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Critical Values of Porosity in Rice Cultures of *Isaria fumosorosea* by Adding Water Hyacinth: Effect on Conidial Yields and Quality

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Abstract Conidia of the entomopathogenic fungus *Isaria fumosorosea* are used to control insect pests in crops. Commercially available mycoinsecticides manufactured with this fungus are produced on a large scale via solid-state cultures (SSC). In order to favour gaseous exchange in SCC, texturizers can be added to increase porosity fraction (ε). This work presents results of water hyacinth (*Eichhornia crassipes*) as a novel texturizer. A mixture of parboiled rice (PR), with a ε=0.23, was used as a substrate, which was then mixed with water hyacinth (WH amendment) as a texturizer at different proportions affecting ε. Strains CNRCB1 and ARSEF3302 of *I. fumosorosea* yielded 1.6 (1.49–1.71)×10^9 and 7.3 (7.02–7.58)×10^9 conidia per gram of initial dry rice after 8 days, at ε values of 0.34 and 0.36, respectively. Improvement of conidial yields corresponded to 1.33 and 1.55 times, respectively, compared to rice alone using WH amendment in the mixtures PR:WH (%) at 90–10 and 80–20. In addition, infectivity against *Galleria mellonella* larvae was maintained. This is the first report of the use of water hyacinth as a texturizer in SSC, affecting ε, which is proposed a key parameter in conidia production by *I. fumosorosea*, without affecting conidial infectivity.

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Introduction

Mycoinsecticides are products based on propagules of entomopathogenic fungi (EF), which are used to control or reduce populations of insect pests in the agricultural sector, being harmless to humans and other mammals [1, 2]. The majority of commercially available mycoinsecticides are based on strains of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea*. These species produce large amounts of aerial conidia in solid-state culture (SSC) [3–5]. The EF *I. fumosorosea* (formerly known as *Paecilomyces fumosoroseus*) infects a wide range of hosts which attack crops of great economic importance [6, 7]. The production of EF for use as biological control agents against insects requires infective units such as aerial conidia or blastospores [8]. Conidia are produced in solid substrates or on the external surfaces of infected insects [9]. In contrast, blastospores form mainly in liquid media cultures (LMC) [1]. One of the techniques commonly used to produce conidia on a large scale is SSC. Advantages of this method of production include that the conidia produced are more tolerant to desiccation, and more stable during the manufacture of complex formulations compared with conidia produced in LMC. Additionally, agroindustrial by-products (cheap raw materials) are generally used as the solid substrate [10].

The growth and metabolism of fungi are influenced by various factors including pH, temperature, moisture content, substrate particle size and gas exchange during solid-state culture [3, 11, 12]. In order to reduce the constraints imposed by limited gas transfer, which are governed by the packing of the substrate within the reactor, texturizers can be added to increase the porosity (ε) of the media, to reduce the compaction of the substrate and to increase the contact area between the substrate and microorganisms, thus favouring gaseous interchange [3, 13, 14]. The agents most commonly used as texturizers include straw, cotton waste, sawdust, wood chips, pruning waste (branches and dry trunks), dry grass, sugar cane bagasse and polystyrene foam [13, 14].

Water hyacinth (*Eichhornia crassipes*) is a weed (undesirable plant) due to its negative environmental impact on different water bodies worldwide [15]. On the other hand, this plant has many beneficial applications such as in the development of handicrafts, biogas production, treatment of oil spills and recycling of organic substances or industrial waste, as well as being used as a fertilizer and animal feed [16]. The use of common water hyacinth as a texturizer in SSC for the production of conidia of entomopathogenic fungi has not been reported.

Although it is important to maximize the production of aerial conidia, it is also essential to ensure that conidia are of a suitably quality (infectivity), since media composition influences conidial yields and quality [17]. Infectivity is the ability of the conidium to enter the host and be distributed and/or reproduce within the host [18] and can be determined via bioassays that provide parameters allowing comparison among strains or treatments [17, 9].

The aim of this study was to evaluate variations of porosity (ε) on the conidial production and infectivity of two strains of *I. fumosorosea* during SSC, using different proportions of rice and water hyacinth (WH amendment) in SSC mixtures.

