the assessment of sporulation of entomophthoralean fungi [14]. Briefly, samples were pre-incubated at 20°C and 100% relative humidity (RH) for 1 h to encourage sporulation, then suspended within the monitor at the same temperature and RH in a 16/8 h light/dark photoperiod. Conidia were discharged onto an acetate sheet mounted on a drum which revolved once in 168 h. Conidia adhered to the acetate sheet due to the presence of a mucous coating, forming a distinct trail with distance along the trail corresponding to the time at which the conidia had been discharged. The acetate sheets were cut into strips corresponding to the trails formed by individual samples and conidia were counted using an Olympus BH-2 light microscope.

The method used here differed from [14] in that image analysis was used to count and measure the conidia. Digital images from the microscope were captured at a resolution of 1.0 μm pixel^{-1} using a JVC TK1280E colour CCD camera connected to a Videologic DVA4000 video adapter mounted in a Macintosh Quadra 650 computer. Image analysis was carried out using the public domain NIH Image programme (developed at the US National Institutes for Health and available on the Internet at http://rsb.info.nih.gov.nih-image). The dimensions of the

image were calibrated by reference to a microscope graticule slide. Background correction of the images was found to be unnecessary, as there was already a high contrast between the conidia and the background and the images were evenly illuminated. At this stage, any obvious artefacts were removed manually. A density slice was then performed to select the conidia on the basis of greyscale (Fig. 1). The conidia appeared hollow, as there was greatest refraction along the edges of the conidia. Individual conidia were readily distinguished from other particles, such as debris and agglomerations of conidia, on the basis of size. Particles with cross-sectional areas inconsistent with individual conidia (<100 μm^2 or >350 μm^2), including clumped conidia ($<5\%$ of conidia present), were automatically excluded from the analysis. Accurate identification of conidia was confirmed by visual examination of the image (Fig. 1c) and any remaining anomalies corrected. The length, breadth and cross-sectional area of each particle were determined automatically, including the 'hollow' interior as part of the particle. Conidia in 10 contiguous fields of view were counted at 5 h intervals based on distance along the spore trail. Conidial volume was calculated based on the assumption that conidia were ellipsoidal [20].

3. Results and discussion

3.1. Comparison of in vitro and in vivo produced biomass

Cadavers produced many more conidia per unit biomass ($P<0.05$, analysis of variance (ANOVA)) and for a longer period of time than either hyphal pellets or mycelial plugs (Fig. 2). Conversely, hyphal pellets and mycelial plugs discharged significantly larger conidia than cadavers (Fig. 3). Taking into account both the number of conidia discharged and their mean volume, the cumulative volume (and hence biomass) of conidia discharged per unit of in vivo grown biomass was substantially greater than that discharged by in vitro grown biomass. Clearly the physico-chemical conditions within the artificial media tested are sub-optimal for production of conidia compared to the in vivo situation. It is well known that differences in nutrient concentration result in different quantities of conidia

