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Effects of drying on the survival of conidiosphores of *Metarhizium anisopliae* var. *acridum* Driver & Milner

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Abstract

Metarhizium anisopliae var. *acridum* Driver & Milner (= *Metarhizium flavoviride* Gams & Rozsypal), isolate CG423, is being developed as a mycoinsecticide against grasshoppers in Brazil. Conidia were harvested and stored in a drying chamber during a study period of 260 d, in a room at ~25 °C. Water content (dry weight) of fresh and dried conidia was 70 and 4%, respectively. Conidia from fresh and dry preparations produced similar *in vitro* germination patterns and were both infective of the American grasshopper *Schistocerca americana* Drury. The drying process greatly enhanced the survival of CG423 conidia, as witnessed by nearly 100% germination rates after more than a 100-d desiccation period. Prolonged 135 and 176-d storage in the drying chamber at room temperature decreased viability to 72.5% and 28.5% respectively. In contrast, fresh conidia sealed in plastic bags and stored at ambient conditions for 52 d were inactive (<10% viable). The low germination rate (28.5%) of conidia exposed to 176 d in dry storage was altered by exposure of the dry conidia to high humidity (96% RH). Dry conidia exposed to a 4-h rehydration period had germination rates of ~50%, whereas 24-h preincubation at high humidity resulted in ~90% germination rates. However, the germination rates were reduced at 204 (81%), 323 (70%) and 260 (23%) days in storage. In addition, preincubation of the CG423 dry conidia in water produced similar positive effects on germination as compared to germination of fresh conidia. At 24h post-inoculation, dry conidia produced significantly lower germination rates (21%) than rehydrated conidia (germination rates > 91.7%, $p < 0.05$). However, after 48h on SDAY plates, the germination rate of dry conidia (88.3%) was comparable to the germination of conidia rehydrated in water, water + Tween-20, or in high humidity.

Key words

Acrididae, entomopathogenic fungi, drying dessication, rehydration, conidia

Introduction

Over the past 2 decades severe outbreaks of the grasshopper *Rhammatocerus schistocercoides* Rehn (Acrididae: Gomphocerinae) have occurred in Brazil (1984 to 1988, 1991 to 1992) (Barrientos 1995), Colombia and Venezuela (1994 to 1996) (Lecoq & Assis-Pujol 1998). During outbreaks in Brazil and Colombia, populations of this insect caused severe damage in various crops including rice, soybeans, maize, sugar cane, and native pasture (Lecoq & Assis-Pujol 1998, Miranda *et al*. 1996). The biological cycle of the univoltine *R. schistocercoides* is similar in Brazil and Colombia: nymphal stages develop during the rainy season, adults during the dry season (Lecoq & Assis-Pujol 1998). In Brazil, this grasshopper is extremely gregarious. The nymphs are capable of dispersing from 10 to a few hundred meters (Lecoq *et al.* 1999), whereas adult swarms may cover 0.1 to 3 km per day (Lecoq & Pierozzi Jr. 1996).

In Brazil, *R. schistocercoides* control is based, presently, on the application of chemical insecticides, mainly malathion and fenitrothion (Cosenza *et al*. 1994). The control strategy involves locating hopper bands, and when needed, treating those bands threatening crops (Miranda *et al.* 1996, Lecoq & Balança 1998). Despite the efficacy of this management approach, there have been continued public concerns about the effects of these chemicals on the environment. As part of an integrated control strategy, the Brazilian government initiated a research project in 1994 to develop alternatives to chemical management.

