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SHORT COMMUNICATION

Method for determining the best hydrophilic-lipophilic balance (HLB) number for a compatible non-ionic surfactant in formulation development for aerial conidia of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae)

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A direct dropping method was developed to determine the best hydrophilic-lipophilic balance number of *Metarhizium anisopliae* conidia at different water content levels during dehydration. Results showed the best HLB number of the tested *M. anisopliae* conidia was 8; dehydration did not change the best HLB number but significantly increased wetting time.

Keywords: biological control; hydrophilic-lipophilic balance number; mycoinsecticides; *Metarhizium anisopliae*; surfactant; wettable formulations

At least 12 species or subspecies of fungi have been employed as active ingredients of mycoinsecticides and mycoacarricides (de Faria and Wraight 2007). *Metarhizium anisopliae* (Metschnikoff) Sorokin commercial products have comprised 58 of the 171 products that are undergoing registration, registered or marketed worldwide. Aerial conidia have been employed as active ingredient in most of *M. anisopliae* based products, such as Bio-Blast™ and Bio-Path™, mycoinsecticides commercialized in the US (Gunner, Agudelo-Silva, and Miller 1995; Rath 1995). Aerial conidia, either fresh harvested or dried of *M. anisopliae* are hydrophobic. Surfactants are used to harvest aerial conidia from culture plates, to prepare conidial suspensions for bioassays and field trials as well as to develop water-based commercial products. A variety of surfactants have been used to make water-based *M. anisopliae* conidial preparations and commercial products, including TDA (polyoxyethylene tridecyl ether), Tween 80 (polyoxyethylene sorbitan monostearate), and Triton X-100 (polyoxyethylene isooctylphenyl ether) (Jin, Grigas, Johnson, Perry, and Miller 1999; Pachamuthu and Kamble 2000; Malsam, Kilian, Oerke, and Dehne 2002; Polar, Kairo, Moore, Pegram, and John 2005). In most publications, selection of a surfactant for aerial conidia of *M. anisopliae* is solely based on compatibility

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between the surfactant and the conidia. Thus, the selected surfactant should reduce water surface tension and not have harmful effects on conidial viability. However, in commercial mycoinsecticide development, selection of a compatible surfactant is only the first step in water-based formulation development. Once a compatible surfactant is selected, further optimization will focus on the formulation physical properties, such as wetting time, suspension stability and uniformity. Non-ionic surfactants have been generally considered to be less toxic to microorganisms than ionic surfactants (Luz and Batagin 2005), which make them ideal candidates for use in water-based conidial suspensions of *M. anisopliae*.

Non-ionic surfactants consist of a molecule that combines both hydrophilic and lipophilic groups (or polar and non-polar groups), and it is the balance of the size and strength of these two opposing groups that Griffin (1949, 1954) called the hydrophilic-lipophilic balance (HLB) number. Griffin (1954) also established a method for calculating the HLB values of non-ionic surfactants. In Griffin's system, surfactants that were lipophilic in character were assigned lower HLB values and surfactants that were hydrophilic in character were assigned higher numbers. There are two methods, swirling and non-swirling methods, for the determination of the time required for complete wetting in wettable formulation development of chemical pesticides (Dobrat and Martijn 1994, reprint 2007). These methods could not be directly applied for measuring the HLB numbers of hydrophobic conidia without modifications. Using the HLB number for selecting surfactants has been applied to almost all industries wherever surfactants are needed. Jin, Streett, Dunlap, and Lyn (2008) reported a rotary shaking method for selecting the best HLB number of a compatible non-ionic surfactant for dried aerial conidia of *Beauveria bassiana* (Balsamo) Vuillemin, and found that wetting time, an easily measured parameter, could be used to optimize the physical properties of a potential formulation. In their report, conidial samples of *B. bassiana* were added to 250 mL flasks, each containing 100 mL of surfactant solution. Flasks were then placed on a rotary shaker and shaken at 125 rpm until all macroscopically visible clusters of conidia were fully suspended, and the wetting time recorded. Due to the difference in conidial physicochemical characteristics between *B. bassiana* and *M. anisopliae*, conidia of *M. anisopliae* can be wetted much faster than conidia of *B. bassiana*. Therefore, rotary shaking becomes unnecessary, and a specially designed devise and method are needed to allow easy loading and then even releasing of conidia. Moreover, conidia of both *B. bassiana* and *M. anisopliae* are usually dried after harvesting to induce dormancy for long-term storage and shelf-life. The relationship between the best HLB number and the conidial water content levels remains unknown. This paper reports a specially designed devise and a direct dropping method for determining the best HLB number of a compatible non-ionic surfactant in formulation development of areal conidia of *M. anisopliae* at different conidial water content levels during dehydration.

