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High level of sporulation of *Metarhizium anisopliae* in a medium containing by-products

Blas Dorta[1]*, Alejandra Bosch[2], Jorge Alfredo Arcas[2], and Rodolfo Jose Ertola[2]

[1] Departamento de Biología Celular, Escuela de Biología, Facultad de Ciencias, Universidad Central de Venezuela, Apartado 21201, Caracas, Venezuela

[2] Centro de Investigación y Desarrollo en Fermentaciones Industriales (CINDEFI), Facultad de Diencias Exactas (UNLP), 47 and 115, (1900) La Plata, Argentina

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Summary. Solid-state fermentation of rice bran or rice-bran-husk mixtures by *Metarhizium anisopliae* proved to be highly successful in spore yield. Optimum sporulation response on bran medium occurred when the initial water activity values ranged from 0.982 to 0.999. When bran was supplemented with 50% rice husk, the spore yield almost doubled, but a narrower initial water activity range (0.997–0.999) was optimum. Under these conditions, spore yields were 5–15 times higher (depending on the strain) than those currently obtained on the basis of rice grain fermentations.

Introduction

The entomopathogenic fungus *Metarhizium anisopliae* is utilized for biological control of many insect pests (Roberts and Yendol 1971; Wraight and Roberts 1987; Alves 1986). Generally, high levels of field inocula are required if this fungus is to be used efficiently as an insecticide (Wraight and Roberts 1987; Alves 1986), so it is important for processes producing infectious material to yield high levels of spores at low cost.

Techniques that have been applied for culturing *M. anisopliae* on defined or semidefined media (Roberts 1966; Barnes et al. 1975; Pontecorvo et al. 1953; Campbell et al. 1978) usually become tedious and expensive for producing large quantities of spores; consequently, their use is restricted in practice to laboratory bioassays. In large-scale field applications using *M. anisopliae*, the need for large quantities of infectious material has prompted the development of inexpensive methods for culturing this fungus, based mainly on the fermentation of rice grains (Aquino et al. 1975; Costa and Clóvis 1974; Costa et al. 1974; Daoust et al. 1983; Daoust and Roberts 1983) or bran (Goettel 1984).

On the basis of rice grain fermentations, *M. anisopliae* spores are currently produced in Brazil on an industrial scale by business companies, grower cooperatives and plantation owners, yielding about 10% fungal mass (Alves 1986), which is considered economically viable. Since rice is commercially valuable as human food, it may be possible to device some cheaper and more highly productive methods for producing *M. anisopliae* spores than those based on rice grain fermentations. In this report we describe a novel and highly productive process for growth and sporulation of *M. anisopliae* based on rice-bran-husk fermentation.

Materials and methods

Microorganism. M. anisopliae isolates A_4, A_{19}, E_6, *M*, and *MT* were kindly supplied by Dr. J. L. Azevedo, Instituto de Genética, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, São Paulo, Brazil; isolate BJ-1110 of our strain collection was originally obtained from Instituto Spegazzini of La Plata, Argentina.

Culture media. Culture media were based on rice bran (bran medium) or rice bran-rice husk mixtures (bran-husk medium). Media were prepared in preweighed 500-ml cylindrical screw-capped glass bottles of 8-cm bore. The water content (on a wet weight basis) varied from 2.8% to 68% for bran medium and from 9% to 49% for bran-husk medium. Media-containing bottles were autoclaved for 20 min at 120° C. After cooling, each bottle was weighed in order to measure any water loss during autoclaving, which was corrected during media inoculation if necessary.

Inoculum and media inoculation. Spore suspensions for inoculum were obtained from 2-week-old cultures grown on rice grains (Alves 1986) by adding sterile 0.1% Tween 80 in distilled water and shaking by hand. Spore counts were made using a Neubauer haemocytometer, and the media were inoculated at a concentration of 10^6 spores/g dry matter. Preparations were hand shaken for spore spreading.

Culture conditions and harvesting technique. Bottles were incubated for 2 weeks at 28° C, under static conditions, in a thermostated room saturated with water vapour and continuous artificial light. These conditions have been reported as optimal for growth and sporulation of *M. anisopliae* on rice grains (Alves 1986). Dur-

* *Present address:* CINDEFI, Facultad de Ciencias Exactas UNLP, Calles 47 y 115, (1900) La Plata, Argentina

Offprint requests to: B. Dorta

ing incubation the bottles remained stationary. After incubation, cultures were suspended for spore harvesting into 25 ml of 0.1% Tween 80/g initial dry matter. Suspensions were stirred for 10 min using a magnetic bar and filtered through a 50-mesh sieve. The process was repeated twice and the filtrates collected were pooled for spore counting as mentioned above. All data were subjected to variance analysis.