The main focus of current research is the development and optimization of entomopathogenic fungi to control grasshoppers. The use of fungi to control grasshoppers is not novel; similar projects have been developed in Africa, Australia, and Mexico (see Lomer *et al.* 2001, Milner *et al.* 2003, Hernández-Velázquez *et al.* 2000). As a result of a survey for native grasshopper active mycopathogens, 7 isolates of the fungus *Metarhizium anisopliae* var. *acridum* Driver & Milner (= *Metarhizium flavoviride* Gams & Rozsypal) were isolated from the grasshopper *Schistocerca pallens* (Thunberg) in northeastern Brazil (Magalhães *et al.* 2001). These isolates were infectious to *R. schistocercoides*, and Vicentini & Magalhães (1996) determined that the isolate CG423 conidia produce penetrant germ tubes 12h post-inoculation (pi), followed by production of internal conidia at 3 d pi, and external sporulation 6 d pi. Initial characterization suggested that the Brazilian isolate CG423 was similar to other *variety acridum* isolates (Magalhães *et al*. 1997, Silveira *et al.* 1998, Driver *et al.* 2000). However, telomeric fingerprinting unambiguously differentiated the Brazilian isolates from the African and Australian *M. anisopliae* var. *acridum* isolates (Inglis *et al.* 1999). Presently the Brazilian isolate is being mass-produced using parboiled rice as substrate (Magalhães & Frazão 1996). Field applications of conidial preparations suspended in a racemic mixture of soybean oil and kerosene at rates of 1×10^{13} and 2×10^{13} conidia/ha caused 80 and 88% mortality rates, respectively, at 14-d post-application (Magalhães *et al.* 2000). Recently, this high dosage was reduced to 5×10^{12} conidia/ha with similar mortality rate (80%) (Faria *et al.* 2002).

An important consideration in developing *M. anisopliae* var. *acridum* as mycoinsecticide in South America is the optimization of post harvest handling of the conidia. Shelf-life of fungal products is universally dependent on temperature. However, predrying and maintaining conidia dry are key steps required for long shelf-life at room temperature (25 to 32 °C) (Moore *et al.* 1996). After 98-d storage at 28 to 32 °C, predried conidia stored as a powder with

silica gel had a high viability (76%), as compared to undried conidia stored without silica which did not germinate. The undried conidia of the Brazilian isolate CG423 also lost their viability within a few weeks when stored at room temperature. Additional studies conducted by Moore *et al.* (1997) determined that dried conidia of *M. anisopliae* var. *acridum* could be damaged if the rehydration phase was rapid, as occurs when dry conidia are directly exposed to free water. They reported that a short equilibration in a saturated atmosphere prior to immersion in water, maintained the viability and infectivity of dried conidia. Further studies by Hong *et al.* (2000) determined that the rate of drying influenced conidial viability: slow desiccation enhanced the survival of dried conidia.

In the following study we examined if drying could be used to increase the shelf-life of primary conidial preparations of the CG423 isolate of *M. anisopliae* var. *acridum*. More specifically, we report on the effects of drying on conidial viability, germination and activity and on the effects of rehydration on viability of dried conidial preparations.

Materials and Methods

Fungal culture.— *M. anisopliae* var. *acridum*, isolate CG423, was isolated from the pallid grasshopper *Schistocerca pallens* (Thunberg) in Brazil (Moreira *et al*. 1996) and has been maintained in the EMBRAPA Entomopathogenic Fungal Culture Collection (Tigano *et al.* 2002). This fungus was grown on Sabouraud dextrose agar amended with 1% yeast extract (SDAY) at 27°C. Conidia were harvested 12 d pi.

Drying and storage.—After harvesting, conidia were transferred to a drying chamber (Auto-desiccator Cabinet, Bel-Art Products®, Pequannock, NJ, USA) and maintained in a room at ~ 25°C. Conidia were stored in the drying chamber throughout the 260-d period. The relative humidity (RH) inside the drying chamber was kept at ~ 5% with the aid of a tray containing 500 g of Drierite (8 mesh) in the bottom. Fresh conidia were sealed in 10 plastic bags measuring 4 × 4 cm (Kapak SealPak, Kapak® Corporation, Minneapolis, MN, USA). These bags were maintained in a second chamber kept at ~25°C.

Moisture content.— Moisture content was quantified by weighing dried conidia (290 mg, 6 d in chamber) and fresh conidia (480 mg), before and after a 48-h incubation in a 90°C drying oven.

Loss of free water.—The kinetics of loss of free water from the conidia in the drying chamber was determined by weighing 3 samples of 400, 370, and 580 mg after 2, 19, 44, 64, 84, 104, and 144h in the drying chamber. Although preferable, it was not possible to work with a larger sample.