Materials and Methods

Microorganisms, Monocolony Culture, and Propagation

The study used two strains of *I. fumosorosea*. The strain CNRCB1 (recently assigned with the key CHE-CNRCB 303) was obtained from the collection of entomopathogenic fungi from the *Centro Nacional de Referencia de Control Biológico* (CNRCB) in Tecoman, Mexico. The strain ARSEF3302 was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) in Ithaca, New York, USA. Both strains were identified as *I. fumosorosea*, with accession numbers in GenBank being HM209049 and HM209050 for CNRCB1 and ARSEF3302, respectively [19].

Monocolony cultures were obtained for both strains by streaking onto Sabouraud maltose agar (SMA) comprising (g L^{-1}) the following: maltose 20, meat peptone 2.5, casein peptone 2.5, yeast extract 0.5, and agar 15 (all constituents; Bioxon, Mexico), contained in 9 x 1.5 cm Petri dishes. The cultures were incubated for 10 days at 28 °C. Subsequently, reactivation through infection of *Galleria mellonella* was achieved, then conidia were taken from the dead insect and reinoculated in Petri dishes with SMA (2 %) medium containing sodium deoxycholate (0.05 %) (Sigma-Aldrich, Auckland, New Zealand); the latter is a growth inhibitor of filamentous organisms and makes it easier to obtain isolated colonies [20]. From this culture, independent colonies were propagated by streaking onto Petri dishes containing 20 mL of oat flour medium. The oat flour medium [21] contained (g L^{-1}) the following: oat flour 33.3 (Grupo Industrial Vida, Zapopan, Jalisco, México), bacteriological agar 15, and meat peptone 10 (both constituents from Bioxon, Mexico City, Mexico). The strains on the Petri dishes were then subject to long-term conservation in sterile deionized water [22]. The sporulating cultures were cut in small blocks (5 mm^2) and placed into the tubes filled with sterile deionized water, then those vials were preserved at 4 °C. Sterile glass tubes of 150× 15 mm with screw caps were used containing 4 mL of sterile deionized water. The preservation was performed every 6 months using this method, since after this time, the viability gradually decreases [22]. Cultures were started taking one vial from the preservation by transferring aseptically a block of inoculum onto a dish of oat flour fresh medium, whose composition was described above. Propagation was carried out in Erlenmeyer flasks (250 mL) containing 50 mL of oat flour medium, previously autoclaved at 15 PSI for 15 min. The flasks were incubated at 28 °C for 10 days and then were used to carry out all of the experiments.

Culture Conditions

The experimental units were glass bottles (80 mL), capped with cotton plugs, with an internal diameter ($\varnothing_i$) of 5 cm and a height (L) of 4.7 cm. Water hyacinth (WH amendment) was used as texturizer, obtained from *Tecnología Especializada en el Medio Ambiente* (TEMA, Mexico). Only dried leaves and stalks of WH amendment were used. Parboiled rice (PR) was used as the main substrate, parboiled rice refers to rice treated by steaming which is then commercially available as “parboiled rice” presentation by Verde Valle™ (Uruguay). Both rice and water hyacinth were passed through sieves with pore diameter 3.36, 2.88, 2.37, 2, 1.19, and 0.80 mm (no. 6, 7, 8, 10, 16, and 20 sieves, respectively); for further experiments, selected particle sizes were used for water hyacinth, 2 mm (width), and for parboiled rice, average of 2 mm (width) by 5 mm (length). The proportions of PR:WH are expressed in percentage of a total of 10 g of solid initial

dry matter. The following proportions of PR:WH (% weight:weight): were tested: 100:0, 90:10, 80:20, and 70:30. Ten grams of solid material was used in each glass bottles, the amount of rice was different depending on the treatment, parboiled rice (g): 10, 9, 8, and 7, for the mixtures PR:WH (%): 100:0 90:10, 80:20, and 70:30, respectively. The mixtures were sterilized in an autoclave at 121 °C for 15 min. The medium was inoculated with conidial suspension (1 mL) containing 1×10^7 conidia mL^{-1}, corresponding to an initial inoculum of 1×10^6 conidia per gram of initial dry matter (conidia $gdim^{-1}$). The inoculum was produced on 50 mL oat flour medium (described in section "Microorganisms, monocolony culture, and propagation"). Sterile distilled water was added to obtain a level of 40 % water content [14]. The contents of each bottle were mixed with a sterile spatula, and then, all experimental units (bottles) were placed in containers which had the following dimensions: 26.6 cm×11.5 cm×20 cm. Every container was provided with hermetic cover having inlet and outlet of air valves. These containers were incubated at 28 °C with a photoperiod of 12:12 (h), the % relative humidity in flasks was 37 % in all mixtures of PR:WH, since they were inoculated and incubated simultaneously under the same conditions.

