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Hydrophobicity-Related Protein Contents and Surface Areas of Aerial Conidia are Useful Traits for Formulation Design of Fungal Biocontrol Agents

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Abstract To clarify the potential use of hydrophobicity-related traits of aerial conidia in formulation design of fungal biocontrol agents, hydrophobicity rates (H_r) and surface areas (S_a) of aerial conidia were assessed with 48 strains of *Beauveria bassiana, Isaria fumosorosea* and *Metarhizium* spp. Inter- or intra-specific variation was large in H_r (59.7–92.2%) and S_a (7.9–25.3 μm^2 conidium^{-1}) measurements, which were significantly correlated ($r^2 = 0.55$). Six isolates of the three fungi with distinguished H_r and S_a were further studied. Conidial wall proteins of these isolates were sequentially extracted with sodium dodecyl sulfate (SDS), formic acid (FA) and trifluoroacetic acid (TFA). Their H_r values were significantly correlated to the contents (P_c) of TFA-soluble, but FA-insoluble, proteins (2.7–44.8 μg per 10^7 conidia; $r^2 = 0.79$) and reduced drastically by the FA/TFA treatments, which eliminated the hydrophobin-based rodlet layers of conidial surfaces. However, the SDS treatments had no effect on either H_r or rodlet layers. The dispersancy of a tested emulsifier to oil formulations of the six isolates in water was adversely correlated to their H_r ($r^2 = 0.94$). The results indicate that both P_c and S_a are inherent hydrophobicity-related traits and can be utilized to select fungal biocontrol candidates for improved formulation and application.

Keywords Fungal biocontrol agents · Conidial hydrophobicity · Cell wall proteins · Oil formulation · Emulsification

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Introduction

Beauveria, *Metarhizium* and *Isaria* are well-known fungal biocontrol agents against arthropod pests, including a large number of candidate isolates that have been developed into mycoinsecticides and mycoacaricides in the world [1–3]. Such biopesticides are usually in the forms of emulsifiable or wettable formulations. As active ingredients of these formulations, aerial conidia are highly hydrophobic no matter how to be produced on solid substrates such as small grains [4]. This feature is an important concern when a fungal formulation is designed for improved field application.

Conidial hydrophobicity of filamentous fungi stems from rodlet layers of conidial surface that consists of hydrophobins [5–7]. Such proteins are associated not only with hydrophobicity but also with morphogenesis, adhesion, antigenicity and defense against host immune reaction [8–10] and can be

dissociated from conidia with agents such as formic acid or trifluoroacetic acid [11–13]. In *B. bassiana*, several hydrophobin-like proteins extracted with formic acid or hydrophobins identified by gene cloning fall in the sizes of 6.5–14.0 kDa [14–16]. A starvation-stress gene (*ssgA*) of *M. anisopliae* was found encoding a hydrophobin [17, 18]. However, no hydrophobin has been identified from *Isaria fumosorosea*. Neither have the amounts of such proteins that may relate to the degree of conidial hydrophobicity been quantified for comparison among or within the mentioned fungi.

For the fungal biocontrol agents, hydrophobicity favors conidial adhesion to insect cuticle, the first step of fungal infection to kill pests [6, 19, 20], but is a barrier to the field spray of a fungal formulation in the form of aqueous dilution. Conidial hydrophobicity can be assessed with several methods [21], often with the method of aqueous-solvent partitioning [22–24]. Apart from inter- or intraspecific variability in hydrophobin content that may affect the hydrophobicity, surface areas of conidia depending on their ellipsoid sizes vary greatly among different fungal species or isolates. For instance, the sizes of *Beauveria*, *Isaria* and *Metarhizium* conidia fall in the ranges of 2–5 × 1–2.5, 4.5–18 × 2.5–5.5 and 2–10 × 1–3.5 μm, respectively [25]. Such a large variation in conidial size or surface area could be another important source of influence on the dispersivity of formulated conidia in aqueous suspension for field spray but has not been elucidated for potential use in fungal formulation design.

In this study, conidial hydrophobicity rates and surface areas measured from 48 isolates of *B. bassiana*, *I. fumosorosea* and *Metarhizium* spp. were compared and correlated. Based on the distribution of their hydrophobicity and surface parameters, six representative isolates were selected from the three fungal groups for further study. The contents of trifluoroacetic acid-soluble cell wall proteins were correlated to the hydrophobicity rates of their conidia. The conidial dispersivity of each of the selected isolates in the aqueous suspension of oil formulation containing different ratios of a selected emulsifier was evaluated. Our goal was to reveal the potential use of the hydrophobicity-related traits for formulation design of the fungal agents.

Materials and Methods

Fungal Isolates

Forty-five isolates of *B. bassiana* (Bb), *M. anisopliae* sensu lato (Ma), *M. acridum* (Mc), *M. majus* (Mm), *M. robertsii* (Mr), *M. anisopliae* var. *acridum* (Maac, likely Mc) and *I. fumosorosea* (If) with different host and geographic origins were obtained from the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; RW Holley Center for Agriculture and Health, Tower Road, Ithaca, NY, USA). Three other isolates, derived from Asian corn borer *Ostrinia furnacalis* (Bb0101) and false-eye leafhopper *Empoasca vitis* (Bb0201 and Ma0201) in China, were also included in the study. All isolates were preserved at −76°C and recovered on the plates of Sabouraud dextrose agar plus 1% yeast extract (SDAY) at 25°C prior to use.

Production of Aerial Conidia

Aerial conidia of all isolates were produced on steamed rice in Petri dishes (15 cm diameter) during a period of one month following a protocol described previously [4, 26, 27]. Rice cultures were grown for 7–9 days at 25°C and then dried under ventilation at 34°C for 1 day. Aerial conidia were harvested through a vibrating sieve and vacuum-dried to ≤5% water content at ambient temperature. The dried conidia were used immediately or stored at 4°C in glass vials for use within 3 months, warranting the viabilities of ≥90%.

