Mortality of *Solenopsis invicta* Workers (Hymenoptera: Formicidae) After Indirect Exposure to Spores of Three Entomopathogenic Fungi

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Abstract

Mortality caused by indirect exposure to *Metarhizium brunneum* and *Beauveria bassiana* (GHA and NI8) to the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), workers was evaluated. Groups of 50 workers were placed in one side of dual-box arenas. The opposite side of the arenas was lined with filter paper squares previously sprayed with unformulated purified spores (10⁶ spores/ml) suspended in 0.2% EthalTDA 3, HLB 8 of the three fungal strains, or untreated filter paper squares as the control. Daily observations were done for 1 wk to determine mortality. Dead ants from each treatment and control were collected, surface cleaned, and placed in PDA media and incubated at 27°C, 60% RH for 7 d to detect fungal growth. The presence of fungal growth in the dead ants confirmed that fungal spores infected workers while walking on the treated paper. In the *M. brunneum* and *B. bassiana* GHA treatments, 51.35 and 56.68% of the workers died, respectively, during days 1 and 2. However, only 9.47 and 35.96% of the mortality could be explained by fungal infection by *M. brunneum* and *B. bassiana* GHA, respectively. Most of the mortality observed in the *B. bassiana* NI8 treatment (84.48%) occurred later (between days 4–6) and most of this mortality occurring during day 4 (89.06%) could be explained by *B. bassiana* infection. Overall mortality was significantly higher in the *B. bassiana* NI8 treatment than the other two fungi tested and control. Potential application of these fungal strains for fire ant control are discussed.

Key words: red imported fire ant, entomopathogen, *Beauveria bassiana*, *Metarhizium brunneum*, infection

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), is one of the most successful invasive ants and it is regarded as one of the World’s worst invasive alien species (Lowe et al. 2000). Native to South America, *S. invicta* has been introduced into many countries and regions, including the United States, Australia, China, the Philippines, Thailand, Taiwan, Hong Kong, Macau, among others (Ascunce et al. 2011). A solution for alleviating the heavy dependence on synthetic insecticide is to implement integrated pest management (IPM) strategies that combine different control practices to overcome the shortcomings of individual practices. Biopesticides, pest control agents based on pathogenic microorganisms or toxic natural products, have become a critical component of IPM in recent years (Chandler et al. 2011).

A tremendous effort has been made over the past few decades to mitigate fire ant problems using biological control agents. These agents include parasitoids, such as phorid flies (Porter 2000, Porter and Briano 2000, Gilbert and Patrock 2002, Gilbert et al. 2008, Callcott et al. 2011), fungi (Broome et al. 1976, Klotz et al. 1994, Briano et al. 1995, Bextine 1998, Bextine and Thorvilson 2002), bacteria (Landry and Phillips 1996, Dedene et al. 2005, Bouwma et al. 2006), microsporidia (Jouvenaz 1984, Oi and Williams 2002, Oi et al. 2004, Fuxa et al. 2005, Oi et al. 2005), viruses (Valles et al. 2004, Valles et al. 2007, Valles et al. 2008, Valles and Hashimoto 2009), and nematodes (Nickle and Jouvenaz 1987, Morris 1989, Drees et al. 1992, Jouvenaz and Martin 1992, Brinkman and Gardner 2000). However, there is not a report of a single biological control agent that has been sufficiently effective against fire ants to become commercially viable.

The entomopathogenic fungi that have been tested include species in the genera *Beauveria* and *Metarhizium* (Bextine and Thorvilson 2002, Fuxa and Richter 2004). Both *Metarhizium brunneum* Petch (formerly *M. anisopliae* F52) (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Balsamo-Crivelli) (Hypocreales: Clavicipitaceae) Vuillemin are EPA-approved insect biological control agents and are currently used in various field applications. Although both have shown great promise in the laboratory, they have been less successful under field conditions. Hence, no ant control products using these fungi are available commercially. Part of the problem is that ants have evolved a number of strategies to combat pathogens (Schmid-Hempel 1998, Cremer et al. 2007); among those are grooming, necrophoric behaviors (Qiu et al. 2014, Qiu et al. 2015), trophallactic
behavior (de Souza et al. 2008 and Qiu et al. 2016), as oral transfer of chemical cues, growth proteins and hormones (Leboeuf et al. 2016). The virulence of a fungus can be reduced by ant defensive alkaloids (Storey 1990) and recently, some nest volatiles of the red imported fire ant have been identified as possessing antimicrobial properties that significantly reduce the germination rate of B. bassiana spores when exposed to these nest volatiles within an artificial ant nest (Wang et al. 2015). Thus, fumigation may be a component of ant social immunity.