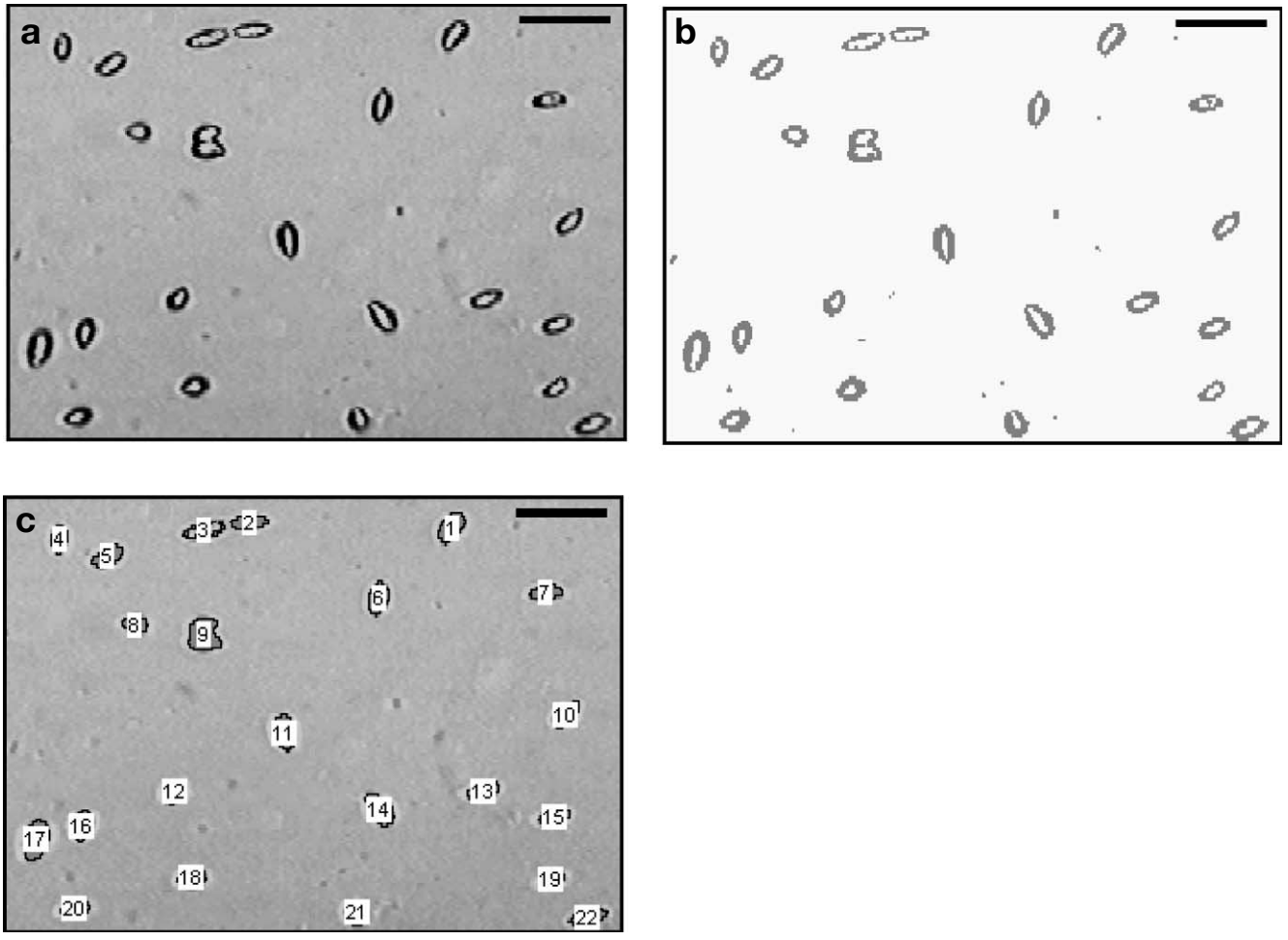

Fig. 1. Detection of conidia of *E. neoaphidis* by image processing. a: Initial image (approximately one quarter of a field of view). b: Selection of regions of interest by density slicing. c: Identification and counting of conidia (particle 9 is intentionally included as an example of clumped conidia which would be removed manually). Scale bar = 50 μm.

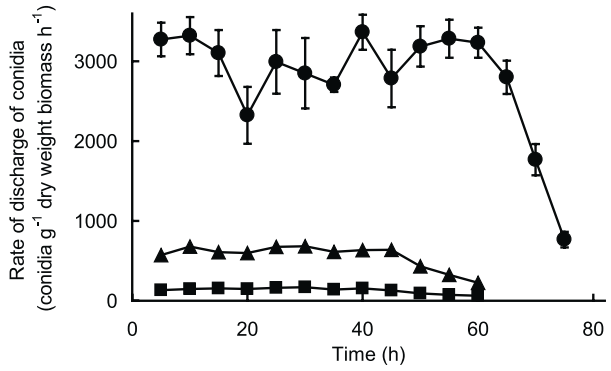

Fig. 2. Comparison of the rate of discharge of conidia of *E. neoaphidis* from aphid cadavers (●), plugs cut from agar plates (■) and pellets from shake-flask culture (▲). The rate of discharge from pellets from fermenter culture (omitted for clarity) did not differ significantly from that of pellets from shake-flask. Error bars represent the standard errors of the means (which are less than the size of the symbols for plugs and pellets), $n = 10$.