Assessment of Conidial Hydrophobicity

Conidial hydrophobicity of each isolate was assessed using the method of aqueous-solvent partitioning [22–24] with some modification. For each isolate, conidia were suspended in PM buffer (per liter: 6.97 g K_2HPO_4, 2.99 g KH_2PO_4 and 0.2 g $MgSO_4{\cdot}7H_2O$; final pH 7.12) containing 0.02% (v/v) Tween 80, and the spore suspension was standardized to 2×10^7 conidia ml^{-1}. Liquid paraffin, which has proven a desired oil vector of fungal formulations [28–30] and thus was used as organic phase in this study, was then added to the spore suspension at the ratio of 40 μl over 4 ml. The buffer was used to neutralize charges of

spore surface, thus excluding their effect on spore distribution in either phase. The mixture in 20-ml standard separation funnel was vortexed for 2 min. When the organic phase was separated from the aqueous phase after vortex, three aliquots were pipetted from the aqueous phase into Neubauer hemocytometers and the conidia left in the aqueous phase were counted under microscope. The hydrophobicity rate (H_r) of the tested conidia was assessed as $H_r = (1-C/C_0) \times 100$, where C_0 was the spore concentration of the aqueous suspension before paraffin was added and C the residual spore concentration in the aqueous phase after the partitioning. The assay of each isolate was repeated four times.

Measurements of Conidial Sizes and Superficial Areas

For each of the 48 isolates, 30 conidia were cross-measured for their lengths and widths under microscope at 1,000× magnification. Based on the shapes of spheroid, quasi-spheroid, elongated spheroid and cylindroid, all measured conidia were considered as ellipsoid; surface area per *capita* (S_a) was computed as square micrometer (μm^2 conidium^{-1}) sing the formula $S_a = 2(2b)^{1/2}(a^2 + b^2)^{1/2}$, where a and b were the length and width of each conidium.

Extraction of Conidial Wall Proteins

To examine cell wall proteins of aerial conidia, six isolates of *B. bassiana* (Bb2860 and Bb2864), *I. fumosorosea* (If4205 and If6032), *M. anisopliae* (Ma456) and *M. majus* (Mm978) were selected as representatives based on the distributions of their H_r and S_a among all the tested isolates. Of those, Bb2860 and Ma456 are excellent candidates against sucking pests such as planthoppers, leafhoppers and spider mites [26–30]. The aerial conidia of each isolate were subjected to sequential treatments with boiling sodium dodecyl sulfate (SDS), iced formic acid (FA) and iced trifluoroacetic acid (TFA) following a basic extraction protocol [15, 31] with some modification below.

For the isolates of *B. bassiana* and *I. fumosorosea*, 100 mg aliquots of aerial conidia were separately suspended in 4 ml SDS buffer [2% (w/v) SDS, 5% (v/v) β-mercaptoethanol], and the suspension was boiled for 10 min. Four cycles were run for extracting the SDS-soluble proteins from the conidia, and the resultant supernatant was stored at 4°C for analysis. Subsequently, the SDS-treated conidia were collected by 10-min centrifuge at 5,000×g after washing twice and then suspended in 2 ml FA on ice for 2 h. The supernatant was mixed with 2 ml dd-H_2O, followed by neutralization with 4 ml 45% (w/v) NaOH on ice. Maintained overnight at 4°C, the supernatant was centrifuged at 5,000×g for 10 min and the deposits were dissolved in 250 µl 2% SDS. After the FA-extracted, SDS-soluble proteins were washed off with 50 ml 2% SDS, the SDS-insoluble pellets collected by 5-min centrifuge at 10,000×g were lyophilized and suspended in 2 ml TFA on ice for 30 min. The suspension was then dried up by evaporation at ambient temperature for removing the TFA and the deposits were resuspended in 50 µl 2% SDS for analysis. For the two isolates of *Metarhizium*, the first two extractions were the same as earlier. Additionally, the FA-treated conidia were further suspended in 2 ml TFA on ice for 1 h extraction. After the TFA was evaporated, the deposits were suspended in 50 µl 2% SDS for the following analyses.

Assessment of TFA-Soluble Protein Contents

For all the selected isolates, the contents (P_c) of the TFA-soluble, but FA-insoluble, proteins extracted from aerial conidia were measured as µg mg^{-1} conidia with a folin-phenol method [32] using bovine serum albumin as a standard. The measurements were then transferred to the unit of µg per 10^7 conidia for standardization due to a wide variation of their conidial sizes.

SDS–PAGE Analysis of Conidial Wall Extracts

The 2% SDS solutions of the SDS-, FA- and TFA-soluble proteins from the sequential extracts of all the selected isolates were boiled for 3 min. Subsequently, 15 µl aliquots of the solutions were loaded on Tris–glycine gels (3% stacking gel and 15% resolving gel) for SDS–PAGE analysis [33]. The stacking gel was run at 12 mA and the resolving gel at 18 mA. After electrophoresis, the gel was stained with 0.12% Coomassie Blue R-250 (0.12 g R-250, 25 ml ethanol, 8 ml acetic acid and 67 ml dd-H_2O)

and visualized in the reagent consisting of 25% (v/v) ethanol and 8% (v/v) glacial acetic acid.

Hydrophobicity Assessment of Treated Conidia

The conidia of the six selected isolates were washed twice with dd-H_2O after each of the treatments with SDS, FA or TFA and suspended again in the PM buffer. All the suspensions were adjusted to the concentration of 2×10^7 conidia ml^{-1}, and conidial hydrophobicity rates were separately measured as described earlier.

