PRODUCTION OF *Trichoderma asperellum* T8a SPORES BY A "HOME-MADE" SOLID-STATE FERMENTATION OF MANGO INDUSTRIAL WASTES

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Dry wastes (dw) generated in processing mangoes, composed (in dry weight) mainly of soluble carbohydrates (71 ± 2%) and fiber (16 ± 1%), were evaluated as substrates in a "home-made" solid-state fermentation (using polyurethane foam as inert support matrix, various C:N ratios, moisture contents, and incubation periods) of *Trichoderma asperellum* T8a, a promising biological control agent against the mango pathogen *Colletotrichum gloeosporioides* (causal agent of anthracnose). Highest spore production (2.5×10^6 up to $76 \pm 3 \times 10^8$ spores g^{-1} dw) occurred after 8 days of incubation [at 28 ± 1 °C, relative humidity of 85 ± 5%, photoperiod of 12h (540 Lux) - 12h (20 Lux)] at a C:N ratio of 26, and a moisture content of 78%. Scanning electron microscopy showed that *T. asperellum* T8a was able to grow on mango industrial wastes and into polyurethane foam. The extensive growth can be related to cellulases secreted by this fungus, liberating glucose from these wastes to its growth. Most (94 ± 1%) of the spores grown on mango industrial wastes survived storage at 4 °C for 7 days and were equally effective as those grown on potato dextrose agar medium (86 ± 4% viable) in biological control tests against *C. gloeosporioides* ATCC MYA 456. Results indicate the potential use of mango industrial wastes as substrates to produce *T. asperellum* T8a spores *in situ* (mango orchards) under a cheap "home-made" solid-state fermentation, reducing problems associated with wastes disposal and permitting the production of a biological control agent against *C. gloeosporioides.*

Keywords: Biological control agent; Cellulase activity; Colletotrichum gloeosporioides; Mango industrial wastes; Proximal analyses; Scanning electron microscopy; Trichoderma

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INTRODUCTION

Total world production of mangoes is about 30×10^6 tonnes per year (FAO 2008). Much of this fruit is consumed raw, while the rest is canned, frozen, or turned into pulp. Most processing is mechanized using high-speed machines that peel and slice fruits to be turned into juices, bottled, canned, dried, frozen, pickled, powdered, pureed, or preserved in other ways. These processes generate high volumes of industrial wastes (de los Santos-Villalobos *et al.* 2011). For example, in the valley of Cauca, Colombia, between 50% and 55% of the total fruit, comprised of the peel, shell, seed, remaining pulp, and fibre is discarded (Ordoñez 2002). Data of this kind have not been gathered in Mexico, but the wastes percentages are undoubtedly similar to those reported for Colombia.

These wastes contain urushiol, which is a contact allergen in the peel; for this reason, mango industrial wastes must not be disposed of in the environment where contact with humans is increased (Oka *et al*. 2004).

Proximal analyses of mango industrial wastes show the following: water content 69% and dry matter 31%, (protein: 7%, ethereal extracts: 5.5%, ash: 3.5%, and carbohydrates: 15.5%) with a pH of about 4.2 (Mejía *et al.* 2007). On this basis, these wastes can probably be used as feed for livestock, and also for the extraction of pectin and vegetable fats (Trejo-Márquez 2010). Of course, highly nutritious mango industrial wastes can also be used in solid-state fermentations, a cost-effective way of growing microorganisms of interest (Martin *et al.* 2002; Silva *et al.* 2002; Couto and Sanromán 2006). Solid-state fermentation also permits large volumetric through-puts, higher concentrations of end-products, less effluent generation, and it allows the use of simple fermentation equipment (Doelle *et al.* 1992; Viniegra-González *et al.* 2003; Hölker *et al.* 2004). For all of these reasons, a broad range of microorganisms has been cultivated on agro-industrial wastes, including *Trichoderma*. This genus has great potential as a Biological Control Agent (BCA) through different mechanisms such as antibiosis, parasitism (production of lytic enzymes), and competition for nutrients and space (El-Kassas and Khairy 2009), against pathogenic fungi that cause significant losses in crop production, such as *Colletotrichum gloeosporioides,* the causal agent of anthracnose (the main mango disease), which is the chief culprit of pre- and post-harvest losses of up to 90% (Spalding and Reeder 1978; Arauz 2000).