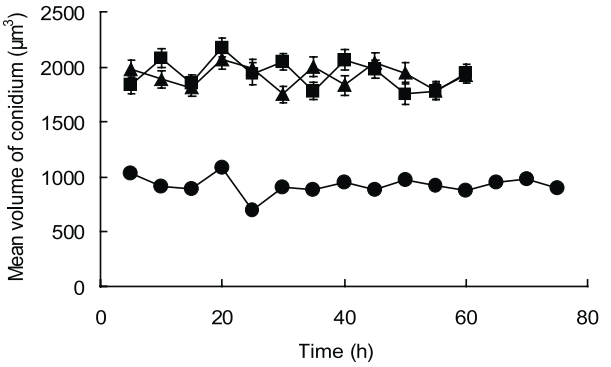

Fig. 3. Comparison of the volume of conidia of *E. neoaphidis* discharged from aphid cadavers (●), plugs cut from agar plates (■) and pellets from shake-flask culture (▲). The volume of conidia discharged from pellets from fermenter culture (omitted for clarity) did not differ significantly from that of pellets from shake-flask. Error bars represent the standard errors of the means (which are less than the size of the symbols for cadavers), $n = 10$.

being produced by many species of filamentous fungi, the best studied example being differences in carbon:nitrogen (C:N) ratio (e.g. [21,22]). The difference in volume between conidia discharged by biomass grown in vitro and in vivo, which agrees with previous studies of *E. neoaphidis* [23], implies a difference in spore physiology. Differences in spore physiology, and consequently in host infectivity, have again been recorded in a range of fungal species as the result of differences in C:N ratio (e.g. [24,25]). Differences in host infectivity between conidia of *E. neoaphidis* produced by biomass grown in vivo and in vitro have been observed previously [23], but there has been no systematic study of the relationship between nutrition, conidium size and infectivity. We speculate that increased spore volume on artificial medium may have resulted from accumulation of storage compounds resulting from one or more nutrients being present in large excess, most probably carbon. Blastospores of the entomopathogenic fungus *Beauveria bassiana* produced in nitrogen-limited (carbon in excess) medium contained endogenous carbon reserves which enhanced spore longevity [26], and growth on a carbon-rich medium also enhanced longevity of conidia of *Metarhizium anisopliae* var. *acridum* (= *Metarhizium flavoviride*) [27]. Production of large conidia containing carbon reserves may therefore improve the efficacy of a biological control agent. However, large conidia are not necessarily desirable: if, as argued in the introduction, biomass is to be distributed in the field in a form which subsequently sporulates, production of conidia with an excessively large aerodynamic diameter might hamper dispersal [28].

Over the sporulation period there was no shift towards discharge of smaller spores (Fig. 3), which would indicate a substantial change in physiology, or a switch to the production of secondary conidia from primary conidia deposited within the original sample. This suggests that the physiology of the conidia was relatively constant during the period of discharge, which would be desirable in material for field application as a control agent. However, Morgan [23] reported that both the speed of germination and aphid infectivity of conidia discharged from *E. neoaphidis*-mycosed aphid cadavers decreased over the period of discharge. More detailed analysis of the size distribution of discharged conidia (Fig. 4) reveals small but significant ($P < 0.05$, χ^2) differences in spore sizes at the beginning and end of the period of discharge. Compared to conidia discharged after 5 h incubation, fewer very small (volume < 400 µm^3) or very large (volume > 2000 µm^3) conidia and more intermediate-sized conidia were discharged after 75 h incubation. Further research will be needed to elucidate the mechanisms responsible, but this is a good illustration of the power of the method in enabling analysis of much larger numbers of spores than would be possible by purely manual means.