Western Blotting of Known Hydrophobins

Two hydrophobins Hyd1 (13.81 kDa) and Hyd2 (11.98 kDa) known in *B. bassiana* [16] were separately expressed as $(His)_6$-tagged recombinant Hyd1 and Hyd2 (ca. 20 kDa for both) in *Escherichia coli* BL21 by gene transformation. Each hydrophobin was purified from 500 ml BL21 culture and homogenized to 1 mg protein ml^{-1} normal saline. The saline was then mixed with Freund's complete adjuvant at the volume ratio of 1:1 and injected into New Zealand white rabbits (4 months old) three times at 14-day interval (2 ml per injection). Rabbit blood was taken 14 days after the final injection. The serums were separated by centrifuge at 3,000×*g* at 4°C, generating the polyclonal antibodies anti-Hyd1 and anti-Hyd2.

The TFA-soluble, but FA-insoluble, proteins in the conidial extracts of the selected isolates were electrophoretically transferred from gels onto the PVDF membranes and analyzed by Western blot using the kit of ProtoBlot alkaline phosphatase system (Novagen). All blots were probed with 2,000× dilution of the anti-Hyd1 or anti-Hyd2 and visualized with goat anti-rabbit IgG-alkaline phosphatase conjugate (Novagen).

Scanning Electronic Microscopy of Treated Conidia

Dried samples of the conidia from the sequential treatments and control (not treated) were covered with evaporated platinum. Possible changes of rodlet layers (presence or absence) on the surfaces of the treated conidia were then observed under scanning electronic microscope (SEM; Hitachi S4800, Ibaraki, Hitachi, Japan).

Assessment of Emulsifier Dispersancy to Aerial Conidia

Aerial conidia of the six selected isolates were separately suspended in the mixtures of 94.5–98% paraffin as oil carrier and 2–5.5% (v/v) fatty alcohol polyethylene glycol ether 'AEO-3' as emulsifier (Xiaoshan Chemical Additives Co., Hangzhou, China), resulting in different emulsifiable formulations at a standardized concentration of 1×10^{10} conidia ml^{-1}. To examine aqueous dispersancy of different emulsifier ratios toward the formulated conidia, 100-fold aqueous dilutions (1×10^8 conidia ml^{-1}) were prepared with four samples (replicates) of each formulation. Each of the dilutions in 15-ml tubes was shaken by wrist action for 0.5 min; three suspension samples were then pipetted into Neubauer hemocytometers for counts of the dispersed conidia in aqueous phase under a microscope. The ratio of the detected spore concentration over the diluted concentration was an index for the dispersancy (D_i) of a given emulsifier ratio to the oil-formulated conidia of each isolate.

Data Analysis

All measurements of H_r, D_i (both transformed to arcsine-squared roots), S_a and P_c (both $\log_{10}$-transformed) were subjected to one- or two-way analysis of variance (ANOVA), followed by linear correlation to each other. The D_i values of each fungal isolate observed over the emulsifier ratios of 2–5.5% (R_e) were fitted to the logistic equation $D_i = K/[1 + \exp(a + r_d R_e)]$, where K is a maximal potential of dispersancy to be achieved by the tested emulsifier for a given isolate, a an intercept for the fitted curve and r_d the rate of dispersancy increase with R_e. The fitted r_d was also linearly correlated to the H_r values of the six isolates. All analyses were performed using an updated version of DPS software [34].

Results

Variability in Conidial Hydrophobicity and Surface Area

The hydrophobicity rates of aerial conidia (Fig. 1a) ranged from 59.7 (Pf6032) to 92.2% (Mm978) and

differed significantly among the tested 48 isolates ($F_{47,144} = 17.9$, $P < 0.01$) and also varied within each fungal group, i.e., 69.5–87.2% (mean: 78.6%) in *B. bassiana* ($F_{13,42} = 12.9$, $P < 0.01$), 77.1–92.2% (83.3%) in *Metarhizium* spp. ($F_{23,72} = 10.6$, $P < 0.01$) and 59.7–79.2% (72.5%) in *I. fumosorosea* ($F_{9,30} = 9.5$, $P < 0.01$). The three means were significantly different from one to another (Fisher's LSD, $P < 0.05$).

Surface areas of aerial conidia (Fig. 1b) computed with measured widths and lengths spanned from 7.9 (If6032) to 25.3 μm^2 $conidium^{-1}$ (Mm978) and differed significantly within each fungal group ($P < 0.01$ in one-way ANOVA). The S_a means (±SD) for the isolates of *Metarhizium* spp., *B. bassiana* and *I. fumosorosea* were 14.4 ± 3.8, 10.1 ± 1.4 and 9.0 ± 0.7 μm^2 ($F_{2,45} = 26.3, P < 0.01$), respectively. The latter two means had no significant difference (Fisher's LSD, $P > 0.05$).

Interestingly, the arcsin-squared roots of the H_r measurements from the 48 isolates were significantly correlated to the $\log_{10}$-transformed S_a estimates (Fig. 2a). The fitted coefficient of determination ($r^2 = 0.55$, $F_{1,46} = 56.5$, $P < 0.0001$) for the linear correlation indicates that 55% of the variation in conidial hydrophobicity is attributed to the surface areas of the tested isolates based on their conidial sizes. The same correlation was also significant for the isolates of *Metarhizium* spp. ($r^2 = 0.45$, $F_{1,22} = 18.2$, $P = 0.0003$) and *B. bassiana* ($r^2 = 0.37$, $F_{1,12} = 7.0$, $P = 0.0214$) but insignificant for *I. fumosorosea* ($r^2 = 0.15$, $F_{1,8} = 1.5$, $P = 0.26$).