There are several ways to improve the virulence of a fungus strain: pressure selection, genetic improvement, and mutation (Jin et al. 1992). Increasing the resistance of fungi to ant defensive chemicals may increase their virulence to ants. Resistance to abiotic stresses can be improved by using a direct evolution method. For example, using an automated continuous culture method called the Elevatorator, which takes advantage of a natural selection-adaptation strategy, a strain of M. brunneum has been successfully improved (de Crecy et al. 2009). The selected strain displayed robust growth at 37°C. In contrast, the unselected strains displayed little to no growth between 35 and 37°C. Similar approaches may be useful to select B. bassiana and M. brunneum for higher resistance to chemical stress inside an ant nest. Those variants may have higher virulence to fire ants. In addition to strain manipulation, improvement to the infectivity of fungi or other pathogens on ants can also be achieved by weakening ant’s immune reactions, making ants more susceptible to infection (Santos et al. 2007). Some ant behaviors, such as grooming and necrophoresis play an important role in defense responses against pathogens. Some chemicals can affect the behavior of social insects at sublethal doses. For example, imidacloprid inhibits self-grooming behavior of the leaf-cutting ant, Acromyrmex subterraneus Forel and make it more susceptible to B. bassiana (Galvanho et al. 2013). Imidacloprid significantly increased the susceptibility of ants, in general, to infection by B. bassiana (Santos et al. 2007).

The objective of this study was to determine if any of the Mississippi native fungal strains currently available at United States Department of Agriculture South East Area, Biological Control of Pest Research Unit (USDA SEA BCPRU), Stoneville, MS have a potential for the management of red imported fire ant workers, due to the fact that there is a history of these entomopathogenic fungi being effective in infecting several agricultural insect pests, and that the spores of these strains have maintained their entomopathogenicity for the past 10 yr making them good candidates to produce a detrimental effect on the red imported fire ant.

Materials and Methods

Rearing Procedures

Three and fifteen fire ant colonies were collected from shoulders of a gravel road South of Washington County, MS in November 2015 and 2016, respectively and transferred to 20-liter plastic buckets. Ant and brood were cleaned from dirt using the traditional dripping-flooding method (Jouveux et al. 1977). Then, ant colonies were individually transferred to trays whose walls were coated with fluon (P. No. 2871C, Bioquip, Rancho Dominguez, CA) to prevent escape. Once in the laboratory, each tray was provided with a culture glass tube containing RO water, a glass tube with 10% sugar, 7 g of each frozen Tenebrio molitor L. (Coleoptera: Tenebrionidae) pupae, and 7 g of frozen house crickets, Acheta domestica (L.) (Orthoptera: Gryllidae) on Mondays, Wednesdays, and Fridays. Water and sugar solutions were provided as required.

Entomopathogen Origin and Production

The three fungal strains, M. brunneum (MB), B. bassiana strain GHA (GHA) and B. bassiana NI8 strain (NI8) were tested. Strains of B. bassiana (NI8 and GHA) were originally isolated from field collected Lygus spp. in the Mississippi Delta (Leland et al. 2005) and initially obtained from the Southern Insect Management Research Unit (SIMRU), Stoneville, MS and stored as dried conidia. These have remained stable at ~-20°C for up to 12 mo as confirmed with periodic germination examination. Conidia from M. brunneum also obtained from SIMRU cultures were aseptically transferred into a solution of 15% Glycerol (Fisher, BP229-1) and stored at ~-20°C, with percentage germination confirmed prior to use.

