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Evaluation of conidia production and mycelial growth in solid culture media from native strains of entomopathogenic fungi isolated from citrus-growing areas of México

Fátima Lizeth Gandarilla-Pacheco, Katiushka Arévalo-Niño, Luis J. Galán-Wong, Carlos F. Sandoval Coronado and Isela Quintero-Zapata*

Instituto de Biotecnología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Av. Manuel L. Barragán Esq. Pedro de Alba S/N, Cd. Universitaria, San Nicolás de los Garza, N. L. México C. P. 66450.

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It is important to know the ability of native strains to carry out the process of sporulation and growth in different conditions and to determine their possible potential as biological control of pests of agricultural importance, mainly in citrus areas. The objective of this study was to evaluate five different solid culture media for the determination of the production of conidia and mycelial growth of twenty-five native isolates from the Mexican states of Sinaloa, San Luis Potosí, Nuevo León and Tamaulipas, and three collection strains using five different solid culture media. The results showed no statistical significant difference between the five media tested for the production of conidia per milliliter and mycelial growth among different isolates. However between isolates a significant difference was found. For conidia per milliliter, the isolate with the highest production of conidia was HIB-4 with an average of 4.85 x 10^9 (spores/ml). With respect to mycelial growth strain Ma presented the highest value with 8.06 cm on average.

Key words: Entomopathogenic fungi, esporulation, conidia, mycelial growth.

INTRODUCTION

Entomopathogenic fungi were defined as obligate or facultative parasites of insects with a high capacity for sporulation and survival, and its greatest advantages are handling, adaptation to different environments, specificity and ability to direct penetration through the integument (Allendes and López, 2007). Almost all insects are susceptible to some diseases caused by these fungi (Alean-Carreño, 2003; Rodríguez et al., 2006). Approximately 100 genera and 700 known species of entomopathogenic fungi have been described, the most important may be mentioned genera *Beauveria*, *Metarhizium*, *Paecilomyces*, *Lecanicillium*, *Hirsutella*, *Aschersonia*, *Erynia*, *Entomophthora*, (Monzón, 2001; Asaff et al., 2002). Currently, worldwide, the most studied species of entomopathogenic fungi are *Beauveria bassiana* and *Metarhizium anisopliae* due to its efficiency and ease of propagation in different substrates (Allendes and López, 2007; Scholte et al., 2004).

A commercial level has developed a wide variety of agar formulations to induce sporulation and the growth of fungi; among the culture media commonly used we can found the Sabaroud dextrose agar (SDA), Malt extract agar (MEA) Nutrient agar (NA) Corn meal agar (CMA) Yeast peptone dextrose agar (YPDA), Potato dextrose agar (PDA), to name a few (Kamp and Bidochka, 2002). Most of these present a media composition based on source of carbon and one nitrogen which generally vary between them and in the proportions in which they are presented as well as in its origin which may be organic or inorganic.

While we have studied the production of spores in liquid culture media of different species of

*Corresponding autor. E-mail: isela.quinterozp@uanl.edu.mx.

entomopathogenic (Jackson et al., 1997; Derakhshan et al., 2008) and natural substrates such as rice, barley, oats, wheat and soybeans, among others (Figueroa et al., 2007; Posada-Flórez, 2008), the initial stages of isolation and morphological characterization of new isolates is still of vital importance for the use of plate assays because the understanding of essential aspects of fungal growth entomopathogenic can be very useful in projects for finding this microorganisms such as potential agents for managing of insect pests.

This detailed knowledge of the nutritional demands for growth and sporulation which are key elements in the stages of mass production and marketing will contribute in high proportion to the selection and continuous improvement of culture media and propagation methods suitable for large scale production of infective spores. In order to determine the ability of five different culture media commonly used in the laboratory to induce sporulation and mycelial growth in the present study we evaluated 25 native strains of three species of entomopathogenic fungi isolated from citrus-growing areas of México.