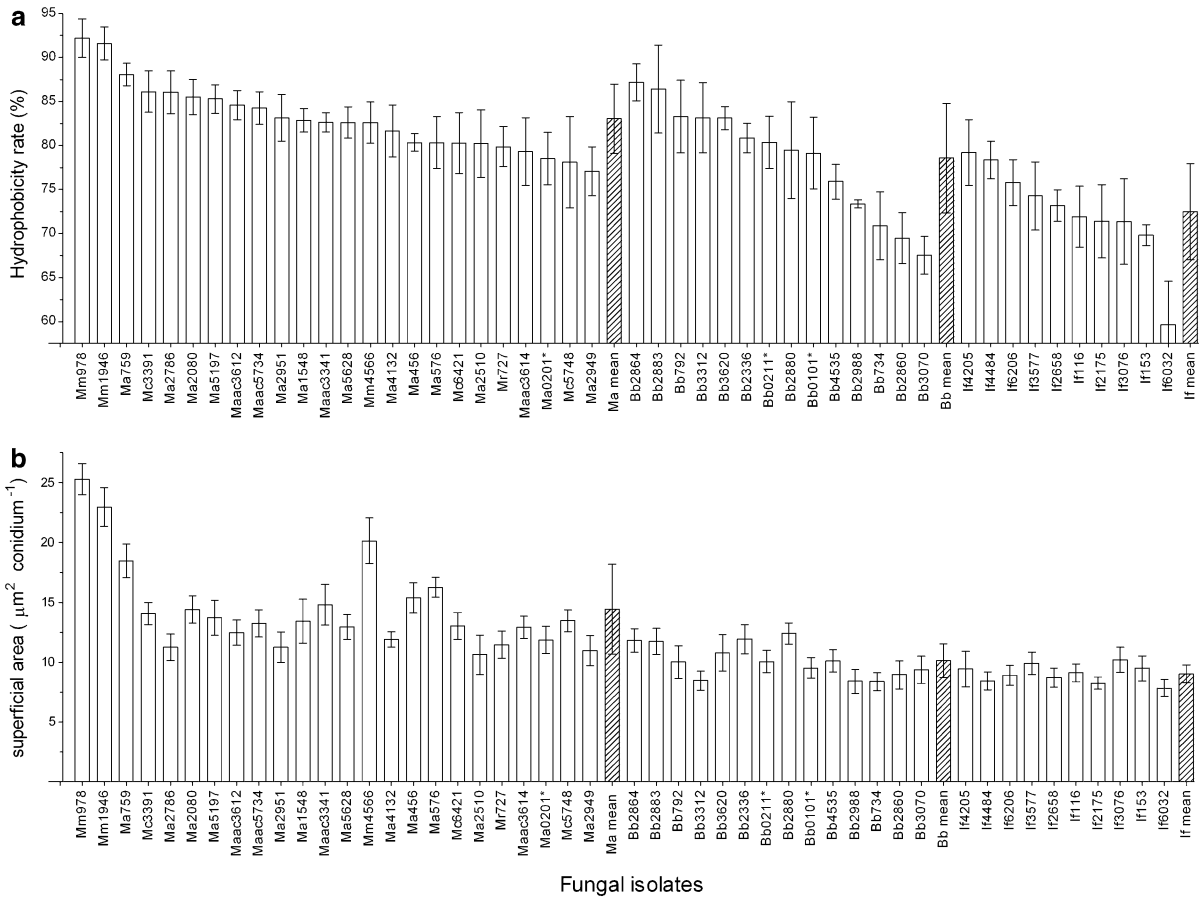

Fig. 1 Hydrophobicity rates (**a**) and superficial areas (**b**) of aerial conidia measured from the 48 isolates of *B. bassiana* (Bb), *M. anisopliae* (Ma), *M. anisopliae* var. *acridum* (Maac), *M. acridum* (Mc), *M. majus* (Mm), *M. robertsii* (Mr) and *I. fumosorosea* (If). *Shading bar* mean of grouped measurements. *Error bars* SD asterisked

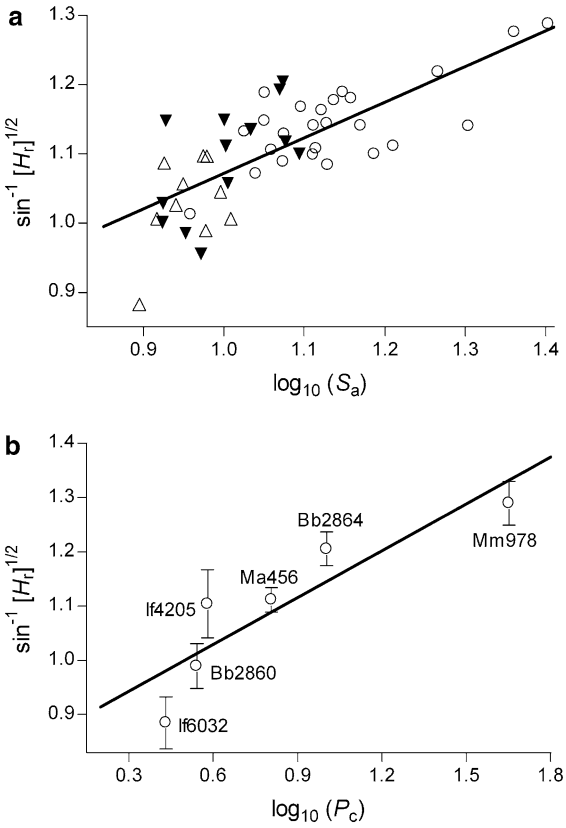

Fig. 2 **a** Correlation of hydrophobicity rates (H_r) to surface areas (μm^2 conidium^{-1}; S_a) of the 48 isolates of *B. bassiana* (*filled inverted triangle*), *I. fumosorosea* (*open triangle*) and *M. anisopliae* (*open circle*) ($r^2 = 0.55$, $F_{1,46} = 56.5$, $P < 0.0001$). **b** Correlation of hydrophobicity rates to trifluoroacetic acid-soluble protein contents (μg per 10^7 conidia; P_c) of six isolates ($r^2 = 0.79$, $F_{1,4} = 15.1$, $P = 0.018$)

Correlation of Hydrophobicity Rates to TFA-Soluble Protein Contents

The contents of the TFA-soluble proteins (P_c) fell in a range of 21.5–91.9 μg mg^{-1} conidia or 2.7–44.8 μg per 10^7 conidia (Table 1). The P_c measurements differed significantly among the tested isolates ($P < 0.01$ in one-way ANOVA).