At present, the use of BCA is a promissory alternative to control anthracnose, reducing the environmental problems generated when traditional practices are used: low efficiency, environmental contamination, and high costs (de los Santos-Villalobos *et al*. 2011). However, the current technology applied to BCA production led to higher costs of production when compared to synthetic fungicides; thus, BCA commercialization has been slow to evolve (Slininger and Schisler 2012), whereby the generation of cheap technology or process is determinant in the successful BCA application in the field.

Thus, the present work studies the production of a BCA on mango industrial wastes under a simple solid-state fermentation system. The aim is to generate a cheap "home-made" alternative to be developed *in situ* (mango orchards) to control anthracnose or other diseases with less negative impact on the environment compared to synthetic fungicides application, as well as to help recycle large amount of wastes generated in mango industrialization.

EXPERIMENTAL

Microorganisms

One fungal strain, T8a, isolated from the rhizosphere of mango orchards located in Tapachula, State of Chiapas in Mexico and belonging to the microbial collection of Environmental Microbiology Laboratory (CINVESTAV-Irapuato, Mexico), showed a potential biological control activity against *Colletotrichum gloeosporioides* ATCC MYA 456, the causal agent of anthracnose.

Biological Control Assays

Confrontation assays were carried out by separately inoculating 1 x 10^5 spores of strain T8a and *C. gloeosporioides* ATCC MYA 456 onto Petri-dishes containing Potato Dextrose Agar (PDA) medium (separation distance 6 cm) and incubating the plates at 28 °C for 7 days (Bell *et al.* 1982). Each value represents the mean of triplicate determinations.

Macro, Microscopic, and Molecular Characterization

Strain T8a (1 x 10^5 spores), was inoculated on petri-dishes containing PDA medium, and incubated at 28°C for 5 days (photoperiods of 12 h). Macroscopic traits of this strain were observed after the incubation period, as well as its microscopic characteristics using Scanning Electron Microscopy (SEM) [low vacuum scanning electron microscope (JEOL, JSM-5910 LV) at 30 Pa and 15 kV)]. Samples were cut-out, mounted on copper stubs, and examined. In addition, samples of strain T8a growing on mango industrial wastes and polyurethane foam were analyzed to observe its growth on these surfaces.

The molecular characterization was carried out growing strain T8a in Potato Dextrose Broth (PDB) medium to obtain mycelia for DNA extraction (Rader and Broda 1985), which was used to perform the Polymerase Chain Reaction (PCR) technique, amplifying the Internal Transcribed Spacer 1 (ITS1) regions (White et al., 1990). DNA sequences were aligned with ClustalX 2.0.12 (Thompson et al. 1997) and analyzed by the Neighbor-Joining method using MEGA 4.0. ITS1 sequences of *Aspergillus flavus* (Accession No. JX028197) was used as the out-group. Stability of clades was assessed with 1000 bootstrap replications. ITS1 sequence obtained from strains T8a was deposited in the NCBI Genbank.

Proximal Analyses

Industrial wastes from the production of 'Tommy Atkins' mangoes were obtained from the pulping industry located in Irapuato, Guanajuato, Mexico. These wastes, containing pericarp fiber and pulp remains, were dried and ground before use. Total solids and moisture content [method 925.09, (AOAC 1990)], ash [method 942.05, (AOAC 1990)], nitrogen [(method 955.04, (AOAC 1990)], protein [(method 954.01, (AOAC 1990)], fat [(method 920.39, (AOAC 1990)], organic matter/carbon (Walkley 1947), fiber (Van Soest and Wine 1967), and pH [method 943.02, (AOAC 1990)] were determined. Nitrogen-free extract was calculated by adding protein, fat, water, ash, and fiber and subtracting this value from 100. All determinations were done in triplicate.