Strains of the target isolates were aseptically transferred onto potato dextrose agar, PDA (39 g/liter) (P. No. 213400, Difco, Fisher Sci. Waltham, MA) and incubated at 27°C for 7 to 14 d. Sterile cotton swabs (Cat. No. 25-806-10WC, Puritan, Guilford, ME) wetted with 0.2% Ethal TDA 3 (polyoxethylene tridecyl ether) HLB 8 solution (Ethox Chemicals, LLC, Greenville, SC) were used to gently remove conidial spores from the surface of the media plate. The swab was then placed into a known volume of 0.2% TDA 3 HLB 8 and agitated to remove the spores from the swab tip. Conidia of both, B. bassiana and M. brunneum are highly hydrophobic with B. bassiana being the more strongly so (Jin et al. 2008, 2009, 2013). However, 0.2% TDA 3 HLB 8 solution can be utilized to successfully suspend both in water-based formulations. A Neubauer Brightline hemacytometer (P. No. 3120A, Hauser Scientific, Horsham, PA) was used to determine spore density along with germination in an effort to determine viability.

Fungi Screening for Repellency and Mortality to Fire Ant Workers

All fungal strains were grown on PDA plates at 28°C for 2 wk, and then conidia were washed with the 0.2% Ethal TDA 3 HLB 8 solution to supply a fresh conidial preparation for each study. The germination of all conidia used in this study was greater than 94%. High concentrations (10⁶ spores/ml) of each fungal spore were suspended in 0.2% Ethal TDA 3, HLB 8 and sprayed onto 5 x 5 cm squares (3 ml per square) of sterile filter paper (P. No. 28320-020, VWR, Radnor, PA). Dual arenas consisting of 7 x 7 x 3 cm plastic boxes were used to perform the bioassays (Fig. 1A). Each dual arena was constructed by joining them at the base on the center of one side with 3 x 1 cm ID long flexible tube (TYGON, Saint-Gobain, Malvern, PA) (Fig. 1A). The sides of each arena were coated with a layer of undiluted fluon and left to dry at room temperature for 24 h. Cotton was then securely placed inside the tygon tubes to prevent ant movement from arena to arena. Ten percent sugar solution and water were separately pipetted into 2 ml plastic centrifuge tubes (Fig. 1C, c and d). The opening of the tubes were covered with a piece of cotton to prevent spillage. One of each tube was placed in one of the arenas and only a T. molitor pupa as a food source (Fig. 1C, e). Fifty fire ant workers of multiple sizes were manually transferred to the arena containing the food using a wooden stick and soft forceps. Once all ant workers were in place, a piece of the fungal spore sprayed filter paper was placed in the empty side of the arena with 5 x 5 cm squares of filter paper sprayed with 3 ml of 0.2% Ethal TDA 3, HLB 8 used as the control (Fig. 1B, b). Finally, the cotton plug covering the tube was removed to allow ant movement. Twelve repetitions per each fungal strain and control were performed. Treatment and control arenas were placed in a controlled environment room at 27°C, 70% RH and 16:8 (L:D) h cycle. After 24 h, the pieces of filter paper were removed from the arenas and placed onto PDA plates to check...
for sporulation. Daily ant mortality was recorded for a period of 7 d. Dead ants were surfaced sterilized with a 0.6% sodium hypochlorite solution for 5 min followed by a 5 min wash in sterile RO water. Each ant was aseptically transferred to 2% water agar plates (P. No. 214530 Difco, Fisher Sci.) and incubated at 27°C and 60% RH for 7 d to check for post-mortem sporulation (Fig. 2). Percentage

Fig. 1. (A) Experimental arenas connected by a flexible tube (a). (B) One side contained a square of filter paper either blank or sprayed with fungal spores (b). (C) The second side contained 50 fire ant workers provided with water (c), a sugar solution (d), and two *Tenebrio molitor* pupae (e) as food.

Fig. 2. Cadaver of a fire ant worker showing *B. bassiana* growth in PDA plate after incubation.
mortality was used to determine which one of the fungal strains was the best candidate for further studies.