The hydrophobicity rates of the extracted conidia (Table 1) were significantly different among the tested isolates ($F_{5,54} = 11.9$, $P < 0.01$) or between the sequential treatments ($F_{2,54} = 360.3$, $P < 0.01$) based on two-way ANOVA. The FA treatment reduced drastically the H_r values of all *B. bassiana* and *I. fumosorosea* isolates (Fisher's LSD, $P < 0.05$), whereas the SDS treatment had no significant effect on H_r (Fisher's LSD, $P > 0.05$). However, the H_r values of the two *Metarhizium* isolates (Ma456 and Mm978) were substantially reduced only by the TFA treatment.

The arcsin-squared roots of the H_r values of the six isolates were linearly correlated to the $\log_{10}$-transformed P_c values (μg per 10^7 conidia) measured from their FA and TFA extracts (Fig. 2b). Up to 79% of the H_r variation ($r^2 = 0.79$, $P = 0.018$) was related to the P_c estimates. However, the correlation was not significant ($r^2 = 0.44$, $F_{1,4} = 3.1$, $P = 0.15$) when the protein contents in the unit of μg mg^{-1} conidia were concerned. This suggests that the hydrophobicity of aerial conidia depend on both their TFA-soluble protein content and conidial size.

Components of Conidial Wall Extracts

The components of conidial wall proteins were diverse in the sequential SDS and FA extracts of the selected isolates, as illustrated by SDS–PAGE profiles (Fig. 3a). After the FA treatment, a very few proteins were retained on conidial walls of the two *Metarhizium* isolates and then extracted well with TFA. In contrast, the FA extraction resulted in no residual wall proteins from the isolates of *B. bassiana* and *I. fumosorosea*. Most of the FA-extracted proteins from these isolates were SDS-soluble, whereas a few were SDS-insoluble until being dissolved with iced TFA.

There were only two TFA-soluble proteins in the FA extracts from each of the two *B. bassiana* isolates. As a result of the Western blotting with prepared antibodies (Fig. 3b), the two proteins were confirmed as the hydrophobins Hyd1 (13.81 kDa) and Hyd2 (11.98 kDa), which were well characterized from the same fungal species (16). However, the TFA-soluble components (10–14 kDa) of the *I. fumosorosea* and *Metarhizium* isolates had no reaction with the antibodies (Fig. 3c, d). No cross-reaction was detected for either anti-Hyd1 with the purified Hyd2 or anti-Hyd2 with the purified Hyd1 of the Bb2860 (Fig. 3e).

SEM Examination of Rodlet Layers on the Surfaces of Treated Conidia

Similar SEM overviews to the conidia sequentially treated with SDS, FA and TFA were attained for the two isolates of each fungus with different H_r and S_a,

Table 1 Hydrophobicity rates of aerial conidia of selected six isolates sequentially extracted with sodium dodecyl sulfate (SDS), formic acid (FA) and trifluoroacetic acid (TFA), and the contents of their TFA-soluble proteins

Selected isolates	Mean ± SD[a]					
	Conidial hydrophobicity rate (%) after extraction				TFA-soluble protein content[b]	
	Untreated (Control)	Hot SDS extracted	FA extracted	TFA extracted	μg mg^{-1} conidia	μg per 10^7 conidia
Bb2860	69.48 ± 2.92d	69.78 ± 3.8d	6.00 ± 7.7c	–	22.51 ± 2.53d	3.48 ± 0.39d
Bb2864	87.17 ± 2.08b	87.49 ± 1.1b	9.00 ± 2.4c	–	25.09 ± 1.53 cd	10.08 ± 0.61b
If4205	79.20 ± 3.71c	79.57 ± 5.2c	3.50 ± 3.4c	–	24.19 ± 1.65 cd	3.80 ± 0.26d
If6032	59.65 ± 4.93e	59.86 ± 4.7e	5.50 ± 5.0c	–	26.25 ± 2.58c	2.71 ± 0.27e
Ma456	80.34 ± 0.99c	80.67 ± 1.7c	80.99 ± 4.4b	8.50 ± 7.0	32.56 ± 1.32b	6.41 ± 0.26c
Mm978	92.20 ± 2.19a	92.20 ± 2.2a	92.99 ± 5.9a	5.75 ± 3.6	91.86 ± 9.18a	44.81 ± 4.48a
F-test	$F_{5,18} = 62.0$	$F_{5,18} = 49.4$	$F_{5,18} = 66.1$		$F_{5,12} = 125.8$	$F_{5,12} = 537.0$

[a] Table entries with different lowercase letters in each column differ significantly (Fisher's LSD, $P < 0.05$)

[b] The FA-extracted, SDS-insoluble proteins of the four isolates of *B. bassiana* (Bb) and *I. fumosorosea* (If) became SDS soluble after being dissolved with TFA, followed by assessment