Cellulase Activity

Enzyme activity was assayed by growing 1 x 10^5 spores of strain T8a in tubes containing 25 mL of liquid synthetic medium (powdered mango peel or cellulose, 10 g; KNO_3, 2.5 g; KH_2PO_4, 1 g; $MgSO_4$, 0.5 g; distilled water to 1000 mL; pH, 6.0) and incubating for 8 days at 24 °C in a rotatory shaker at 180 rpm. Every 24 hours, the culture was filtered through Whatman No. 1 paper, and the biomass was determined after drying at 60 to 70 °C. Then, the filtered medium was centrifuged at 10,000 x g for 30 min and the supernatant again passed through glass fiber filters. These supernatants were used to determine cellulase activity according to Ghose (1987) modified as follows: 1 mL of appropriately diluted enzyme solution was added to 2 mL of 0.1 M citrate phosphate

buffer (pH 4.4), and then cellulose (5 mg) was added; the reaction was incubated at 50 °C for 60 min. Released reducing sugars were measured at 565 nm using the modified 3,5-dinitrosalicylic acid (DNS) method (Miller 1959), using glucose as a standard. One unit of activity (U) was defined as the amount of enzyme that released one micromole equivalent of glucose per minute under the specified assay conditions. All determinations were done in triplicate.

Spore Production by Strain T8a

Spores were raised in glass petri-dishes using polyurethane foam as the inert support matrix. Carbon nitrogen (C:N) ratios of 6 to 34 were reached by adding commercial ammonium sulfate (purity 20.5%; the main nitrogen source used by farmers in mango production in Mexico). The moisture content ranged from 64 to 94% and harvest times of 1 to 9 days were all tested. All replicates used 2 g powdered mango industrial wastes (particle size < 800 µm) as the dry wastes (dw). Glass petri-dishes containing wastes and polyurethane foam under different treatments were sterilized at 121 °C (1 atm) for 15 min and cooled down to room temperature before the inoculation with 2.5 x 10^6 spores g^{-1} dw (28 ± 1 ºC, 85 ± 5% relative humidity, cycles of 12 h at 540 Lux followed by 12 h at 20 Lux). Under these growth conditions, the impact of different C:N ratios, moisture contents, and harvest times on the spore production were evaluated. Spores were extracted from the mango industrial wastes and polyurethane foam by washing with water containing 1% (v/v) of Triton X-100, vortexing for 5 min, followed by centrifugation at 5,000 x g for 10 min at room temperature. Extraction was repeated three times and the concentration of spores was determined using a microscopic counting chamber. Spores harvested from conditions that maximized production were stored at 4 °C for 7 days and then tested for viability [the number of colony-forming units (CFU)] by plating out on PDA. Biological control capacities using confrontation assays against *C. gloeosporioides* ATCC MYA 456 were performed as illustrated above. All experiments were replicated.

Statistical Analysis

Data were analyzed by one-way analyses of variance (ANOVA) test and Tukey-Kramer method (P = 0.05) using JMP-SAS software v. 8.0.2.

RESULTS AND DISCUSSION

Strain T8a and its Biological Control Activity

Anthracnose is the main disease of world-wide mango production, caused by the phytopathogenic fungus *C. gloeosporioides*, generating pre- and postharvest losses of up to 90% (Arauz 2000). The traditional alternatives include physical and chemical methods, which present disadvantage such as low efficiency, environmental contamination, and high costs (de los Santos-Villalobos *et al*. 2011). Similar problems have been reported in the production of economically important crops: tomato, barley, maize, wheat (Lucas 2010). Thus, the generation and/or optimization of more efficient, eco-friendly, and cheaper alternatives are determinant to produce BCA, increasing the crop production. BCA are promissory microorganisms for disease control in pre and postharvest. Thus, the use of BCA as an alternative is becoming popular throughout the world (Sharma *et al*.

2009). We studied the impact of parameters (C:N ratios, moisture contents, and harvest time) under a "home-made" solid-state fermentation in the spores production of a BCA (strain T8a) against *C. gloeosporioides* isolated from mango rhizosphere.

Fungal colonies of strain T8a, growing on petri-dishes containing PDA medium, showed characteristics such as fast growth (24 ± 1.7 mm^2 h^{-1}) and formation of concentric green and white rings, when petri-dishes inoculated were incubated under photoperiod of 12 h (Fig. 1a). The microscopic characterization using SEM showed the presence of *Trichoderma* typical phyalides (Fig. 1b), as reported by Barnet and Hunter (1978). This information suggests that strain T8a belongs to the *Trichoderma* genus; the molecular characterization was conducted to confirm the taxonomic classification of this strain. The sequence analysis of ITS1 region amplified showed that strain T8a belongs to *Trichoderma asperellum* (Fig. 2). This species has been frequently isolated from root-free soil, rhizosphere of various plants, healthy plant tissues, and dead wood. The broad habitat suggests its outstanding environmental opportunism ranging from saprotrophy to biotrophy (Guigón-López *et al*. 2010). For these reasons, *T. asperellum* has been reported as a powerful antagonist of other fungi, as it is able to parasitize them (necrootrophic hyperparasitism or) or inhibit their growth and development (Hoyos-Carvajal *et al*. 2008).