The strain F52 of *M. anisopliae* used in these studies was provided by the USDA-ARS-MSA, Southern Insect Management Research Unit, Stoneville, Mississippi. The fungus was first grown on Sabouraud Dextrose Agar plates at 26°C for 2 weeks, and then 4–5-mm discs cut from the growing edge of the *M. anisopliae* culture were transferred to 1-L flasks, each containing 500 mL of Streptomycete Productive Medium (SPM) broth (10 g glucose, 10 g yeast, 4 g K_2HPO_4, 2 g KH_2PO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4$, 0.2 g KCL, and trace minerals including

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0.002 g $FeCL_2$, 0.002 g $MnSO_4$ and 0.002 g $ZnSO_4$ in 1 L of deionized water). Sterilization was conducted at 121°C for 30 min prior to culture discs being transferred into the liquid culture flasks. The flasks were then placed on a rotary shaker and shaken at 125 rpm for 3 days at room temperature. For aerial conidia production, $SacO_2$ bags (Rozenstraat 1A, 9810 Eke, Belgium), each containing 1 kg of long grain enriched parboiled rice (Producers Rice Mill, Inc., Stuttgart, AK) and 600 mL DI water were autoclaved at 121°C for 70 min, and cooled overnight to room temperature.

The autoclave process was conducted on two consecutive days before 50 mL of *M. anisopliae* culture broth was injected into each bag. Then, mixing of the bags was conducted by hand-shaking. Inoculated bags were incubated at 26–28°C. Heavy conidiation started after 5–6 days of incubation and the rice surface turned dark green in about 8–10 days. The bags were cut open after 10 days of incubation, and the rice with *M. anisopliae* aerial conidia from each bag were equally divided and poured into two paper bags and allowed to dry overnight at room temperature. Aerial conidia were harvested by sieving through a 100 mesh screen (149 μm). The conidia were then placed in desiccators that contained 'Drierite' (Acros Organic, USA, One Reagent Lane, Fair Lawn, NY). The amount of Drierite added was calculated before this study to maintain the relative humidity (RH) level in the desiccators below 5% at room temperature. Conidia samples were taken from the desiccators to determine their water content levels at different times during the 3 days of dehydration. Water content levels of the conidia samples were determined using an HB 43 Halogen Moisture Analyzer (Mettler-Toledo GmbH, Laboratory & Weighing Technologies, CH-8606 Greifensee, Switzerland). The initial conidial water content level of the conidia was 50%.

The surfactant used in this study was Ethal TDA (polyoxyethylene tridecyl ether, Ethox Chemical, LLC, Greenville, SC) with different HLB numbers. TDA surfactant under HLB 8 is not commercially available and therefore TDA 3 (HLB = 8) and TDA 9 (HLB = 13) were used to make TDA mixtures of HLB 9, 10, and 11 according to Griffin's (1949) formula. The wetting study was performed using TDA mixtures with HLB numbers of 8, 9, 10, and 11 to determine the best HLB number of TDA surfactant.