Germination.—In storage tests, viability of fresh conidia was determined at 1, 5, 11, 18, 24, 31, and 52 d post-harvest. For dry conidia, viability tests were performed daily in the first month and thereafter at 50, 80, 96, 104, 135, 151, 170, 176, 204, 232, and 260 d. Conidial viability was determined by plating conidial preparations (10^3/ plate) on SDAY plates. Conidia were considered to germinate if the germ tube was equal to or greater than conidial width at 24h post-inoculation. Measurements of at least 300 conidia or germ tubes/plate were collected from digital images. There were 2 replicates per treatment. Although preferable, it was not possible to work with a larger number of replicates. Means from dry and fresh conidia were compared with the Mann-Whitney Rank-Sum Test.

Rehydration.— Dry conidial preparations were rehydrated over 12h using 1 of 3 methods. In the first, conidia were exposed to a high relative humidity (RH = 96%) in a sealed container containing a saturated solution of potassium permanganate. In the second, approx. 10^8 conidia were suspended in 1 ml of aqueous Tween 20 (0.05%) and vortexed for 1 min. Finally, approx. 10^8 conidia were suspended in 1 ml of pure water and vortexed for 3 min. Preparations were then applied to SDAY medium and germination evaluated at 12, 24, 32, 36, and 48h intervals. Data were submitted to analysis of variance, and the means compared with a t-test.

Bioassay.— Fresh and dry (21 d in desiccator) conidial preparations were bioassayed against lab-reared 3rd instar nymphs of *Schistocerca americana* (Drury). Conidia were suspended in aqueous Tween 20 (0.05%) and vortexed for 1 min and diluted to a final concentration of 6.67×10^5/ml. Germination tests indicated >90% viability for the assayed conidial preparations. Insects were inoculated, with a deposition of 2000 conidia contained in a 3µl suspension, at the insertion of the hind leg. The insects were transferred to cages and fed leaves of romaine lettuce daily. There were 10 insects per cage and 3 cages per treatment. Mortality was recorded daily and tissue samples examined under the microscope to confirm the presence of fungi in dead insects.

Results and Discussion

The drying process collapsed the cell wall of CG423, producing conidia with a shriveled topography (Fig. 1). This shriveling might be partially caused by use of metabolites during storage by dry conidia. Fresh conidia, after harvest from the plates, had a moisture content of ~70%. The drying chamber employed in this study effectively removed the majority of free water (99%) within the initial 19h (Table 1). It took an additional 75h to remove the remaining 1% of free water. (Similarly, Hong *et al.* (2000), using different conditions, dried the IMI 330189 conidia to ~10% moisture content within 20 min.) After 7 d in the drying chamber, the moisture content of conidia was ~4%, the optimal moisture content for storage of isolate IMI 330189 (Moore *et al.* 1995).

The removal of free water from the conidia did not adversely influence germination (Table 2). Both dry and fresh conidial preparations produced detectable germ tubes at 12h. Within 15h both preparations exhibited over 70% germination. In fact between 15 and 18h the dry conidia exhibited significantly higher germination rates than did freshly harvested conidia. Microscopic analysis demonstrated that the drying process did not influence the swelling process nor the subsequent germ tube-elongation step. Conidial swelling, an event marked by the gradual elongation of the conidia, took 10 to 12 h. Fresh and dried conidia measuring initially 4.8 ± 0.1 µm in length, swelled to 6.3 ± 0.1 and 6.5 ± 0.1 µm, respectively, after 13h on SDAY plates. The development of germ tubes from conidia was not delayed by the drying event. Over time the rates of germ tube elongation between 12 and 19h postinoculation averaged ~1.3 µm/h for both the dried and fresh conidial preparations.

The drying process greatly enhanced the survival of CG423 conidia as witnessed by nearly 100% germination rates after more than a 100-day desiccation period (Fig. 2). In comparison, CG423 conidia stored without drying sealed in plastic bags, had a median survival time of 17 d postharvest, as determined by linear regression ($R = 0.88$; $F = 20$; $n = 8$). Prolonged 135 and 176-d storage of dry conidia in the drying chamber at room temperature decreased viability to 72.5% and 27% respectively, and the median survival

Table 1. Loss of free water by *Metarhizium anisopliae* var. *acridum* conidia in the drying chamber for 6 d at ~ 27°C.