Conidia Kinetics

Conidia production was carried out in 21 bottles per mixture of PR:WH with three bottles as negative controls (with no inoculum). Three bottles per mixture were taken each 24 h (replicates), and further experiments were performed at different times (three experiments).

Data from conidia counts were obtained using the whole 10 g of solid initial matter contained in each bottle. Solid matter from those samples was transferred to a glass beaker, and the conidia harvested using a 0.05 % Tween 80 solution (Amresco, Ohio, USA) for 10 min on a magnetic stirrer [19, 23]. Different volumes of Tween 80 (0.05 % solution) were required for each mixture employed, due to the fact that the bed height increased in accordance with the percentage of water hyacinth. Sixty milliliters of Tween 80 (0.05 % solution) was used to harvest conidia for the PR:WH (%): 100:0 and 90:10 mixtures, while 70 and 80 mL were used for the PR:WH (%): 80:20 and 70:30 mixtures, respectively. The extracts were then filtered through sterile 10×10 cm gauze to eliminate the solids and obtain conidial suspensions. Serial dilutions were made, and the conidia were counted in a Neubauer chamber (Marienfield, Lauda-Königshofen, Germany), with a light microscope (BOECO) using a×40 objective lens. The production levels were reported directly as conidia per gram of initial dry rice, C_r (conidia $gidr^{-1}$).

Porosity and Bulk Density

In order to evaluate initial porosity, 10 g of sterilized material was packed into glass bottles, and water was added to achieve an initial moisture of 40 % as water content in each mixture of PR:WH (using triplicates). Subsequently, each bottle was filled with mineral oil (REASOL™, Mexico), and the porosity fraction (ε) was calculated according to Mitchell et al. [24]:

$$\varepsilon = \frac{(V_t - V_s)}{V_t}$$

where ε is the porosity fraction (dimensionless); V_t is the total volume (mL), namely, this is the volume occupied by the sample including the void volume; V_s is the volume occupied only

by the sample (mL); and the difference between V_t and V_s is the void volume (mL). The void volume was determined by the volume of mineral oil necessary to cover the height of the substrate bed; such volumes were (mL) as follows: 12.7, 21.2, 34, and 45 corresponding to PR:WH (%) values of 100:0, 90:10, 80:20, and 70:30, respectively.

The bulk density is defined as the mass of particles of a material divided by the total occupied volume. The total volume includes particle volume, interparticle void volume, and internal pore volume. The bulk density was calculated with the following expression [24]:

$$\rho_b = \frac{W_s}{V_t}$$

where ρ_b is the bulk density (g mL^{-1}); W_s is the weight of the sample (g); V_t is the total volume occupied by the sample (mL).

Evaluation of Infectivity (Bioassays)

The insect *Galleria mellonella* is a common pest in apiculture or beekeeping processes affecting the economy of this activity around the world [25]; moreover, this is an insect model to test and compared virulence of fungi [26]. Infectivity parameters obtained by bioassays allow the evaluation of the quality of conidia produced by entomopathogenic fungi [9]. The infectivity of the conidia of both strains was evaluated using *G. mellonella* larvae (Petmmal, Mexico) weighing between 62.2 and 71.6 mg. The infectivity tests were performed with conidia obtained on day 8. Seven Petri dishes were used (five dishes for infected larvae and two dishes as a negative control) with each containing ten larvae. Conidial suspensions (20 mL; 5×10^7 conidia mL^{-1}) were prepared using the aerial conidia produced in each mixture of PR:WH. Larvae were submerged for 5 s in those suspensions from each treatment [27]. The control larvae were submerged only in Tween 80 (0.05 % solution) for 5 s. After treatment, the larvae were fed with *Wax Worm* (PETMMAL, Mexico) diet to avoid death by starvation and incubated with a photoperiod of 12:12 (h) at 28 °C. Mortality was recorded every 24 h for 12 days.