To evaluate the potential of *T. asperellum* T8a as a BCA against *C. gloeosporioides* ATCC MYA 456, biological control assays were carried out and we observed that strain T8a completely overgrew the pathogen (Fig. 1c), which suggests mycoparasitism as a main biological control mechanism (Bell *et al*. 1982).

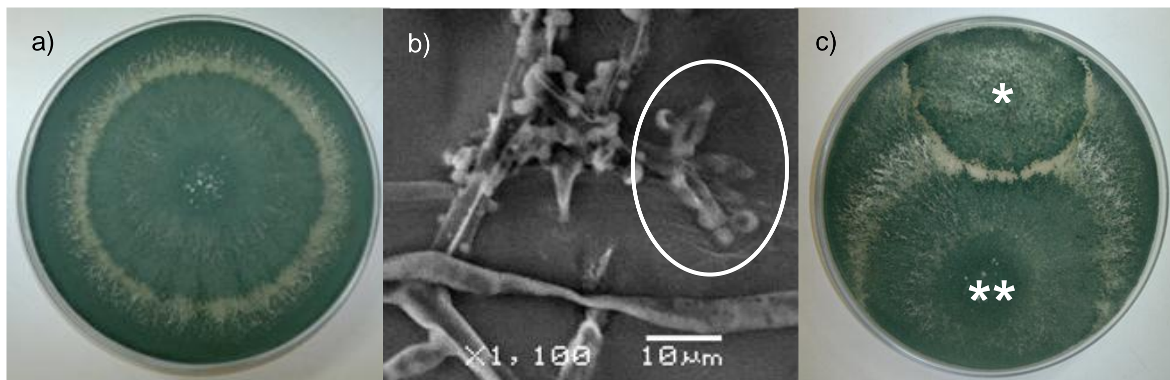

Fig. 1. Cultural and biological control characteristics of *T. asperellum* T8a. a) Macroscopic morphology of strain T8a growing onto petri-dishes containing PDA after 5 days of inoculation. The formation of concentric green and white rings were observed when these petri-dishes were incubated under photoperiod of 12 h. b) Microscopic morphology of strain T8a using SEM, observing *Trichoderma* typical phyalides (white circle). c) Biological control assay (through mycoparasitism) onto petri-dishes containing PDA medium, after 7 days, at 28 °C. One (*) and two (**) asterisks indicate the inoculation of *C. gloeosporioides* ATCC MYA 456 and the strain T8a, respectively.

Proximal Analyses of Mango Industrial Wastes & *T. asperellum* T8a Growth

At present, the BCA production is expensive due to: a) limited technology (companies need to shift their synthetic fungicide production systems to BCA production systems) and expensive substrates to produce those BCA (Soccol and Vandenberghe (2003). Thus, mango industrial wastes were studied to explore their potential use as substrates in the spores production of *T. asperellum* T8a.