MATERIALS AND METHODS

Microorganisms

The native isolates of entomopathogenic fungi were obtained from citrus growing areas of México. The method used for isolation and characterization of the different species of entomopathogenic fungi were described by Galán-Franco et al. (2011). For this study were selected twenty-five isolates from the states of Sinaloa (*B. bassiana*: HIB-1, HIB-2, HIB-3, HIB-4, HIB-5, HIB-6, HIB-7, HIB-16, HIB-17,); San Luis Potosi (*B. bassiana*: HIB-8, *Isaria fumosorosea*: HIB-9); Nuevo León (*B. bassiana*: HIB-10; *M. anisopliae*: HIB-11, HIB-12; *I. fumosorosea*: HIB-20, HIB-21, HIB-26, HIB-29, HIB-30, HIB-33) and Tamaulipas (*B. bassiana*: HIB-13, HIB-14, HIB-15, HIB-18; *I. fumosorosea*: HIB-32), and three strains from to the collection of the Institute of Biotechnology, FCB-UANL (*B. bassiana*: GHA), (*I. fumosorosea*: Pfr-612), (*M. anisopliae*: Ma).

Preparation of inoculum

For each of the assessments we used the strains described before which were stored in cryogenic state (10% glycerol at -80°C) in the laboratory L-6 from the collection of the Institute of Biotechnology, FCB-UANL. Strains were thawed at room temperature in approximately 30 to 60 minutes, then were inoculated as streak in the potato dextrose- agar medium and allowed to incubate at 25 ± 2°C for a period of 14 days.

Culture medium

Medium A

Potato dextrose agar (PDA) DIBICO® formulates grams per liter of bidistilled water: agar 15.0, dextrose 20.0, potato infusion 4.0, pH 5.6 ± 0.2.

Medium B

Potato dextrose agar (PDA) DIBICO® formulates grams per liter of bidistilled water: agar 15.0, dextrose 20.0, potato infusion 4.0, pH 5.6 ± 0.2, supplemented with 0.5% casein peptone BIOXON®.

Medium C

Potato dextrose agar (PDA) DIBICO® formulates grams per liter of bidistilled water: agar 15.0, dextrose 20.0, potato infusion 4.0, pH 5.6 ± 0.2, supplemented with 0.5% yeast extract DIBICO®.

Medium D

Yeast dextrose agar: composed of anhydrous dextrose (4%) Jalmek®, yeast extract DIBICO® (1%) and bacteriological agar DIBICO® (1.5%).

Medium E

Sabouraud dextrose agar DIBICO® formulates grams per liter of bidistilled water: agar 15.0, dextrose 40.0, special peptone 10, pH 5.6 ± 0.2.

All solid media were prepared according to nutritive property adding 1000 ml of bidistilled water to each, sterilized at 121°C to 15 lbs. pressure for 15 min.

Inoculation media

For inoculation in each of the mediums described earlier were obtained uniform sizing each of the strains and placed in the center of 100 x 15 mm Petri dishes. We incubated at 25 ± 2°C for 14 days. Each of the culture media was assessed for triplicate.

Evaluating production of conidia

After 14 days of incubation in each culture medium, each strain was added 10 ml of bidistilled water with 0.1% Tween 80 and a bacteriological loop surface scraping was performed to obtain a concentrated solution of conidia. To determine its concentration, the total counts in a Neubauer chamber using dilutions from the concentrated solution was utilized.

Evaluating mycelial growth

After 14 days of incubation, the diameter of the colonies in centimeters using a vernier was measured.

Statistical analysis

The results were analyzed using IBM® SPSS ® v.19. Kolmogorov-Smirnov test was initially performed to verify the normality of the data. We performed a one-way analysis of variance (ANOVA) and a comparison of Scheffé means at the 0.05 significance level.

RESULTS

Conidial production and mycelial growth by effect of culture medium

The average production of conidia obtained in different culture media ranged from 6.7×10^6 to 5.8×10^9 spores/

ml, corresponding to the minimum being isolated HIB-3 in medium A (PDA) and the maximum for the HIB-4 isolated in the medium D (ADY). But according to the results of the ANOVA, no significant difference (F = 0.817, df= 4 P = 0.517) exists between the five media tested for the production of conidia per milliliter between the different isolates (Table 1).

The average mycelial growth obtained in different culture media ranged from 3.13 to 8.50 centimeters, the minimum for the isolated HIB-18 in medium B (PDA supplemented with 0.5% peptone from casein) and the maximum strain in Ma media D and E (ADY, ADS). But according to the results of the ANOVA no significant difference was found (F=1,087, df= 4, P= 0.366) between the five media evaluated for mycelial growth among different isolates (Table 2).