In order to compare infectivity of conidia harvested from different treatments, specific parameters were estimated according to the model proposed previously [17].These include the time at which the first dead larva appeared (t_0, delay time), the half lethal time (LT_{50}, the time at which 50 % mortality is reached) and the percentage of survival (S) were calculated and employed in a decay model previously described by Rodríguez-Gómez et al. [17]. The model uses a first-order decay equation with the indicated delay time:

$$\begin{aligned} Y &= 100; && \text{If } 0 \le t \le t_0 \\ Y &= (100-S)e^{-k(t-to)} + S; && \text{If } t > t_0 \end{aligned}$$

where Y is the survival (%) at time t; k is the specific death rate ($days^{-1}$); t_0 is the time required for the first larval death (days); and S is the estimated asymptotic survival level (%).

Statistical Analysis

For the statistical analysis, mean conidia production values and infectivity parameters obtained for each porosity were employed. Analysis of variance (Tukey test) was used to compare

results obtained with each treatment and each given strain using one-way analysis with significance level, $p<0.05$ with the help of SPSS program (SPSS, Chicago, IL).

Results and Discussion

Conidia Production and Porosity

The addition of water hyacinth to the mixture significantly ($p<0.05$) increased the porosity fraction (ε) or empty space (Fig. 1), it was found a linear correlation (Y=0.0551*X+0.1989; $R^2=0.947$), and consequently significantly ($p<0.05$) reduced the packing density (Y= −0.1663*X+0.8653; $R^2=0.931$). Conidial production kinetics are presented, for CNRCB1 and for ARSEF3302 (Figs. 2 and 3), for both strains in all PR:WH mixtures, maximal conidia production was observed on day 8. For strain CNRCB1, when the conidia production was reported on the basis of gram of initial dry rice (C_r), levels were significantly different ($p<0.05$), with the highest values (up to 1.33 times) for the 90:10 and 80:20 mixtures. Similarly, for strain ARSEF3302, the 80:20 mixture supported the highest levels of conidial production, with an increase of 1.55 times over the control level (PR:WH=100:0). However, with both strains, the 70:30 mixture had similar conidiation level as compared to control PR:WH=100:0 ($p>0.05$).

The production of conidia by *I. fumosorosea* strains has been reported to be 1×10^9 conidia per gram of initial solid substrate [4, 28]. Present results were found to be in the range from 1.3×10^9 to 6.9×10^9 conidia per gram initial dry rice. This is a good result because it shows that taking the proposed dose 1×10^{13} conidia sprayed per hectare [8], it would require only from 1.5 up to 8 kg of substrate (rice grains) per hectare to produce the recommended dose of conidia for a single treatment in crop fields.

The water hyacinth was incorporated as a texturizer to avoid compaction of the substrate based on rice, to increase the porosity fraction (ε) of the bed and to promote oxygen transfer.

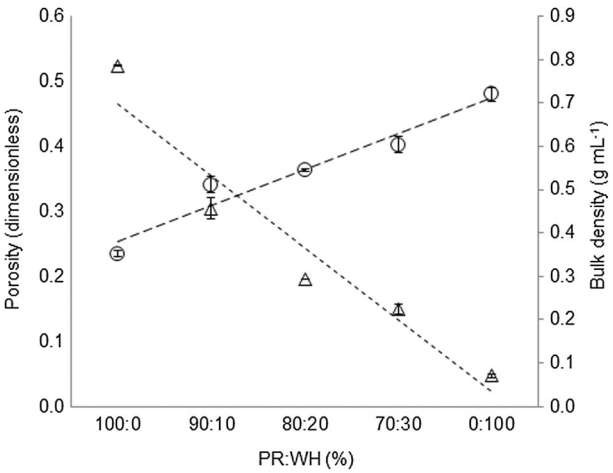

Fig. 1 Porosity (*circles*) and bulk density (*triangles*) in mixtures of parboiled rice (PR) and water hyacinth (WH amendment). Linear correlation was indicated with the *dashed lines* ($R^2=0.947$ for porosity and $R^2=0.931$ for bulk density)