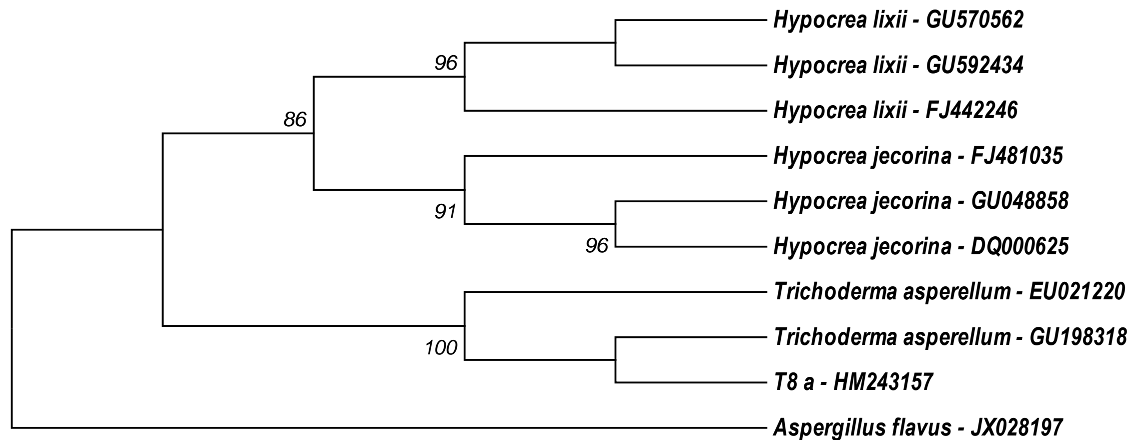

Fig. 2. Phylogenetic relationships of *T. asperellum* T8a inferred by neighbor-joining analysis of ITS1 sequences. *Aspergillus flavus* (Accession No. JX028197) was used as the out-group. The numbers given over selected branches indicate the percentage of 1000 bootstrap re-sampled data sets supporting the clade to the right of the branch, and are given only for values >50. *Hypocrea lixxi* and *Hypocrea jecorina* are the teleomorph of *Trichoderma harzianum* and *Trichoderma reesei*, respectively.

Proximal analyses of mango industrial wastes (Table 1) suggested that they are both suitable and sustainable substrates for raising microorganisms of agricultural importance. Similar findings have been reported for wastes of cabbage, wheat straw, banana, corncob, and grapes (Chahal *et al.* 1996; Ojumu *et al.* 2003; Baig 2005; Bai *et al.* 2008; Das and Ghosh 2009). Mango industrial wastes are largely composed of nitrogen-free extract indicating high contents of soluble and available carbohydrates suitable for supporting the growth of fungi such as *T. asperellum* T8a. This species has been recognized for its wide metabolic capacity leading to use several composts from agricultural wastes for its growth (Trillas *et al*. 2006).

Table 1. Proximal Analysis of Mango Industrial Wastes

Determination	Wet Weight (%)
Humidity	79 ± 1
Total solids	21 ± 1
Ash	0.6 ± 0.0
Protein	1.9 ± 0.0
Fat	0.3 ± 0.1
Nitrogen-free extract	15 ± 2
Fiber	3.4 ± 0.2
Cellulose	2.1 ± 0.1
Hemicellulose	0.7 ± 0.1
Lignin	0.6 ± 0.0
Organic Matter	18 ± 0.5
Carbon	10 ± 0.3
Nitrogen	0.3 ± 0.0
C:N ratio	33 ± 1
pH	4.0 ± 0.0

Cellulose, which is present in these wastes, is an important substrate, since cellulases secreted by the fungi can liberate glucose and use it to aid their growth when soluble and available carbohydrates in this system have been consumed by the microorganism (Jackson and Bothast 1990). Cellulase activity in supernatants of *T. asperellum* T8a was indeed observed 2 days after inoculation but remained low when cellulose was the sole carbon source. Cellulose activity reached another peak on the seventh day (Fig. 3), which can be explained by inhibition of the cellulase activity by the end product glucose released from cellulose, especially on the second day; this activity was recovered on the seventh day, by which time that glucose had been consumed (Mandels and Reese 1960). In contrast, a sharp peak in cellulase activity was observed on the sixth day in the presence of mango industrial wastes, suggesting that their high content of available carbohydrates stimulated the growth of *T. asperellum* T8a during 5 days after inoculation, and then cellulase activity was stimulated to release glucose. Activity reached the highest level in the stationary phase when growing on mango industrial wastes, which proved to be a better substrate than cellulose to produce biomass of this strain, 160 ± 10 and 27 ± 6 mg mycelial dry weight, respectively. Similar results have been reported by studies on *Trichoderma reesei* observing that available carbohydrates such as glucose and fructose are superior to cellulose as a growth-stimulating carbon source (Messner and Kubicek 1991).