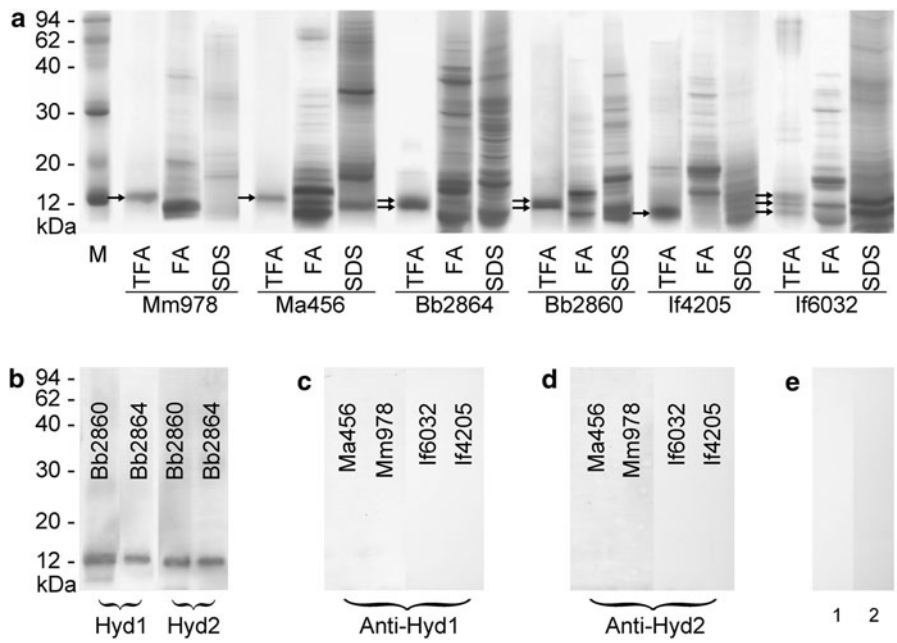

Fig. 3 Analysis of conidial wall extracts of *B. bassiana* (Bb2860 and Bb2864), *M. anisopliae* (Ma456), *M. majus* (Mm978) and *I. fumosorosea* (If4205 and If6032). **a** SDS–PAGE profiles of the wall proteins sequentially extracted with boiling sodium dodecyl sulfate (SDS) and iced formic acid (FA). The FA-extracted conidia of Mm978 and Ma456 were further extracted with iced trifluoroacetic acid (TFA) while the FA-extracted, SDS-insoluble proteins of other isolates were dissolved with iced TFA before analysis. Arrows indicate major bands of TFA-soluble proteins. **b–d** Western blots of the TFA-soluble hydrophobins Hyd1 and Hyd2 in the Bb2860 and Bb2864 FA extracts or of the TFA extracts of Ma456, Mm978, If4205 and If6032, detected by the antibodies anti-Hyd1 and anti-Hyd2, respectively. **e** Western blots of anti-Hyd1 and anti-Hyd2 for cross reactions of the purified recombinant proteins Hyd2 (*lane 1*) and Hyd1 (*lane 2*), respectively

and thus half of them were shown in Fig. 4. The rodlet layers on conidial surfaces were well retained after the SDS treatment irrespective of the fungal species or isolates. The subsequent FA treatment removed such hydrophobic structures from the conidia of *B. bassiana* and *I. fumosorosea*. However,

the same surface structure of *M. anisopliae* was removed only by further TFA extraction.

Aqueous Dispersancy of Emulsifier to Oil-Formulated Conidia

Listed in Table 2 are the measured dispersancy indices (D_i) of 2–5.5% emulsifier toward the oil-formulated conidia of the six isolates with distinguished H_r, S_a and P_c values. In aqueous dilutions, the D_i values generally increased with the emulsifier ratio but varied greatly among the isolates even at the same emulsifier ratio ($P < 0.01$ in one-way ANOVA). As a result, emulsifying the oil-formulated conidia in water was easy for If6032, followed by Bb2860 and If4205. In contrast, the emulsification was most difficult for Mm978, followed by Bb2864 and Ma456.

The D_i trends over the tested emulsifier ratios fit well the logistic equation with high coefficients of