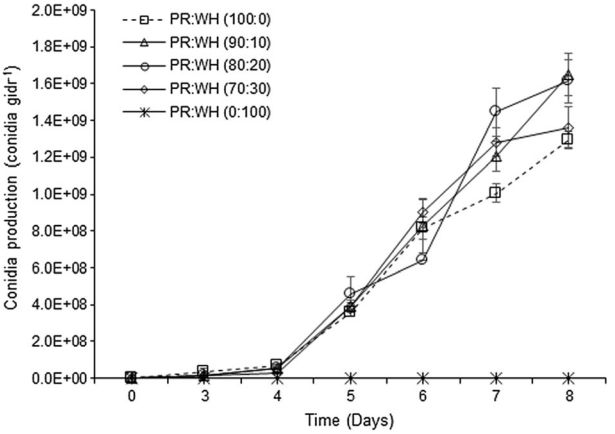

Fig. 2 Conidia production for 8 days by *Isaria fumosorosea* CNRCB1 in mixtures of parboiled rice (PR) and water hyacinth (WH amendment). C_r (conidia per gram initial dry rice)

The impact of this texturizer on conidia production was then analyzed. When 30 % texturizer was used, conidia production per gram of initial dry rice was lower compared with the other treatments and did not differ significantly from that in the control with the mixture containing PR:WH 100:0. This pattern was also observed with *Beauveria bassiana*, with higher proportions of texturizer (sugar cane bagasse) reducing conidia production per gram of total solid matter. In contrast, there was no significant difference when conidia production was reported per gram of wheat bran (the main substrate) [14]. In the present study, both strains of *I. fumosorosea* grew mainly on the rice grains (the rice was the unique substrate), there was negligible growth of mycelium on the water hyacinth, neither growth or conidiation of this microorganism was observed in the water hyacinth particles (Figs. 2 and 3). Arzumanov et al. [29] reported similar results in a SSC system using rice and sugar cane bagasse (50:50),

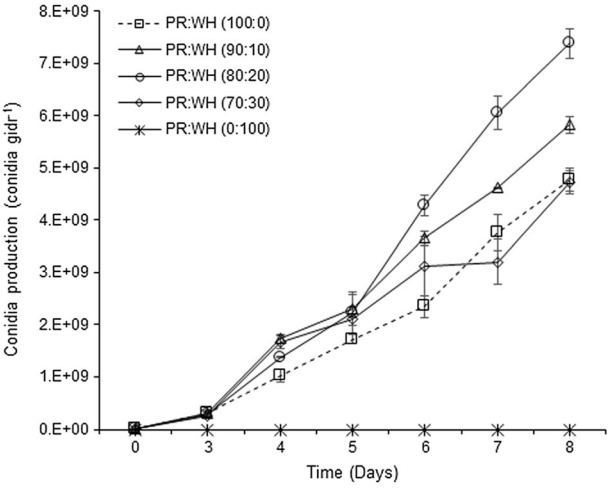

Fig. 3 Conidia production for 8 days by *Isaria fumosorosea* ARSEF3302 in mixtures of parboiled rice (PR) and water hyacinth (WH amendment). C_r (conidia per gram initial dry rice)

observing that *Metarhizium anisopliae* grew almost exclusively on the grains of rice. For both strains of *I. fumosorosea*, experiments were conducted using only water hyacinth, and only a negligible growth was observed without sporulation. This shows that *I. fumosorosea* and *M. anisopliae* prefer rice as the main carbon source, possibly because the production of the lignocellulolytic enzymes, required to hydrolyze components of either water hyacinth or sugar cane baggase, is not favoured by these fungi.

The results of Figs. 1, 2, and 3 seem to support that extreme porosity values ($\varepsilon \approx 0.24$ and 0.48) are less favourable for condial production than intermediate porosity values ($\varepsilon \approx 0.35 \pm 0.05$). Details on the statistical analysis of conidial production as a function of porosity in three separate experiments, each one with three replicates, are shown in Table 1. It should be noted that the negative control (PR:WH=0:100) without fungal growth and consequently, negative conidiation, showed that this effect was not related to the alternative use of water hyacinth as a substrate ($\varepsilon = 0.48 \pm 0.01$).