### Preliminary Evaluation of \( B. \text{ bassiana } \) NI8 Formulation in Confined Fire Ant Mounds

Because \( B. \text{ bassiana } \) NI8 showed to be the most promising of the tested fungal strains in killing fire ant workers in the dual arenas, this fungal strain was chosen to be preliminarily tested for its efficacy in killing fire ant mounds. Twelve fire mounds and mounds about (12 cm high × 12 cm wide) were collected alongside roads in Washington Co. MS. The mounds were individually transferred into 45.1 × 38.7 × 27.3 cm string Light boxes (Part No. 7875LWRD, Homz, Chicago, IL) in which sides were previously coated with Fluon to prevent escapes. All boxes with ants were placed on the floor of a greenhouse at 27°C and 35% RH and 16:8 (L:D) h cycle. A glass vial with RO water, a glass with 5% sugar solution, and \( T. \text{ molitor } \) pupae were provided as food source. Three empty 50 ml glass vials per nest were gently inserted into the soil to allow ants to move brood and queen into them, and 100 g hydrated (4 g per 250 ml RO water) polymer (T-400 Terra Wet, San Diego, CA) was manually inoculated into the soil to maintain moisture. After 24 h, each nest was connected to a 36 × 24 × 10 cm rectangular tray which sides were also coated with Fluon. A 5-cm-wide thick cardboard stripe attached to a piece of wire was used as a bridge to allow the ants to forage for food, water, and to remove dead ants from nest. Six trays were used as treatment and six as control.

The \( B. \text{ bassiana } \) NI8 spore formulation consisted of 0.250 g of NI8 spore powder mixed with 30 ml of 0.2% TDA (HLB8) to wet and suspend. The suspension was then poured into 100 ml of 6% sucrose and manually mixed until it turned homogenous. The resulting solution was sprayed onto 440 g of corn grits (Jim Dandy Enriched Quick Grits) using a Fluid Bed (Model STREA-1, Niro Inc., Columbia, MO), bottom spray with short booster tube technique with the following settings, Nozzle: 1 bar, fan: 6, and 40°C. The formulation was placed in 30 ml plastic centrifuge tubes and stored in the dark at 4°C until use.

Ants were allowed to settle and regroup for 4 d after the mounds were collected; then dead ants present in the foraging tray were removed. Five-hundred milligrams of the formula was weighed into 10-cm-diameter Petri dishes and placed on the far end of foraging tray to allow ants to carry it to the nest. Control boxes were provided with formula without spores. Fresh formula was added every other day for a period of 30 d. Every 2 d the vials placed in the nest box were checked with an augmentation lens to determine larval mortality.

### Preliminary Field Evaluation of \( B. \text{ bassiana } \) NI8 Formulation

A preliminary field test consisted of the treatment of 35, 1-yr-old fire ant nests housed at the base of different types of trees and flower beds in an area surrounded by buildings. An adjacent parking lot landscaped with grass medians, with oak trees and shrubs was also treated. An area close to a greenhouse was used as the control which housed 15 ant mounds.

Three holes of about 5 cm deep were made directly on the sides of each ant mound with the bottom of the centrifuge tube. Approximately 5 g of the fungal formulation described above was placed into the hole and then covered with soil to protect spores from sunlight in September 2016. Weekly application was done for 1 mo, then every 2 wk for another month. Treated area and control were inspected for mound growth and evidence of dead ants every 2 d until the end of December 2016. Control area remained untreated and was monitored as above. Ant mounds that showed no ant activity were excavated for evidence of brood or other signs of life. Additional monthly observations continued through 2017 until March 2018, but no new applications were done.

### Data Analysis

Contingency table analysis was used to determine differences in mortality among treatments and control by analyzing data consisting of total number of live and dead individuals from each treatment at the end of the 7-d experiment. Analysis of means (ANOM) for proportions of JMP software ver. 11 was used to determine significant deviations from the 95% confidence levels of the mean in mortality rates among treatments and control (Nelson et al. 2005, SAS Institute 2013a). Survival analysis was used to determine differences in daily mortality distribution patterns from day 1 to 7 among the treatments and control. Data of the number of individuals that did not die at the end of the experiment were censored. The Weibull distribution model was used to compare treatment mortality across time using JMP software ver. 11 (SAS Institute 2013b).