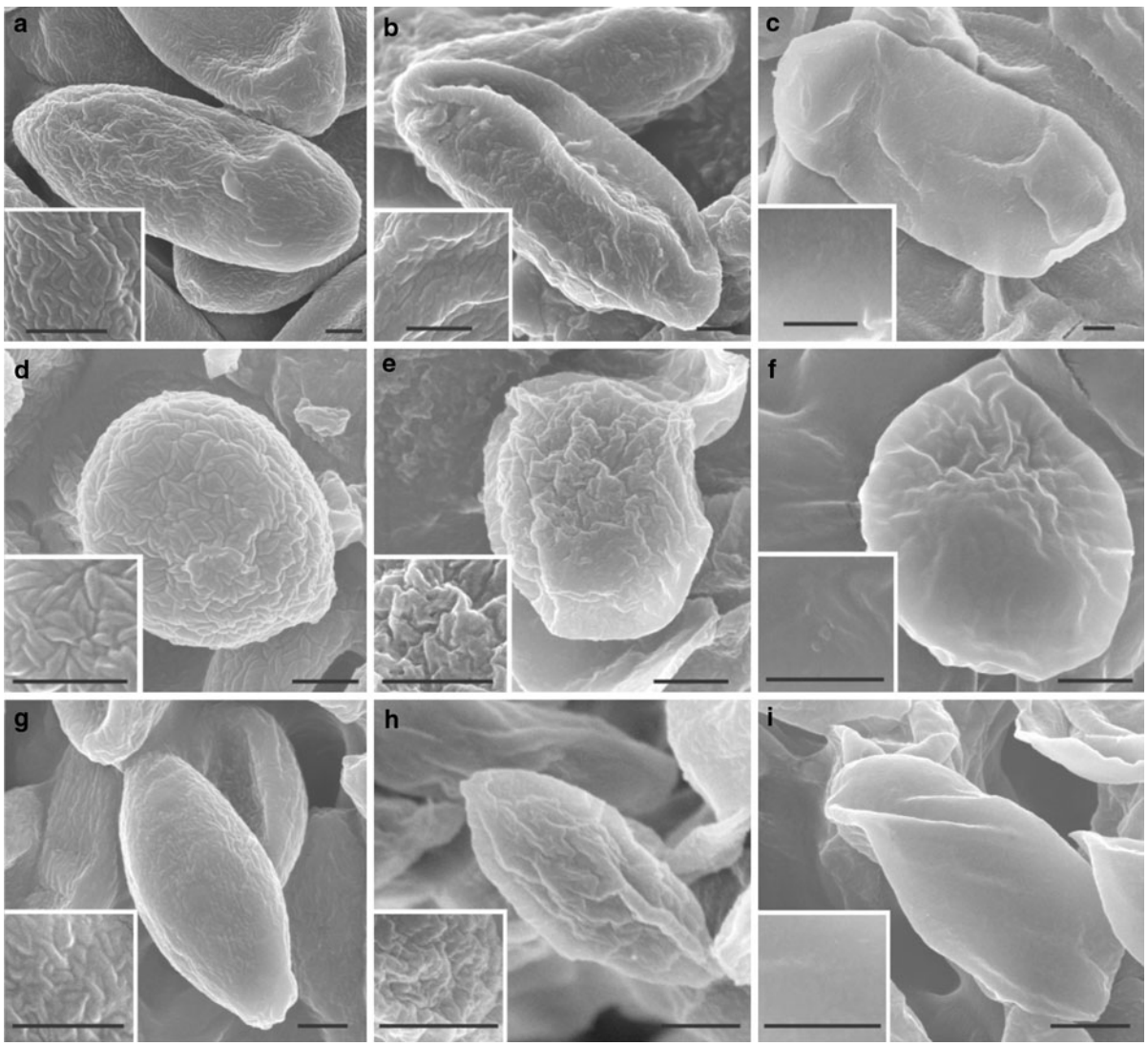

Fig. 4 SEM views of the surface changes of treated conidia. **a–c** *M. anisopliae* (Ma456). **d–f** *B. bassiana* (Bb2860). **g–i** *I. fumosorosea* (If6032). Aerial conidia were sequentially treated with sodium dodecyl sulfate (**e**, **h**), formic acid (**b**, **f**, **i**) and trifluoroacetic acid (**c**) or not treated as control (**a**, **d**, **g**). Note that rodlet layers (detailed in *left bottom corner*) were removed from conidial surfaces by final treatment with formic acid (**f**: Bb2860; **i**: If6032) or trifluoroacetic acid (**c**: Ma456). *Scale bars* = 0.5 μm

Table 2 The aqueous dipersancy of the emulsifier AEO-3 to the oil-formulated conidia of the isolates of *B. bassiana* (Bb), *M. anisopliae* (Ma), *M. majus* (Mm) and *I. fumosorosea* (If) with different degrees of conidial hydrophobicity

	Aqueous dispersancy index (% ± SD)[a]						*F* test[b] ($F_{5,12}$)
	Bb2860	Bb2864	Ma456	Mm978	If4205	If6032	
Ratio of emulsifier (%)							
2.0	5.0 ± 3.3ab	0.7 ± 0.4c	0.7 ± 0.2c	0.1 ± 0.0c	3.5 ± 0.2b	8.0 ± 3.7a	14.6
2.5	18.1 ± 3.2a	2.1 ± 0.9c	1.8 ± 0.8c	0.4 ± 0.1d	8.4 ± 0.8b	16.3 ± 3.5a	69.4
3.0	31.1 ± 13.4a	6.6 ± 2.8bc	2.6 ± 1.3 cd	1.1 ± 0.5d	15.2 ± 3.9b	39.7 ± 10.4a	23.7
3.5	43.1 ± 5.1b	7.2 ± 3.7d	5.3 ± 2.4d	1.4 ± 0.2e	28.3 ± 6.0c	62.0 ± 7.7a	77.6
4.0	47.8 ± 5.5b	9.8 ± 4.2c	7.1 ± 1.3c	1.3 ± 0.4d	30.6 ± 9.2b	92.9 ± 5.9a	90.4
4.5	46.8 ± 14.5b	12.8 ± 2.3 cd	19.1 ± 6.8 cd	3.9 ± 0.4d	36.4 ± 8.2bc	81.3 ± 19.2a	17.1
5.0	55.6 ± 4.7b	21.9 ± 2.9d	23.6 ± 3.8d	3.5 ± 0.8e	36.8 ± 11.6c	87.1 ± 2.7a	100.2
5.5	54.2 ± 4.8b	27.3 ± 2.1d	33.7 ± 10.1 cd	6.1 ± 0.3e	43.2 ± 11.4bc	96.0 ± 3.6a	46.4
Fitted parameters[c]							
K	52.8351	73.6002	46.8699	26.8259	40.9053	91.6937	
a	6.1843	5.4125	7.4496	6.0389	5.7615	7.7087	
r_d	−2.1725	−0.8913	−1.5182	−0.8695	−1.7797	−2.4759	
r^2	0.9814	0.9804	0.9853	0.9279	0.9797	0.9470	

[a] Table entries followed by different lowercase letters in each line differed significantly (Fisher's LSD, $P < 0.05$)

[b] All the F tests were significant at $P < 0.01$

[c] The parameters were estimated by fitting the logistic equation $D_i = K/[1 + \exp(a + r_d R_e)]$ with the measured dispersancy indices (D_i) over the ratios of emulsifier (R_e) in each of the fungal formulations. All coefficients of determination (r^2) were significant at $P < 0.001$

determination ($0.93 \leq r^2 \leq 0.98$; Table 2). The maximal potential of the emulsifier dispersancy to the oil formulation ranged from 26.8 (Mm978) to 91.7% (If6032). The fitted rates of dispersancy increase over the emulsifier ratios were inversely correlated to the H_r values of the six isolates ($r^2 = 0.94$), as illustrated in Fig. 5.