Drying hours	Loss of water (%)[1]	± $s_{\bar{x}}$
2	78.8	11.0
19	98.8	0.4
44	99.5	0.3
64	99.7	0.2
84	100.0	0.0
104	100.0	0.0
124	100.0	0.0
144	100.0	0.0

[1]Based on weighting 3 samples of 400, 370, and 580 mg at 0 h.

Table 2. Time course for percent germination (±$s_{\bar{x}}$)[1] of dried and fresh conidia of *Metarhizium anisopliae* var. *acridum* at 27°C.

Hours	Dry conidia[2]	Fresh conidia[3]	P
0	0.0	0.0	
12	4.7 ± 0.7 a	4.3 ± 0.3 a	0.67
13	15.3 ± 0.9 a	22.0 ± 5.2 a	0.70
14	44.3 ± 2.6 a	42.0 ± 1.5 a	0.48
15	89.0 ± 1.2 a	73.7 ± 2.0 b	<0.01
16	93.7 ± 1.2 a	81.0 ± 3.0 b	<0.01
17	91.7 ± 1.2 a	89.0 ± 1.2 b	<0.03
18	96.3 ± 0.7 a	89.0 ± 1.0 b	0.10

[1]Averages (from 3 replicates) followed by the same letter in rows are not significantly different according to a t-test. [2]Fifteen d in the drying chamber at 5% RH. [3]From 12-d culture.

Table 3. Percent germination of *Metarhizium anisopliae* var. *acridum* dry conidia after different times of rehydration in high humidity air (96%) at ~ 27°C.

Hours of rehydration	Germination (%)[1]	± $s_{\bar{x}}$
Control	27.3	2.3
2	38.7	4.9
4	49.3	5.0
6	60.0	4.2
8	61.7	1.8
12	74.7	6.2
16	93.0	2.0
24	87.0	2.1

[1]After storage in dry chamber for 176 d.

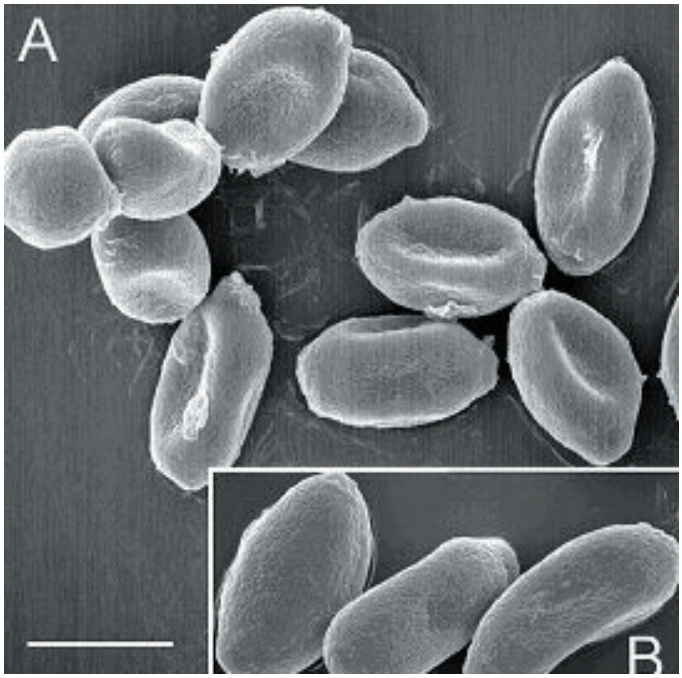

Fig. 1. Scanning electron micrographs of (A) dry and (B) fresh conidia of *Metarhizium anisopliae* var. *acridum*. Bar = 3 µm. Dry conidia were from 260 d storage and fresh conidia from 15 d old culture.

time was 136 d (R = 0.94; F = 158; n = 14). Alternatively the same fresh conidia, stored without drying, sealed in plastic bags at ~ 25°C for 52 d, were inactive (< 10% viable). The results with the CG423 strain are in agreement with those reported for the well-studied IMI330189 isolate. Previously, Hedgecock *et al.* (1995) reported that conidia of the isolate IMI 330189 dried to 5% moisture, could be stored at 25°C for at least 120 d and still maintain >80% germination rates. As with the CG423 strain, Moore *et al.* (1996) found that dried preparations stored for longer periods (135 d) at 27 to 37°C had lower germination rates (42%).