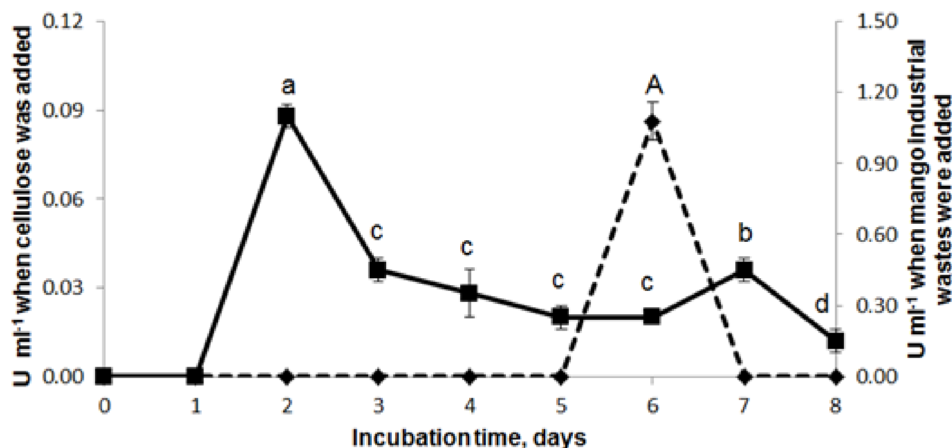

Fig. 3. Cellulase activity of *T. asperellum* T8a growing in liquid medium with cellulose (continuous line) or mango industrial wastes (dotted line) added as the sole carbon source. Means with the same lowercase letter (cellulase activity using cellulose) and uppercase letter (cellulase activity using mango industrial wastes) are not significantly different according to ANOVA test (P = 0.05).

Spores Production of *T. asperellum* T8a under Solid-State Fermentation

The information mentioned above showed that *T. asperellum* T8a can grow using mango industrial wastes as substrates under liquid fermentation, but without producing spores. Thus, spores production by this strain using mango industrial wastes under a "home-made" solid-state fermentation was evaluated, because this system possess several biotechnological advantages, such as higher fermentation productivity and stability of products, lower catabolic repression, and lower water demand (Hölker *et al*. 2004).

Our solid-state fermentation system was especially focused on those determinant parameters to develop this alternative *in situ* (mango orchards) with the aim to diminish the high economical cost, above mentioned, in the spores production of BCA. For this reason, C:N ratios, moisture contents, and the optimum time to harvest spores provided

by the proximal composition analyses of mango wastes were studied. Highest spore numbers (2.5 x 10^6 up to 57 ± 3 x 10^8 spores g^{-1}dw) were observed at C:N ratios of 26 (incubated for 6 days with a moisture content of 88%) and as shown Table 2, a median C:N value that corresponds to similar reports for several *Trichoderma harzianum* strains (Serrano-Carreon *et al.* 1992; Agosin *et al.* 1997; Dahlan 2007). Similar reports indicated that C:N ratio is a determinant factor affecting fungal sporulation (Elson *et al*. 1998). In addition, in many cases, the optimal value of C:N ratio for sporulation is different to the optimal value for mycelial growth, as these values depend on the fungal species and/or isolates (Gao and Liu 2009). *Trichoderma asperellum* T8a was unable to grow under the same conditions mentioned above, using dry whole peel as substrate, probably because smaller size wastes provides large surface area, which helps to increase the nutrient uptake by microorganisms (Maurya *et al*. 2012).

Similarly, the highest cellulose activities were observed at C:N ratios ranging from 22 to 30 (Table 2), suggesting that degradation of cellulose was a determining factor in the induction of mycelial growth and spore production, when the content of available carbohydrates was low (Ellouz *et al.* 1995; Xia and Shen 2004) (Fig. 4).

Table 2. Spore Production and Cellulase Activity of *T. asperellum* T8a 6 days after Inoculation at Different C:N Ratios and a Moisture Content of 88%

C:N (Ratio)	Spore Production (spores x 10^8 g^{-1}dw)	Cellulase Activity (U g^{-1} dw)
6	0.03 ± 0.01 f	0.8 ± 0.1 b
10	14 ± 3 e	0.2 ± 0.1 c
14	28 ± 1 d	0.3 ± 0.1 c
18	29 ± 3 c, d	0.2 ± 0.0 c
22	43 ± 0.3 b	1.6 ± 0.1 a
26	57 ± 3 a	1.0 ± 0.1 b
30	38 ± 4 b, c	1.8 ± 0.2 a
34	2.4 ± 0.8 f	0.3 ± 0.0 c

Means with the same letter (spores production and cellulase activity) are not significantly different according to ANOVA test (P = 0.05).