Production of conidia and mycelial growth among isolates

The average production of conidia obtained between isolates ranged from 1.85 x 10^7 to 4.85 x 10^9 spores/ml, corresponding to the minimum being isolated HIB-3 and the maximum for the HIB-4 isolated. The comparison of conidia production of the different strains showed highly significant statistical differences between strains (F=8,727; df=27, P≤0.0001). A test was performed for comparison of Scheffé means at 0.05 significance (Table 3) and it was found that HIB-4 strain had the highest conidia production between the strains tested with 4.85x10^9 spores / ml, while strains HIB-2, HIB-3 and HIB-29 had the lowest production of conidia with 4.70x10^7, 1.85x10^7 and 1.95x10^7, respectively.

The average mycelial growth between strains ranged from 3.62 to 8.06 centimeters, the minimum for the isolated HIB-18 and the maximum strain Ma. The comparison of mycelial growth among different isolates showed highly significant statistical difference between strains (F=24,498; df=27, P≤0.0001). A test was performed for comparison of Scheffé means at 0.05 significance (Table 4), which showed that the Ma strain present the greatest mycelial growth with 8.06 cm while the HIB-18 strain had the lowest with 3.62 cm.

DISCUSSION

Sporulation is a process that involves the development of sexual or asexual spores and associated structures. It is considered that this mechanism of reproduction is controlled by genetic, hormonal, nutritional and environmental factors. The survival of fungal species depends in part on its ability to produce large quantities of viable conidia under different environmental conditions and even physical and nutritional requirements are more stringent than those required for mycelial growth (Moore, 1996). For the development of sporulation there are two major nutrient requirements that are a source of carbon and nitrogen source which is required for synthesizing amino acids, proteins, and nucleic acids necessary for the construction of protoplasm (Pérez and Ramírez, 2000). Glucose is the carbon source most widely used by the fungi followed by fructose, mannose, galactose, sucrose, lactose, maltose and polysaccharides such as starch, cellulose, pectin (Griffin, 1996), chitin (Hegedus et al., 1990), trehalose, sorbitol and mannitol (Bidochka et al., 1990). The most nitrogen preferred sources by fungi are organic nitrogen as urea and casein hydrolyzate (Griffin, 1996), peptone (Humphreys et al., 1989), yeast (Hegedus et al., 1990) corn soaking liquid (Blachere et al., 1973) and casamino acids (Jackson et al., 1997). But it also mentions the use of inorganic sources of nitrogen (Bidochka et al., 1990).

In this study we evaluated the production of conidia and mycelial growth from 25 native strains of entomopathogenic fungi as a result of different culture media widely used in the laboratory to induce growth and sporulation of various types of fungi, potato dextrose agar and Sabouraud dextrose agar which include glucose as carbon source in different proportions and three modifications by the addition of casein peptone and yeast extract as nitrogen sources. In the results no significant difference in the production of conidia and mycelial growth among growth media were evaluated. There are several factors related to the nitrogen sources that might have influenced these results. For example, casein peptone is a pancreatic digest containing all the amino acids found in the casein; moreover of fractions peptic long, and some minerals such as calcium, magnesium, potassium and sodium in small quantities, however lacks a defined content of vitamins in composition, which may have some effect on the development of sporulation and the formation of reproductive structures that requires certain concentrations of minerals or vitamins (Griffin, 1996).

The yeast extract has proven to be a source of nitrogen more efficient in promoting the mycelial growth and sporulation, however, also been reported as a limiting factor for growth and sporulation depending on the concentration used. At low concentrations of yeast (0.25 to 0.50%) the mycelial growth is below the optimum and the sporulation is low while at very high concentrations of yeast (1.75 to 2%) the mycelial growth is high and this inhibits sporulation (Vimala-Devi, 1994). The lack or deficiency of certain growth factors such as vitamins or minerals, or the concentration of yeast extract used in this study that was 0.5 to 1% could have a significant impact for the production of conidia and mycelial growth.

In addition, Moore (1996) also mentioned that fungi may differ in their ability to use various sources of carbon and nitrogen for reproduction, since the mycelial growth and conidial production may be favored by monosaccharides such as glucose or fructose, however some

Table 1. Average production of conidia by the effect of different culture media tested at 14 days of incubation under laboratory conditions (25±2°C).