The low germination rate (27%) of conidia exposed to 176 d in dry storage was altered by exposure of the dry conidia to high humidity (96% RH) (Table 3). Dry conidia exposed to a 4-h rehydration period had germination rates of ~50%, whereas 24h pre-incubation at high humidity resulted in a ~90% germination rates (Table 3). However, the germination rates were reduced at 204 (81%), 232 (70%) and 260 (23%) d in storage (Fig. 3). Prior studies by Moore *et al.* (1997) found that germination rates of dried preparations of IMI330189 isolate could be increased from 24% to 73%, with 5h rehydration at high humidity. However, most of the increase was found within 5 min.

Table 4. Cumulative mortality and confirmed mycosis of *Schistocerca americana* infected with *Metarhizium anisopliae* var. *acridum*.

	Percent mortality[1]			Percent confirmed mycosis[2]		
Days	Dry conidia	Fresh conidia	Control	Dry conidia	Fresh conidia	Control
1	16.7	13.3	0.0	0.0	0.0	0.0
2	20.0	30.0	0.0	0.0	0.0	0.0
3	43.3	36.7	3.3	13.3	3.3	0.0
4	56.7	53.3	3.3	30.0	10.0	0.0
5	73.3	80.0	3.3	46.7	30.0	0.0
6	96.7	90.0	3.3	60.0	40.0	0.0
7	100.0	93.3	3.3	63.3	43.3	0.0

[1]From 3 replicates of 10 insects. [2]From the total test insects.

Preincubation of the CG423 dry conidia in water produced similar positive effects on germination as compared to germination of fresh conidia. At 24h post-inoculation, dry conidia produced significantly lower germination rates (21%) than rehydrated conidia (germination rates > 91.7%, $p < 0.05$). However, after 48h on SDAY plates the germination rates of dry conidia (88.3%) were comparable to the germination of conidia rehydrated in water, water + Tween-20, or under high humidity. The ability to rehydrate by direct transfer in water was unexpected. Previous research has demonstrated that direct transfer of dry conidia to water is deleterious. For example, Moore *et al.* (1997) found that rehydration of *M. anisopliae* var. *acridum* conidia in water reduced germination from 76% (when dry conidia were previously rehydrated in saturated water atmosphere before immersion in water) to 24.5% (when conidia were presoaked without rehydration).

The biological activity of *M. anisopliae* var. *acridum* conidia against *S. americana* nymphs was not affected by the drying process. Both dry and fresh conidial preparations produced high mortality (>95%) 7 d after inoculation (Table 4). At this time there were more insects with mycosis caused by dry conidia (63.3%) than by fresh conidia (43.3%). However, there was no significant difference between these 2 treatments ($t = 1.3$, $df = 4$, $p = 0.25$).

In summary, fresh and dried conidia of *M. anisopliae* var. *acridum* produced similar *in vitro* germination patterns and were both infectious to *S. americana*. However, the germination velocity of dry conidial preparations was reduced by prolonged (260-d) storage. Interestingly, the germination speed of dry conidia can be recovered by rehydration in water or by exposing them to high humidity. These findings can be used in the formulation of this pathogen. For field application, CG423 strain could be produced and dried for long term storage.