Viability measurements. After harvesting, germination was examined. A sample of 0.1 ml suspended spores was plated on Difco (Detroit, Mo, USA) Y_pS_c agar coated slides to be able to count about ten spores per field under microscopical examination (400×). Slides which were placed into moist saturated petri dishes were incubated at 28° C for 48 h, with continuous artificial light. Germination was recorded by examining about 500 spores for the presence of visible germ tubes.

Water activity. Water activity of bran and bran-husk media, was measured at 28° C using a Novasina Thermoconstanter Humidat-TH2 (Zurich, Switzerland).

Results and discussion

Water availability is a very important parameter in solid substrate fermentations (Grajek and Gervais 1987; Oriol et al. 1988; Gervais et al. 1988). Our experiments were carried out at various initial water activities (a_w) for measuring the sporulation response of BJ-1110 and E_6 strains on bran and bran-husk media. Sorption isotherms of each medium were determined (Fig. 1). A non-linear regression analysis was made on the fitting abilities of various two-parameter equations (Iglesias and Chirife 1982) as applied to each experimental set of data. Smith's equation best describes the experimental sorption data, being for bran medium:

$$wc = -0.00746 - 9.7651 \ln(1 - a_w) \quad (1)$$

and for bran-husk medium:

$$wc = 3.8977 - 6.8077 \ln(1 - a_w) \quad (2)$$

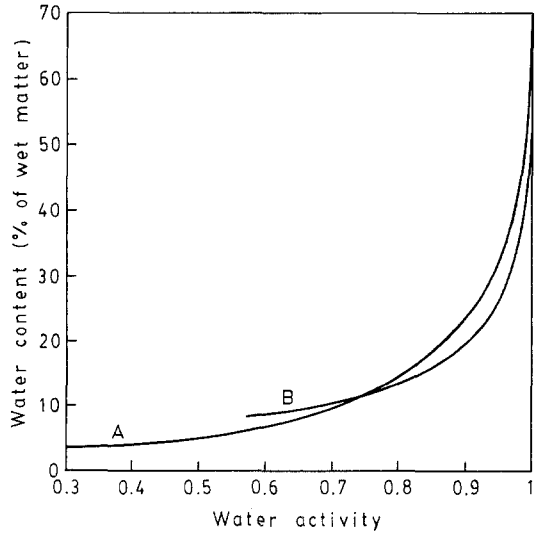

Fig. 1. Sorption isotherms of bran (*A*) and bran-husk (*B*) media, as fitted from Eqs. 1 and 2 (see text). Husk content (on a dry weight basis) in the bran-husk medium was 50%

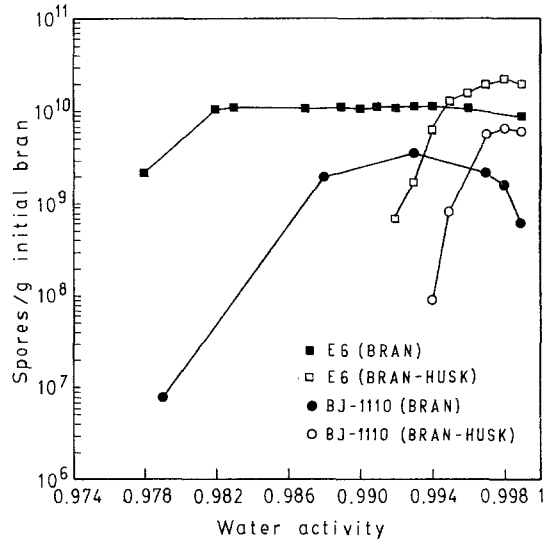

Fig. 2. Sporulation response of strains E_6 and BJ-1110 of *Metarhizium anisopliae* on bran and bran-husk media prepared at various initial water activities. Husk content (on a dry weight basis) in the bran-husk medium was 50%

where *wc* is the water content. The correlation coefficients were higher than 0.995. These equations were used for calculating the initial *wc* at which the corresponding media were to be prepared to give a_w values ranging from 0.835 to 0.999.

The *wc* for bran medium becomes almost 20% higher than that of bran-husk medium at the same a_w values beyond 0.990, meaning that rice husk enhances water availability in the culture media. Similarly, lignocellulosic supports improve the water availability in solid substrate fermentations (Sato et al. 1982; Oriol et al. 1988).

For bran-husk medium, the optimum sporulation response of BJ-1110 and E_6 strains occurred when the initial a_w values ranged from 0.997 to 0.999 (Fig. 2), meaning that high water availability was required in this medium. Nevertheless, a wider a_w range was observed for bran medium, where the concentration of soluble nutrients becomes higher than in bran-husk medium, as to be expected from the diluting effect of added husk. The minimum a_w for growth of fungi is often affected as a consequence of variations in several physical and chemical factors, including nutrient availability (Corry 1978).