Fig. 4. Six days of solid-state fermentation of *T. asperellum* T8a growing on mango industrial wastes with different C:N ratios, a) 6, b) 14, c) 26 and d) 34, and a moisture content of 88%.

Once the C:N ratio was fixed at 26, the moisture content was optimized on the sixth day. The moisture content is a critical factor for cell growth and spores production under solid-state fermentation, increasing the outcome of the process. In addition, in solid-state fermentation, the quantity of water in the system depends of the substrate water retention capacity. This quantity should be sufficient for the growth of micro-organisms through carriage of enzymes, nutrients and metabolites, as well as in the solubilization of oxygen, without destroying the solid structure or reducing the porosity

of substrate or support (Oriol *et al*. 1988). In our case, no significant impact was observed on spore production when different moisture contents were evaluated (Table 3), probably because the maximum water absorption capacity of wastes and polyurethane foam was constant in each treatment, independent of initial water content. A moisture content of 75% was sufficient to saturate the solid-state fermentation system. Similar findings have been reported for rice, corn bran, and wheat bran, observing a narrow moisture range when increased amounts of water were added (Cavalcante *et al.* 2008). In addition, Bai *et al*. (2008) reported that spores production of *T. viride* under solid-state fermentation of winery wastes were not increased using a moisture range of 66 to 72%.

Table 3. Effect of Different Moisture Levels on Spore Production of *T. asperellum* T8a (C:N Ratio Fixed at 26, 6 Days Incubation)

Moisture Content (%)	Spore Production (spores x 10^8 g^{-1}dw)
0	0.01 ± 0.002 e
64	20 ± 2 d, e
78	51 ± 7 a, b, c
84	30 ± 7 c, d
88	60 ± 11 a, b
90	48 ± 3 b, c
92	76 ± 11 a
93	39 ± 11 b, c, d
94	63 ± 6 a, b

Means with the same letter (spores production) are not significantly different according to ANOVA test (P = 0.05).

Nevertheless, a C:N ratio of 26 and a moisture content of 78% (the lowest that induced the most spore production) were fixed to follow the kinetics of sporulation. Eight days after inoculation turned out to be the best time to harvest spores (Table 4), coinciding with complete coverage of the substrates by the fungus. The spore production was very slow after 8 days of fermentation, suggesting that fermentation may be completed in this time. Spore production by *T. asperellum* T8a (76 x 10^8 spores g^{-1}dw, after 8 days) was higher and faster compared to that obtained by *T. harzianum* using rice bran, wheat straw, sugarcane bagasse under solid-state fermentation reaching 40, 2, 3 x 10^8 spores g^{-1}dw, after 15 days of inoculation (Lakshmi and Chandra 2004). The faster mycelial and spores production by strain T8a can be attributed to its fast growth as well as easily available nutrients from mango industrial wastes (composition of these wastes and *T. asperellum* T8a cellulase activity) (Rossi-Rodrigues *et al*. 2009).

T. asperellum T8a was unable to grow in the absence of the polyurethane foam as an inert support matrix (data not shown), probably because of the low rates of oxygen transfer and/or catabolite repression by the high level of soluble carbohydrates (Viniegra-González *et al.* 2003).

Scanning electron microscopic observations of *T. asperellum* T8a growing on mango industrial wastes and the polyurethane foam under optimal conditions to produce spores (C:N ratio of 26, moisture content of 78%, harvest 8 days after inoculation) suggests that the structure allows oxygen and soluble carbohydrates to pass through its pores, generating zones with optimal concentrations of both oxygen and nutrients for the growth and sporulation of this strain (Fig. 5).

Table 4. Effect of Harvest-Time on the Yields of *T. asperellum* T8a Spores (C:N Ratio Fixed at 26, the Moisture Content at 78%)

Harvest Time (days)	Spore Production (spores x 10^8 g^{-1}dw)
1	0.02 ± 0.004 e
2	0.03 ± 0.02 e
3	0.08 ± 0.04 e
4	3.9 ± 0.01 d, e
5	12 ± 0.1 d
6	41 ± 3 c
7	60 ± 2 b
8	76 ± 3 a
9	77 ± 6 a

Means with the same letter (spores production) are not significantly different according to ANOVA test (P = 0.05).