A direct dropping method was employed to determine the best HLB numbers for aerial conidia of *M. anisopliae*. A three-prong extension clamp was mounted on a standard laboratory support stand, which held a glass funnel tightly. The top opening diameter of the glass funnel was 8 cm and the tube diameter at the apex was about 1 cm. A 250 mL beaker containing 100 mL of 0.2% surfactant solution in deionized water was then positioned in the center under the tube at the funnel apex. The end opening of the tube at the apex was 1 cm above the edge of the beaker. A 2.5 × 7.5-cm microscope glass slide was held flush against the end opening of the tube at the apex tightly. With the glass slide in place, a 0.1-g conidial sample was added into the funnel. It was necessary to tap the top of the funnel lightly to ensure that all conidia were in the tube at the apex of the funnel on the glass slide. Quickly and evenly, the glass slide was removed away from the tube opening allowing the conidia to fall into the wetting solution, and timing was started. Wetting time was recorded when all macroscopically visible clusters of conidia were fully suspended in the surfactant solution, and the HLB number that resulted in the shortest wetting

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time was the best HLB number. The best HLB numbers for *M. anisopliae* conidia were determined at conidial water content levels at 50, 17, 9 and 5%, respectively.

This experiment was a randomized complete block with factorial treatment structure of 4 TDA HLB numbers and 4 conidial water content levels. There were three replicate blocks, and two samples were taken for each replicate. Analysis of variance (ANOVA) was performed using a general linear mixed model and the Least Square *Post Hoc* tests to identify significant differences among treatments at 0.05 levels (Littell, Milliken, Stroup, Wolfinger, and Schabenberger 2006).

Our results indicated that TDA HLB number 8 was the best one we found for the tested conidia of *M. anisopliae* regardless of the conidial water content levels (Figure 1). The interaction between TDA HLB numbers and conidial water content levels was not significant ($F = 1.62$, df $= 78$, $P = 0.1244$). The main effect of HLB numbers on wetting times was significant ($F = 91.91$, df $= 78$, $P < 0.0001$,), and TDA HLB 8 required less wetting time than TDA solutions with other HLB numbers. Griffin (1949) indicated that surfactants with lower HLB numbers (4–6) were mostly used as emulsifiers (water-in-oil), while those with higher HLB numbers (13–15) were detergents, and surfactants with HLB numbers between 7 and 9 were superior candidates for wetting agents. Understanding these HLB guidelines, other generally used laboratory surfactants, Tween 80 (HLB = 15), Triton X-100 (HLB = 13.6) and Span 80 (HLB = 4.3) (Gennis and Strominger 1976; Kruglyakov, Mal'kov, and Sedova 2002) should be excluded from the selection of wetting agents for water-based formulations of hydrophobic conidia because their HLB numbers were either in the range of detergents or in the range of water–oil emulsifiers (Griffin 1949). HLB number 8 was within the range of wetting agents (HLB = 7–9), and therefore provided the least wetting time compared with other TDA solutions that had different HLB numbers. Fast wetting is one of the most important characteristics that a successful commercial product must have for field application. Jin et al. (2008) showed that the fastest wetting times resulted in the highest conidia concentrations

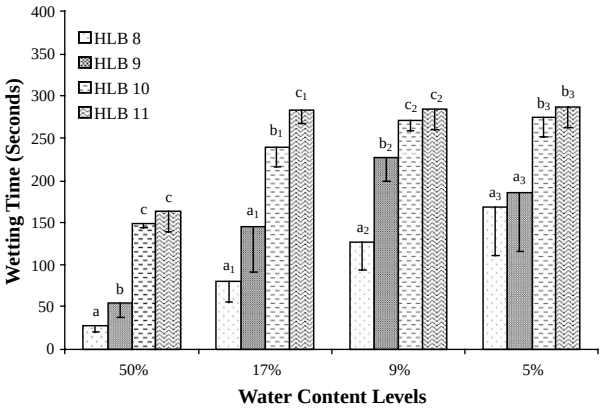

Figure 1. Times required for wetting 0.1 g aerial conidia of *Metarhizium anisopliae* at different water content levels in 100 mL of 0.2% polyoxyethylene tridecyl ether (TDA) solutions of different hydrophilic-lipophilic balance (HLB) numbers employing a non-swirling/direct dropping method. Values are the means of three experiments. Means with different letters within each conidial water content level are significantly different ($P < 0.05$).