The spore yield for bran medium was about half that for bran-husk medium. This difference could be ascribed to the high degree of agglomeration shown by the bran medium, where porosity was lower than that of the bran-husk medium. Space availability is one of the various factors limiting fungal biomass concentration in solid substrate fermentations (Laukevics et al. 1985).

Requirements for growth and sporulation of *M. anisopliae* on the bran-husk medium are mainly supplied by bran (see composition in Table 1), because, as observed in our laboratory, autoclaved washed husk is unable to support mycelial growth. Crude fibre and silica

Table 1. Composition[a] of rice bran

Compound	Content (% on dry weight basis)
Non-*N*-extract	57.72
Fibre	8.00
Protein[b]	14.44
Lipid	8.93
Ash	10.91

[a] Determined according to the AOAC Official Methods of Analysis (1984)
[b] Calculated as total $N \times 6.25$

are the main components of husk (Hawkey 1974). Silica is a solid structure in the outer epidermis and a dispersed system inside the husk (De Lhoneux et al. 1988), so it behaves as a relatively inert support. Furthermore, rice husk increases the media porosity, which would enhance gas transfer.

Husk amount (**H**) and % of husk content (**h**) in bran-husk medium were evaluated in connection with the sporulation response of the BJ-1110 strain. Table 2 shows that when **H** was varied, total spore production was significantly affected, reaching the highest spore count (5.6×10^{10}) for 10 g husk. Nevertheless, variance analysis indicated that there was a significant interaction between **H** and **h**, meaning that the husk content effect depended on the amount of husk used. In total spore production no significant differences were observed for low **H** values (5 g) when **h** was varied. On the other hand, for higher **H** values, significant differences were observed when **h** was varied, reaching the highest spore production at **h** values of 45.4%–50.0% and 55.5%–62.5% for 10 and 15 g husk, respectively. Based on those results it was decided to use the 10 g bran-10 g husk medium (**H** = 10 g and **h** = 50.0%) in order to measure the sporulation response of other *M. anisopliae* strains. For E_6, A_{19}, A_4 and *MT* strains, this medium is highly successful in spore yield, as compared to BJ-1110 strain (Table 3). Spore yields (expressed as spores/g bran) varied from 5.8×10^9 to 15.3×10^9 depending on the strain. Viability values were higher than 85%.

Table 2. Husk amount and husk content effects on the sporulation response of strain BJ-1110 on bran-husk medium

H					
5 g		10 g		15 g	
h (%)	Total spores $\times 10^{10}$	**h** (%)	Total spores $\times 10^{10}$	**h** (%)	Total spores $\times 10^{10}$
45.4	3.95 A	45.4	7.48 A	55.5	6.43 A
41.6	3.80 A	50.0	5.93 AB	62.5	6.09 A
50.0	3.15 A	41.6	5.44 BC	45.4	4.29 B
55.5	2.49 A	55.5	5.25 BC	50.0	3.30 BC
62.5	2.27 A	62.5	3.92 C	41.6	2.41 C
$\tilde{x} = 3.13$ C		$\tilde{x} = 5.60$ A		$\tilde{x} = 4.50$ B	

H = husk amount; **h** = husk content. Means within columns (based on three replicates) that are followed by the same letter are not significantly different by Tukey's procedure ($P < 0.05$). Means among columns are significantly different according to the same test. Initial water activities (a_w) were estimated to be about 0.998

Table 3. Sporulation response of various *Metarhizium anisopliae* strains on bran-husk medium[a]

	Strains					
	E_6	A_{19}	A_4	*MT*	*M*	BJ-1110
Spore yield[b]	15.3 A	12.0 B	9.3 B	6.1 C	5.9 D	5.8 D
Viability (%)	99	88	99	98	97	85

[a] Medium containing 10 g bran + 10 g husk at an estimated initial a_w of about 0.998
[b] Spore/g initial bran ($\times 10^9$)
Means (based on eight replicates) that are followed by the same letter are not significantly different by Tukey's procedure ($P < 0.05$)

The reported production methods of *M. anisopliae* spores, based on rice grain fermentations, yield about 10^9 spores/g rice (Alves 1986). Yields obtained as spores/g bran, in the bran-husk medium (depending on the strain used), are 5–15 times higher than those usually obtained on rice grains (Table 3). Given that the mean thickness of such a bran-husk medium was 3.6 cm (data not shown), the calculated apparent volume of the culture bed, according to the size and shape of the fermentor used, was about 0.18 l. This makes the volumetric yield $3.2–8.5 \times 10^{11}$ spores/l, i.e., 1.2–3 l culture medium, with a mean thickness of 3.6 cm, is required for producing the 10^{12} spore inoculum currently applied per hectare (Alves 1986) in large-scale field applications.