### Results and Discussion

The contingency table analysis revealed significant differences in mortality among treatments and control \((\chi^2 = 123.11, df = 3, P < 0.0001)\). The total mean proportion of dead individuals was 0.3017 and the 95% deviation level was ± 0.04. The control group was below the 95% lower deviation level (LDL) with a value of 0.175 and treatment NI8 exceeded the 95% upper deviation level (ULD) with a value of 0.4616. Treatments MB and GHA did not exceed either LDL or UDL with values of 0.308 and 0.261, respectively (Fig. 3). These results can be interpreted as the control group having significantly lower mortality than all the treatments and treatment NI8 having significantly higher mortality than the rest of the groups (Nelson et al. 2005). Individual comparisons of these proportions using the Z-test confirm the results as the mortality in the control was significantly lower than that in treatments MB, GHA, and NI8 with Z values of 5.39, 3.63, and 10.65 and P values of <0.0001, 0.0002, and <0.0001, respectively. Also mortality in treatment NI8 was significantly higher than treatments MB and GHA, with Z values of 5.46 and 7.21, respectively, both with \(P < 0.0001\). These results indicate that ants that were indirectly exposed to unfumigated spores from the three pathogens experienced higher mortality

![Fig. 3. Graphic analysis of means (ANOM) showing significant departures from the 95% deviation level (±0.04) from the mean (0.3017).](image-url)
than ants that were not exposed to any entomopathogen as the control groups, but unformulated NI8 spores produced significantly more mortality than the other two pathogens in ant groups within a period of 7 d of indirect exposure.

Mortality occurred earlier in treatments MB and GHA and more than 40% of the total mortality occurred during day 1 in these two treatments (Table 1). In treatment NI8 mortality occurred later, more than 80% of the total mortality observed in this treatment occurring during days 4–6 (Table 1). Survival analysis confirmed significant differences in mortality distribution among treatments and control ($\chi^2 = 105.15$, df = 3, $P < 0.0001$) (Fig. 4).

Entomopathogenic fungi were isolated from ant cadavers in treatments MB, GHA, and NI8. Fungi from treatment MB were identified as *M. brunneum* and fungi from treatments GHA and NI8 as *B. bassiana*. From a total of 185 cadavers from treatment MB, *M. brunneum* was isolated from 19 of the cadavers or 10.27%. *B. bassiana* was isolated from 48 and 132 ant cadavers from a total of 157 and 277 or 30.57 and 47.65% in treatments GHA and NI8, respectively. No entomopathogenic fungi were isolated from ant cadavers from the control group, but *Aspergillus* sp. appeared in 17 cadavers from day 5 (Fig. 5). Most of the mortality in treatment MB occurred between days 1 and 2 (51.35%), but only 9.47% could be attributed to *M. brunneum*. Similarly, in treatment GHA, 56.68% of the total mortality occurred during days 1 and 2, but only 35.96% could be attributed to *B. bassiana*. On the other hand, 84.48% of the total mortality in treatment NI8 occurred during days 4, 5, and 6 and 51.71% of that mortality could be attributed to *B. bassiana*. Most remarkably, 89.06% of the mortality occurring during day 4 in the NI8 treatment could be attributed to *B. bassiana* (Fig. 5).

Although treatments MB and GHA showed significantly higher mortality of ant workers than the control, most of this mortality occurred earlier and most of it could not be explained by entomopathogen infection (Fig. 5). Mortality occurring in these two treatments may be better explained by toxicity of the fungal spores of *M. brunneum* and *B. bassiana*. *M. brunneum* produces destruksins, which are known to possess insecticidal characteristics (Strasser et al. 2000, Schank and Vainstein 2010). Suzuki et al. (1970) isolated Destruxins C and D and Desmethyldestruxin B from *M. brunneum* showing insecticidal effects. In addition, *M. brunneum* spores can secrete lipases, proteases and chitinases that can compromise the insect cuticle (Schank and Vainstein 2010). Damage to the cuticle by *M. brunneum* spores may produce severe dehydration and death to insects if exposed to high concentrations of the spores. Kučera and Samšiáková (1968) isolated two proteases from *B. bassiana* cultured in an artificial medium, which were toxic to *Galleria mellonella*. *B. bassiana* is known to produce beauvericin (Gupta et al. 1995) and bassianolide (Suzuki et al. 1977), which have insecticidal effects of varying intensity depending of the insect species (Strasser et al. 2000). Production of toxins by entomopathogenic fungi differ among strains and are typically much more abundant when fungi are grown in culture media or in commercialized formulations (Strasser et al. 2000). In contrast, the NI8 treatment produced little mortality during days 1–3 and produced higher mortality during days 4–6, most of which (51.71%) could be explained by fungal infection. Although the overall mortality induced by fungal infection was relatively low in this study, the fact that ant workers were infected by indirect contact with the spores and not by direct spraying is significant. Also, the experimental design did not warrant that all ant workers were exposed to fungal spores and only those workers that explored the side containing the treated filter paper had the opportunity of being infected. Siebeneicher et al. (1992) observed increased mortality (over 80%) in red imported fire ant workers after walking on a surface contaminated with *B. bassiana* spores. Although *B. bassiana* spores were detected in the head, antennae, abdomen, and legs of ant workers, the authors determined that the most likely route of infection was through the tarsi were germination was detected. Another route of infection was by ingestion of conidia, but this was observed only in the larger workers and last instar larvae, which were able to ingest conidia because of their larger size. The authors recommended the selection of strains with smaller conidia that could be ingested by worker ants (Siebeneicher et al. 1992).