Strain code	Culture medium (conidia/ml)				
	A	B	C	D	E
HIB-1	$2.6x10^8$	$2.7x10^8$	$2.1x10^8$	$3.3x10^8$	$5.8x10^8$
HIB-2	$3x10^7$	$2.8x10^7$	$5.9x10^7$	$1.8x10^8$	$2.8x10^7$
HIB-3	$6.7x10^6$	$8.8x10^6$	$8.8x10^6$	$5x10^8$	$8.2x10^6$
HIB-4	$4.1x10^9$	$8.6x10^9$	$3.2x10^9$	$9.3x10^9$	$2.8x10^9$
HIB-5	$3.2x10^8$	$3.2x10^7$	$3.4x10^8$	$3.4x10^8$	$3.5x10^8$
HIB-6	$1.6x10^9$	$2.0x10^9$	$4.8x10^9$	$5.8x10^9$	$5.2x10^9$
HIB-7	$2.0x10^8$	$2.7x10^8$	$2.0x10^7$	$2.0x10^8$	$2.0x10^8$
HIB-8	$2.2x10^7$	$3.5x10^7$	$2.4x10^7$	$1.5x10^8$	$2.3x10^8$
HIB-9	$2.3x10^8$	$2.2x10^8$	$4.5x10^8$	$4.6x10^8$	$6.1x10^8$
HIB-10	$3.4x10^8$	$3.3x10^8$	$3.6x10^8$	$3.9x10^8$	$2.9x10^9$
HIB-11	$1.2x10^9$	$4.2x10^9$	$1.2x10^9$	$1.1x10^8$	$1.6x10^8$
HIB-12	$2.6x10^9$	$2.1x10^9$	$3.2x10^9$	$1.4x10^8$	$1.2x10^8$
HIB-13	$4.4x10^7$	$3.0x10^7$	$5.5x10^7$	$2.5x10^8$	$1.3x10^8$
HIB-14	$6.0x10^8$	$4.9x10^7$	$6.5x10^7$	$2.8x10^8$	$2.3x10^8$
HIB-15	$3.2x10^8$	$4.2x10^7$	$3.2x10^8$	$2.8x10^8$	$1.8x10^9$
HIB-16	$6.5x10^7$	$4.4x10^7$	$3.7x10^7$	$3.3x10^8$	$4.0x10^7$
HIB-17	$3.1x10^8$	$5.8x10^7$	$6.0x10^8$	$3.9x10^8$	$3.9x10^8$
HIB-18	$4.5x10^7$	$5.7x10^7$	$5.1x10^8$	$5.4x10^7$	$2.6x10^8$
HIB-20	$6.8x10^8$	$6.3x10^8$	$4.0x10^8$	$2.9x10^8$	$4.5x10^8$
HIB-21	$2.5x10^8$	$1.4x10^8$	$2.9x10^8$	$3.9x10^8$	$1.7x10^8$
HIB-26	$1.8x10^7$	$6.8x10^7$	$2.7x10^8$	$5.3x10^7$	$2.1x10^8$
HIB-29	$1.8x10^7$	$1.7x10^7$	$2.9x10^8$	$2.2x10^7$	$1.5x10^7$
HIB-30	$2.4x10^7$	$1.0x10^7$	$2.8x10^8$	$7.7x10^7$	$2.0x10^8$
HIB-32	$2.3x10^8$	$3.5x10^8$	$4.0x10^8$	$1.4x10^8$	$4.1x10^8$
HIB-33	$5.0x10^7$	$6.3x10^7$	$3.3x10^7$	$2.9x10^7$	$2.6x10^8$
GHA	$2.6x10^8$	$2.0x10^8$	$3.4x10^8$	$5.5x10^8$	$1.6x10^8$
Pfr-612	$1.05x10^8$	$1.0x10^8$	$1.82x10^8$	$1.66x10^8$	$2.0x10^8$
Ma	$6.4x10^8$	$3.4x10^8$	$1.8x10^8$	$1.8x10^8$	$1.3x10^8$
Mean±SD	$1.65x10^8$±0.72	$1.42x10^8$±0.76	$2.05x10^8$±0.68	$2.80x10^8$±0.59	$2.35x10^8$±0.63

of the nitrogen sources which allow good mycelial growth do not favor sporulation, such as asparagine and the ammonium compounds that inhibit the mycelial growth and sporulation due to the accumulation of ammonia during the somatic growth, which alkalinized the medium inhibits this process.