3.2. Comparison of conidia from different phases of growth in batch fermenter culture

The number of conidia discharged by biomass pellets from liquid batch fermenter culture differed significantly

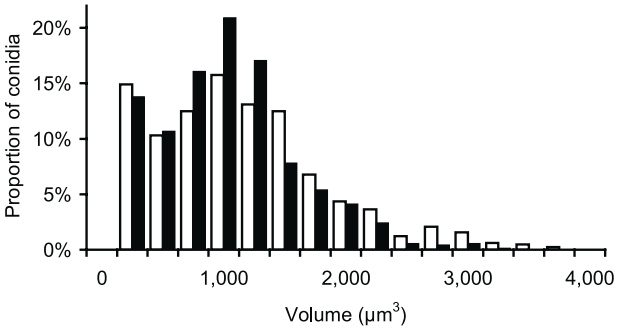

Fig. 4. Comparison of the distributions of volumes of conidia of *E. neoaphidis* discharged by aphid cadavers after 5 h (open bars) and 75 h (filled bars) incubation.

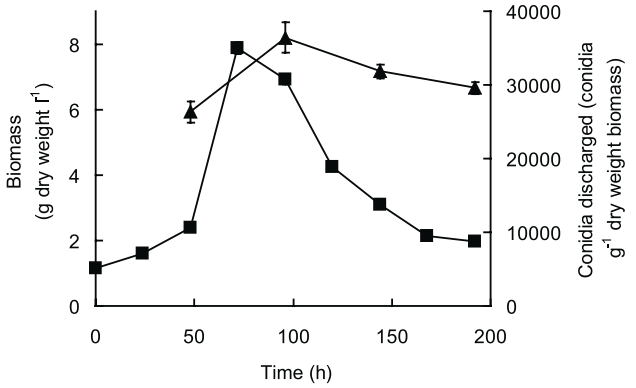

Fig. 5. Comparison of the total number of conidia discharged from biomass pellets of *E. neoaphidis* taken from the lag (48 h), late exponential/stationary (96 h), early (144 h) and late (196 h) decline phases of liquid batch fermenter culture (▲). Biomass concentrations within the fermenter culture are shown for comparison (■). Standard errors of the means are less than the size of the symbols, $n = 3$.

between phases of growth ($P < 0.05$, ANOVA) (Fig. 5). Biomass harvested at the end of the exponential growth phase discharged the greatest number of conidia, with decline and lag phase biomass discharging the fewest conidia. In the exponential growth phase nutrients are in excess and growth is at its most active, therefore optimal production of conidia is expected. It has previously been shown that addition of complex carbon sources to *E. neoaphidis* biomass formulated within alginate beads increased the number of conidia discharged [29], although this had no significant effect on infectivity.

Neither the number of conidia g^{-1} biomass nor the mean volume of conidia discharged by pellets taken from the late exponential phase of batch culture differed significantly between shake-flask and fermenter culture (data not shown). Pellets from shake-flask cultures discharged significantly more conidia per unit biomass than plugs cut from plate cultures ($P < 0.05$, ANOVA+Tukey HSD) (Fig. 2). A possible explanation for this is that the biomass from shake-flask culture was in exponential growth phase whereas, behind the peripheral growth zone (≈ 3 mm wide on colonies of *E. neoaphidis* on SDEM, [30]), the concentrations of nutrients present may have become severely depleted [31], thus the plate culture may have been in the equivalent of decline phase.

3.3. Conclusions

Image analysis is clearly a useful tool for quantifying and characterising conidia of *E. neoaphidis* and, potentially, other fungi. It is possible to gather more data to increase statistical power, and data can be gathered that would not be feasible using manual microscopy and counting techniques. It should be noted that the relatively large size of conidia of *E. neoaphidis* suited them to this type of analysis. It was also much more straightforward to develop a method of analysis for pure preparations of conidia than for preparations contaminated by other species or large quantities of debris, as might be obtained from field samples; however, this is potentially possible if an algorithm can be constructed to distinguish the spores of interest from the remaining material.

If fermenter culture is to be used for production of *E. neoaphidis* biomass in a form which will sporulate after application to the field, the data presented above suggest that cultures should be harvested at the end of the exponential growth phase. The data also show that there is scope for improvement in the yield of conidia through refinement of the growth medium, and that the method presented here will be of value in optimising and standardising the physiological status of biomass for field application.

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