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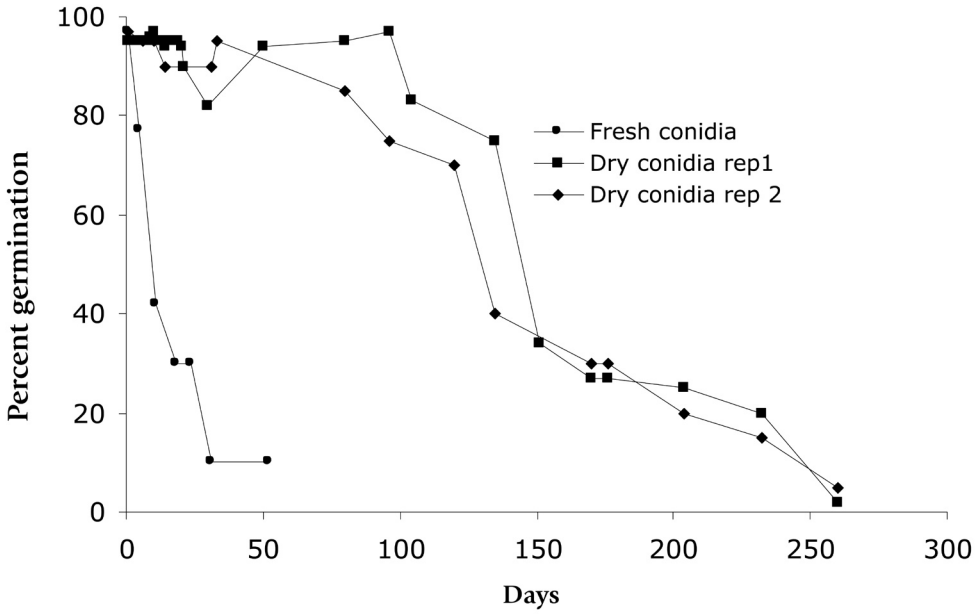

Fig. 2. Germination of *Metarhizium anisopliae* var. *acridum* fresh and dry conidia. Fresh conidia were sealed in plastic bags and stored in a (switched off) drying chamber. Dry conidia were stored in a drying chamber in low-humidity air (≤ 5%). Drying chambers were maintained at room temperature of ~ 25°C.

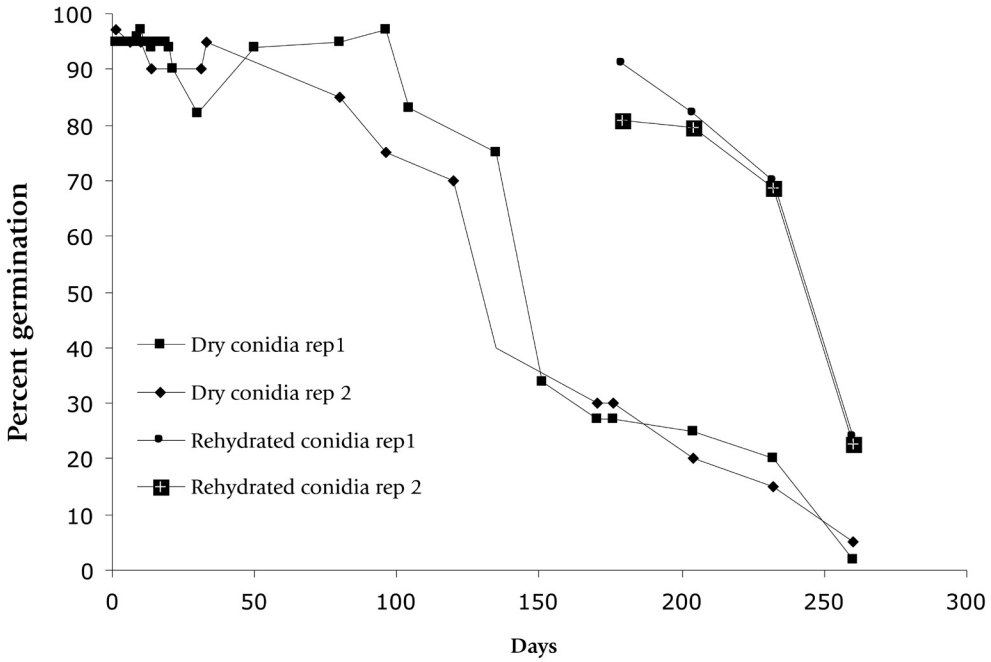

Fig. 3 Germination of *Metarhizium anisopliae* var. *acridum* dry and rehydrated conidia. Dry conidia were stored in a drying chamber in low-humidity air (≤ 5%) and at ~ 25°C; rehydrated conidia were kept in high humidity (96%) during 24h prior to plating and incubating at 27°C.