The *B. bassiana* NI8 formulation was carried into the ant nests and consumed by the ants in the greenhouse experiments using

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**Table 1.** Daily and cumulative mortality of *S. invicta* workers in groups of 50 workers (12 groups per treatment) after indirect exposure to unformulated spores of three entomopathogens and a control with no exposure.

| Day | Control | MB | GHA | NI8 |
|-----|---------|----|-----|-----|
|     | Daily | Cumul. | Daily | Cumul. | Daily | Cumul. | Daily | Cumul. |
| 1   | 8     | 8    | 81   | 81   | 65   | 65   | 10    | 10    |
| 2   | 6     | 14   | 14   | 95   | 24   | 89   | 19    | 29    |
| 3   | 2     | 16   | 8    | 103  | 10   | 99   | 8     | 37    |
| 4   | 50    | 66   | 11   | 114  | 16   | 115  | 64    | 101   |
| 5   | 29    | 95   | 33   | 147  | 12   | 127  | 96    | 197   |
| 6   | 3     | 98   | 25   | 172  | 15   | 142  | 74    | 271   |
| 7   | 7     | 105  | 13   | 185  | 15   | 157  | 6     | 277   |
confined ant colonies. It took between 2.5 to 3 mo for all the ant workers and brood confined in the plastic containers to die in the treatment group. All the ant colonies in the treatment group were confirmed dead by the end of the 90 d experimental period. All the ant colonies in the confined control groups were alive at the end of the same period.

Preliminary direct field mount treatments done in September 2016 appeared to be effective in killing fire ant workers and brood. By December 2016, all treated ant mounds were inactive while ant mounds in the control area were still active. Excavated inactive treated mound showed no brood or living workers. Monitoring continued for all 2017 to February 2018. Up to the end of February 2018, ant activity in the treated area was limited to three small mounds which appear to be from new founding queens. Ant activity in the control area was high coming from 10 very well established ant mounds, even after this area was chemically treated during spring 2017.

Siebeneicher et al. (1992) reported that baits were not as effective as carriers of *B. bassiana* due the inability of fire ant workers to ingest the conidia. The formulation used in the preliminary greenhouse and field tests in this study, based on spore-sprayed grits, functions in a similar way as a bait because ants tend to carry the contaminated grits into the nest and feed it to the larvae. Measurements of *B. bassiana* NI8 and GHA were done using an Olympus BX60 compound microscope fitted to a Leica DFC 420 digital camera and analyzed with Leica Suite V4.4 imaging system. The conidial size of *B. bassiana* NI8 and GHA was similar to that reported for most commercial strains derived from ATCC-74040 (2–3 μm) (Wright and Chandler 1995), but larger than that reported by Siebeneicher et al. (1992) (1–2 μm). Because conidial size was similar between strains NI8 and GHA and larger than that reported by Siebeneicher et al. (1992), conidial size does not explain the higher virulence observed in the NI8 strain. The higher virulence of the NI8 strain of *B. bassiana* is probably due to other characteristics of the strain and requires further investigation. Based on the results from this study we conclude that *B. bassiana* strain NI8 is a promising entomopathogen of the red imported fire ant and deserves further studies to improve formulation efficacy and delivery for effective biological control of the red imported fire ants in field conditions.

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