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References

Alves SB (1986) Fungos entomopatogênicos. In: Alves SB (ed) Controle microbiano de insetos. Editora Manole LTDA, São Paulo, Brasil, pp 73–126

AOAC (1984) Official methods of analysis, 40th edn. Association of Official Analytical Chemistry, Arlington, Ma, USA

Aquino MLN, Cavalcanti VAIB, Sena RC de, Queiroz GF de, (1975) Nova tecnologia de multiplicacão de fungo *Metarhizium anisopliae*. Bol Tec CODECAP, Refice, 4:1–79

Barnes GL, Boethel DJ, Eikenbary RD, Criswell JT, Gentry GR (1975) Growth and sporulation of *Metarhizium anisopliae* and *Beauveria bassiana* on media containing various peptone sources. J Invertebr Pathol 8:212–221

Campbell RK, Perring TM, Barnes GL, Eikenbary RD, Gentry

RC (1978) Growth and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* on media containing various amino acids. J Invertebr Pathol 31:289–295

Corry JEL (1978) Relationships of water activity to fungal growth. In: Beuchat LR (ed) Food and beverage mycology. Avi Publishing Company, Westport, Conn, pp 45–82

Costa MDM, Clóvis DM (1974) Um novo meio de cultura para o fungo entomógeno *Metarhizium anisopliae* (Metsch) Sorokin parasito da cigarrinha das pastagens. Bol Inst Biol Bahia 13:57–60

Costa MDM, Matta EAF, Magalhães CD, Matos DP (1974) Nova técnica para producão em larga escala do fungo *Metarhizium anisopliae* (Metsch) Sorokin, em laboratório. Bol Inst Biol Bahia 13:85–89

Daoust RA, Roberts DW (1983) Studies on the prolonged storage of *Metarhizium anisopliae* conidia: effect of growth substrate on conidial survival and virulence against mosquitoes. J Invertebr Pathol 41:161–170

Daoust RA, Ward MG, Roberts DW (1983) Effect of formulation on the viability of *Metarhizium anisopliae* conidia. J Invertebr Pathol 41:151–160

De Lhoneux B, Gerlache L, Clemente A, Roda-Santos ML, Menaia JAG, Fernandes TH (1988) Ultrastructural characterization of rice husk submitted to different pretreatment to optimize its fermentation. Biol Wastes 23:163–180

Gervais P, Bensoussan M, Grajek W (1988) Water activity and water content: comparative effects on the growth of *Penicillium roqueforti* on solid substrate. Appl Microbiol Biotechnol 27:389–392

Goettel MS (1984) Simple method for mass culturing entomopathogenic hyphomycete fungi. J Microbiol Methods 3:15–20

Grajek W, Gervais P (1987) Effect of the sugar-beet pulp water activity on the solid state culture of *Trichoderma viride* TS. Appl Microbiol Biotechnol 26:537–541

Hawkey R (1974) Rice husk utilization. In: Barber S, Tortosa E (eds) Rice husk utilization. Proceedings of the Rice By-products Utilization International Conference, 30 September–2 October, Valencia, Spain, c1977:59–66

Iglesias HA, Chirife J (1982) Handbook of food isotherms: water sorption parameters for food and food components. In: Schweigert BS, Hawthorn J (eds) Food science and technology, a series of monographs. Academic Press, New York, pp 262–264

Laukevics JJ, Apsite AF, Viesturs US, Tengerdy RP (1985) Steric hindrance of growth of fungi in solid substrate fermentation of wheat straw. Biotechnol Bioeng 27:1687–1691

Oriol E, Raimbault M, Roussos S, Viniegra-Gonzalez G (1988) Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger*. Appl Microbiol Biotechnol 27:498–503

Pontecorvo G, Roper JA, Hemmons LM, Mc Donald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141–238

Roberts DW (1966) Toxins from the entomogenous fungus *Metarhizium anisopliae*. I. Production in submerged and surface cultures, and in inorganic and organic nitrogen media. J Invertebr Pathol 8:212–221

Roberts DW, Yendol WG (1971) Use of fungi for microbial control of insects. In: Burges HD, Hussey NW (eds) Microbial control of insects and mites. Academic Press, London, pp 125–149

Sato K, Nagatani M, Sato S (1982) A method of supplying moisture to the medium in a solid-state culture with forced aeration. J Ferment Technol 60:607–610

Wraight SP, Roberts DW (1987) Insect control effort with fungi. In: Pierce G (ed) Development in industrial microbiology, vol 28. Society for Industrial Microbiology, pp 77–87