Regarding the production of conidia and mycelial growth among different strains were found high significant differences in their ability to use different culture media. According to the results reported in this study, a strain of *B. bassiana* showed the highest conidia production between the strains tested, while two other strains of the same species showed the lowest levels which is consistent with that reported by James (2001) who observed an increase germination efficiency of *B. bassiana* and *P. fumosoroseus* with the addition of peptone and yeast extract. Smith and Grula (1981) reported that *B. bassiana* requires exogenous carbon sources for germination and exogenous sources of nitrogen for the initial stages of hyphal growth and maintenance. Wrona and Gleason (2005) concluded that a carbon source can activate germination in some microorganisms, but a source of nitrogen is essential for germ tube elongation and hyphal growth. Some of the amino acids of said nutritional sources can induce the expression of genes which control the sporulation, as this process requires the activation of over 80 genes (Roncal and Ugalde, 2003).

The events related to fungal mycelial growth, that begins with the emergence of germ tube and end with the growing hyphae and branching to finally form a young mycelium, are also related to genetics and molecular regulation that determines the expression or repression of morphological features (Papagianni, 2004). Som

Table 2. Average mycelial growth effect of different culture media tested at 14 days of incubation under laboratory conditions (25±2°C).

Strain code	Culture medium (cm)				
	A	B	C	D	E
HIB-1	4.21	3.40	3.86	3.85	4.08
HIB-2	3.76	4.51	4.15	3.68	3.85
HIB-3	4.78	4.21	4.13	4.21	3.66
HIB-4	4.53	5.03	4.11	5.76	4.83
HIB-5	4.21	4.25	4.23	3.56	4.26
HIB-6	4.25	5.33	5.08	6.45	4.83
HIB-7	4.76	4.48	3.88	4.18	4.76
HIB-8	5.90	5.36	5.01	6.11	5.41
HIB-9	7.33	7.56	7.33	7.76	7.48
HIB-10	3.48	4.65	5.68	5.10	5.41
HIB-11	5.78	5.10	5.71	5.91	5.75
HIB-12	5.70	5.75	5.83	6.73	6.20
HIB-13	4.18	5.35	5.28	5.36	5.25
HIB-14	4.66	4.16	4.86	5.01	4.11
HIB-15	5.21	6.28	6.46	5.71	5.48
HIB-16	4.28	4.25	4.75	4.53	4.98
HIB-17	5.08	5.03	4.90	4.31	5.03
HIB-18	3.18	3.13	4.11	3.43	4.23
HIB-20	7.36	7.26	8.03	7.46	6.81
HIB-21	7.48	7.00	7.18	7.91	7.66
HIB-26	6.20	7.28	7.00	6.53	6.63
HIB-29	6.16	6.46	7.13	7.76	7.66
HIB-30	4.93	6.23	5.78	7.06	7.73
HIB-32	5.81	6.31	6.66	7.21	6.98
HIB-33	6.08	7.23	7.55	7.83	7.83
GHA	4.51	5.70	5.75	6.71	5.53
Pfr-612	4.08	5.73	7.08	6.75	5.95
Ma	7.81	7.70	7.80	8.50	8.50
Mean±SD	5.20±1.24	5.52±1.26	5.69±1.32	5.90±1.52	5.74±1.38

microorganisms do not require nitrogen sources to germinate. James (2001) reported that some isolates of *M. anisopliae* germinated very well when using glucose as the sole source of nutrition. Similarly Li and Holdom (1995) observed that *M. anisopliae* conidia germination were the same in both agar with a nitrogen source (individual amino acids) and glucose agar without any source of organic nitrogen, and even reported a good mycelial growth *M. anisopliae* on soluble starch for mass production, indicating that the response of each microorganism nutrient sources is different. These results coincides with the reported for the strain Ma of *M. anisopliae* which had the highest mycelial growth among different strains tested in this studied.