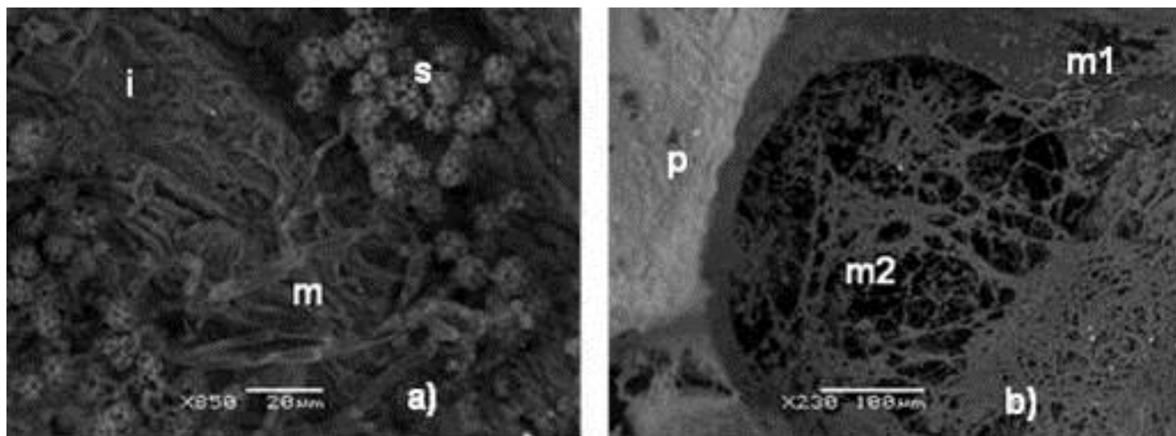

Fig. 5. Scanning electron micrographs of *T. asperellum* T8a after 8 days of culture on: a) mango industrial wastes (i: industrial wastes of mango, m: mycelia of *T. asperellum* T8a, and s: spores of *T. asperellum* T8a) and b) polyurethane foam (m1: mycelia of *T. asperellum* T8a, m2: mycelia of *T. asperellum* T8a in the pores of the polyurethane foam, and p: polyurethane foam as inert support matrix), in solid-state fermentation (C:N ratio 26 and moisture content 78%).

Viability of Spores Produced under Solid-State Fermentation

Viability of spores was determined by collecting them from conditions that maximized their production using mango industrial wastes and PDA medium and storing them for 7 days at 4 °C. Almost all spores (94 ± 1%) grown on mango wastes survived one week of storage at 4 °C, while only 86 ± 4% remained alive when grown on PDA medium. This suggests that the high levels of carbohydrates present in the mango wastes served as osmo-protectants (Thomas *et al.* 1994; Argüelles 2000). In addition, spores of *T. asperellum* T8a produced using this system can be applied to the field using the mango industrial wastes (substrates in solid-state fermentation) as a carrier, increasing the viability and germination of the spores in soil. *Trichoderma* species are cosmopolitan saprophytic fungi that need organic matter to grow (Gams and Bissett 1998).

Evaluation of the spores grown on mango wastes or on PDA in a confrontational assay against *C. gloeosporioides* ATCC MYA 456 showed similar percent inhibitions and degrees of mycoparasitism following storage (89 ± 2% and 91 ± 2% inhibition, respectively). In other words, mango industrial wastes help to prolong the shelf life of *T. asperellum* T8a spores (agents to control anthracnose) when they are produced. In

addition, this system of spore production (optimized to be used *in situ*) suggests an economical alternative that can be used for the biological control of plant pathogens in the field as well as the use of high volumes of mango industrial wastes.

CONCLUSIONS

1. Solid-state fermentation of *T. asperellum* T8a on mango industrial wastes is an effective way of producing spores that can be used as a biological control agent against anthracnose. C:N ratios of these wastes and the duration of the solid-state fermentation have more effect on spore production than moisture content.
2. Use of mango industrial wastes to produce antagonistic fungi will reduce problems associated with wastes disposal and permit the production of a biological control agent (*T. asperellum* T8a) against *C. gloeosporioides* (the causal agent of Anthracnose).
3. With appropriate refinement, the system should permit low-cost production of *T. asperellum* T8a spores, perhaps even on local farms.

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