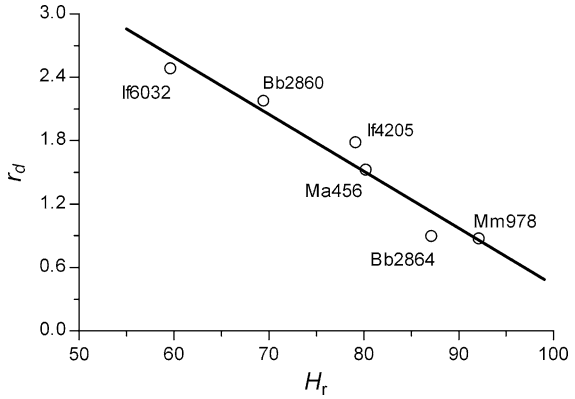

Fig. 5 Linear inverse correlation of the dispersancy increase rates (r_d) of the emulsifier to the hydrophobicity rates (H_r) of *B. bassiana* (Bb), *M. anisopliae* (Ma), *M. majus* (Mm) and *I. fumosorosea* (If) ($r^2 = 0.94$, $F_{1,4} = 62.1$, $P = 0.0014$)

Discussion

As presented earlier, both TFA-soluble protein content and surface area depending on conidial size are important traits for the conidial hydrophobicity of the tested fungal agents and thus useful for improving fungal formulation and field spray. Several aspects on fungal formulations are discussed later.

First of all, fungal biocontrol candidates are usually selected based on their virulence against target pests [1, 2] and tolerance or resistance to environmental stresses such as high temperatures [35, 36], solar UV irradiations [37–39] and fungicide sprays [40]. Apart from the inherent features associated with their biocontrol potential, our results highlight significant contributions of surface area and TFA-soluble protein content to conidial hydrophobicity (Fig. 2). Both traits are also inherent as is well known for fungal virulence and stress tolerance and thus need be taken into account for formulating a fungal candidate.

The TFA-soluble proteins examined in this study are apparently hydrophobins, although some of them are FA extractable. First, this was fully proven with

the two isolates of *B. bassiana* by the Western blots of the polyclonal antibodies (Fig. 3b), which were prepared based on known Hyd1 and Hyd2 [16]. The fact that a few FA-extracted, SDS-insoluble proteins from the two *I. fumosorosea* isolates became SDS-soluble after being dissolved with TFA indicates their same hydrophobic feature as observed from *B. bassiana*. The residue proteins of the *Metarhizium* isolates after the FA treatment were well extracted with TFA. The TFA-soluble proteins of both *M. anisopliae* and *I. fumosorosea* in the sizes of 10–14 kDa, although not examined by Western blot due to unavailable hydrophobin-encoded genes, could be biochemically similar to Hyd1 and Hyd2. This is because those proteins can be dissociated into the SDS–PAGE visible monomers with TFA only [11–13] and their molecules fall within the sizes of known hydrophobins [8]. Moreover, the hydrophobin-based rodlet layers [5, 14] were removed by FA for *B. bassiana* and *I. fumosorosea* or TFA for *M. anisopliae* (Fig. 4), accompanied by drastic H_r decreases (Table 1). These provide further support for the deduction. Thus, a source of variation in conidial hydrophobicity comes mainly from the content of the TFA-soluble proteins (hydrophobins) and the surface area per conidium. Thus, fungal candidates with desired hydrophobicity can be readily selected from the strains with high virulence and stress tolerance based on the two measurable traits.

From practical point of view, some degree of conidial hydrophobicity is essential for a fungal formulation because it favors conidial adhesion to insect cuticles [6, 19]. However, oil formulation of aerial conidia with high hydrophobicity cannot be readily emulsified into aqueous dilutions for qualified field sprays. The emulsifier tested in this study is usually added to the oil formulation at about 5% for field spray against sucking pests and proven biologically compatible with the conidia of the tested fungal species [28–30, 41]. This emulsifier ratio resulted in 55% dispersancy to the oil formulation of Bb2860 conidia with lower hydrophobicity, but less than 5% to the same formulation of Mm978 conidia with maximal hydrophobicity (Table 2). For each of the six isolates with different degrees of conidial hydrophobicity, the fitted K value provides a potential dispersancy of the used emulsifier toward the oil formulation. For instance, the aqueous dispersivity of the oil-formulated conidia predicted with the fitted equations can be enhanced to 51.2 and 46.4% by increasing the emulsifier ratio to 7 and 8% for the very hydrophobic isolates Bb2864 and Ma456, respectively, but to only 26.8% by elevating the ratio to 15% for the most hydrophobic isolate Mm978. With our experiences, about 50% dispersancy of the same oil formulation emulsified in water ensures acceptable field sprays by conventional sprayers for pest control [42]. A ratio of the tested emulsifier to achieve this dispersancy is ideally around 5%, not exceeding 8%, at a reasonable cost. The same principle may suit to other types of emulsifiers or wetting agents with different potential of aqueous dispersancy to a fungal formulation. The hydrophobicity-related traits elucidated in this study would help understand dispersancy potential of different emulsifiers or wetting agents to a formulation of selected fungal candidates and thus determine their use in the formulation design.

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