Several experiments have been devoted to elucidate the influence of nutrition on mycelial growth and spore production in order to choose the components and concentrations optimal to increase production on a large scale. Gao and Liu (2010) examined the effects on growth and sporulation in liquid medium and solid for *P. lilacinus* and *M. anisopliae* using various combinations of carbon and nitrogen sources different, they concluded that the results were highly variable between strains and each strain present certain nutrition requirements for the development of mycelial growth and sporulation. Safavi et al. (2007), studied the effect of nutrition on the virulence of three isolates of *B. bassiana* and one of *M. anisopliae* culturing in seven culture media with different proportions of carbon/nitrogen (C / N). They reported that the mycelial growth and the production of conidia showed differences between different species and strains. Liu and Chen (2002) they found that some carbon and nitrogen sources were good for growth in liquid and solid for *Hirsutella rhossiliensis*, while some could not be utilized either in liquid or solid.

The present study and the above investigations have

Table 3. Average yield of conidia per strain at 14 days of incubation under laboratory conditions (25±2°C).

Strains code	Average yield of conidia per strain (conidia/ml)
HIB-1	$3.10x10^{8abc}$
HIB-2	$4.70x10^{7a}$
HIB-3	$1.85x0^{7a}$
HIB-4	$4.85x10^{9c}$
HIB-5	$2.10x10^{8abc}$
HIB-6	$3.45x10^{9bc}$
HIB-7	$1.38x10^{8abc}$
HIB-8	$5.77x10^{7ab}$
HIB-9	$3.70x10^{8abc}$
HIB-10	$8.70x10^{8abc}$
HIB-11	$6.40x10^{8abc}$
HIB-12	$7.80x10^{8abc}$
HIB-13	$7.55x10^{7ab}$
HIB-14	$1.65x10^{8abc}$
HIB-15	$2.89x10^{8abc}$
HIB-16	$6.70x10^{7ab}$
HIB-17	$4.40x10^{8abc}$
HIB-18	$1.14x10^{8abc}$
HIB-20	$4.68x10^{8abc}$
HIB-21	$2.30x10^{8abc}$
HIB-26	$8.20x10^{7abc}$
HIB-29	$1.95x10^{7a}$
HIB-30	$1.00x10^{8\ abc}$
HIB-32	$2.85x10^{8abc}$
HIB-33	$6.00x10^{7ab}$
GHA	$2.80x10^{8abc}$
Pfr-612	$1.45x10^{8abc}$
Ma	$2.50x10^{8abc}$
Mean±SD	$2.00x10^{8}$±0.56

*Different letters represent significant differences in the Scheffé Multiple Range test (P≤0.05).

Table 4. Average radial growth per strain at 14 days of incubation under laboratory conditions (25 ± 2°C).

Strains code	Average radial growth per strain (cm)
HIB-1	3.88^{ab}
HIB-2	3.99^{abc}
HIB-3	4.20^{abc}
HIB-4	4.85^{abcde}
HIB-5	4.10^{abc}
HIB-6	$5.19^{abcdefg}$
HIB-7	4.41^{abcd}
HIB-8	$5.56^{abcdefg}$
HIB-9	7.49^{gh}
HIB-10	4.86^{abcde}
HIB-11	$5.65^{abcdefg}$
HIB-12	$6.04^{bcdefgh}$
HIB-13	5.08^{abcdef}

Table 4 Contd.

HIB-14	4.56^{abcd}
HIB-15	$5.83^{abcdefgh}$
HIB-16	4.56^{abcd}
HIB-17	4.87^{abcde}
HIB-18	3.62^{a}
HIB-20	7.38^{fgh}
HIB-21	7.45^{fgh}
HIB-26	6.71^{defgh}
HIB-29	7.03^{efgh}
HIB-30	6.35^{cdefgh}
HIB-32	6.59^{defgh}
HIB-33	7.30^{fgh}
GHA	$5.64^{abcdefg}$
Pfr-612	$5.92^{abcdefgh}$
Ma	8.06^{h}
Mean±SD	5.61±1.27

*Different letters represent significant differences in the Scheffé Multiple Range test (P≤0.05).

sought help to elucidate the effect of different sources of carbon and nitrogen and the relationship between these elements as high factors impact on growth and sporulation of entomopathogenic fungal and aided the selection of the best strains that may have potential as biological control agents.

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