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after a fixed mixing time. The quantity of suspended conidia in a surfactant solution is a critical issue for commercial product development because higher conidial concentration should improve efficacy. For commercial product development, conidia of *M. anisopliae* are always dried to a certain water content level, usually below 7% to induce dormancy for prolonged storage or shelf-life. It is unknown if the best HLB number of a compatible non-ionic surfactant changes as a function of conidial water content. Our results showed that reducing conidia water content levels did not result in changes of the best HLB number (Figure 1). However, the wetting times were significantly increased as conidia water content levels were reduced ($F = 53.6$, df $= 78$, $P < 0.0001$). Dehydration of conidia can result in reduced size, altered shape, and crenellated surfaces, but this does not necessarily change surface or other physicochemical characteristics. Our data supported this idea as conidia with lower moisture levels had the same best HLB number as the conidia with higher moisture levels. Dried *M. anisopliae* conidia are more stable and have a longer shelf life than undried conidia, however conidial vigor can be reduced with prolonged storage time due to the loss of reserved materials, such as sugars. Conidial vigor, as distinguished from viability, is a quantitative trait relating to the rapidity of conidial germination and germ tube growth (Jin, Hayes, and Harman 1992), and is one of the important characteristics of a biological control agent. Conidial vigor can be weakened by processing and long-term storage, and can cause slow germination that is poorly synchronized. Thus, dried conidia can have a lower germination rate with respect to time than the fresh ones without a loss in viability. Jin et al. (1999, 2008) reported that surfactants that had the best HLB numbers improved the germination rate of dried conidia of *M. anisopliae* and *B. bassiana* with lower vigor after long-term storage due to faster wetting, better water absorption, and accelerated activation.

In this report we introduced a method for determining the best HLB number of a compatible non-ionic surfactant in formulation development of aerial conidia of *M. anisopliae*. Mycoinsecticide development is different from that of chemical pesticides. Selection of the right method to determine the best HLB number of a compatible non-ionic surfactant is usually based on a variety of factors, such as species or strains and even mass production methods that govern the surface physicochemical characteristics of conidia of the entomopathogenic fungi (Hegedus, Bidochka, and Khachatourians 1990; Hegedus, Bidochka, Miranpuri, and Khachatourians 1992; Jin et al. 2008). According to Griffin's theory (1954), HLB number and correct chemical group are the two most important factors to be concerned with in selecting the proper surfactant for any application. TDA is a convenient non-ionic surfactant employed in determining the best HLB number because its HLB numbers can be tailored to a wide range of values and tested, and it is also compatible with both *M. anisopliae* and *B. bassiana* (Jin et al. 1999, 2008). Our previous study reported that employing TDA as the wetting agent, the rotary shaking method should be used for water-based product development of *B. bassiana* (Jin et al. 2008) because if direct dropping method was used, the dried conidia of *B. bassiana* floated on the surface of the surfactant solutions for hours, even days to achieve total wetting (Jin, unpublished data). Under this circumstance, the rotary shaking method was employed to accelerate the testing procedure for accurate results. Results from this study showed that direct dropping method was the preferred technique for determining the best HLB number for the development of *M. anisopliae* conidia

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based wettable formulations because conidia were wetted quickly; the procedure was simple and easy to conduct, making rotary shaking method unnecessary.

Using the HLB number to determine the best HLB number of a compatible non-ionic surfactant is a powerful tool for the development of biological pesticides when hydrophobic conidia are employed as an active ingredient. The HLB classification system offers some indication of physiological characteristics of a biological pesticide and reduces the labor required for selection of the best surfactants for both laboratory studies and product development of hydrophobic biological control agents (Jin et al. 2008). However, commercial formulation development of biological pesticides is a complicated process; consideration should be given to the final assembly for a successful product.

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