The incorporation of the water hyacinth was effective to increase the ε value, reaching a critical level between 0.34 and 0.36, which increases surface area and improves exchange gaseous according to Arzumanov et al. [29]; this was related to higher conidia production. When fungi grow on a solid substrate, the packing density [3] and the height of the bed affect conidia production [13], thus a high packing density may limit the aeration and therefore the exchange of gases at the bottom of the bed. This situation could adversely affect the growth of the fungus [11].

The mechanistic explanation of why porosity has an optimal value for conidial production requires a study of the relationship between respiratory and growth functions that are beyond the aim of this work. Miranda et al. [30] and Miranda et al. [31] have shown that porosity of

Table 1 Effect of porosity in the conidia production of the CNRCB1 and ARSEF3302 strains of *Isaria fumosorosea*

Strain	ε[a] (dimesionless)	Experiment 1 C_r[b] Conidia gidr^{-1} 1×10^9	Experiment 2 C_r[b] Conidia gidr^{-1} 1×10^9	Experiment 3 C_r[b] Conidia gidr^{-1} 1×10^9
I. fumosorosea CNRCB1	0.24	1.2±0.04[e]	1.53±0.06[d]	2.37±0.43[d]
	0.34	1.6±0.11[c]	2.5±0.10[c]	4.17±0.10[c]
	0.36	1.6±0.11[c, d]	2.47±0.12[c]	3.93±0.27[c]
	0.40	1.3±0.11[d, e]	1.37±0.04[d]	2.59±0.14[d]
I. fumosorosea ARSEF3302	0.24	4.7±0.21[e]	4.46±0.37[d]	1.97±0.07[e]
	0.34	5.8±0.16[d]	6.02±0.15[c]	4.08±0.12[d]
	0.36	7.3±0.28[c]	6.89±0.75[c]	5.07±0.25[c]
	0.40	4.7±0.22[e]	3.77±0.13[d]	2.1±0.16[e]

Three replicates were analysed at day 8

[a] Porosity

[b] Conidia per gram of initial dry rice

[c, d, e] Means in the same column with different letters indicate a significant difference ($p<0.05$)

polyurethane as inert support has a remarkable biphasic effect on the production of reactive oxygen species and the induction of superoxide dismutase of *Aspergillus terreus.* This is an interesting area of research because it points on the importance of the structural properties of solid support that go beyond the average composition of solid substrates and highlight on possible physiological effects linked to oxygen mass transfer and occupation of the interstitial space by the mycelial mat as suggested by earlier work [32, 33]. As a practical consequence, present results indicate the need to measure porosity as a fermentation parameter that could change the yield of conidia per gram of initial substrate. They also show the possibility of modifying this structural parameter by the appropriate blending of grains easy to breakdown and fibers which are not consumed. Namely, rice grains blended with dried water hiacynth fibers.

In order to analyze whether conidia extraction may be affected by using different amount of Tween 80, a simultaneous experiment was carried out where 80 mL of Tween 80 (0.05 % solution) was used for the extraction of conidia of each bottle. For CNRCB1 strain, no significant difference ($p<0.05$) was found in the extraction of conidia using different volumes of 0.05 % Tween 80 solution (60, 70, or 80 mL) or an equal volume (80 mL) in every treatment with different amounts of texturizer (Electronic Supplementary Material Table S1). For ARSEF3302 strain, significant difference ($p<0.05$) was found only in the treatment PR:WH (%): 100-0 (Electronic Supplementary Material Table S2). However, the profiles of better production of conidia in the 90-10 ($\varepsilon=0.34$) and 80-20 ($\varepsilon=0.36$) mixtures were always consistent in both strains.

Conidia Infectivity Bioassays

For both strains, infectivity tests on *Galleria mellonella* were performed with conidia obtained on day 8 as described in "Material and Methods". Figure 4 shows the typical stages of infection of *G. mellonella* larvae by *I. fumosorosea*. The parameters t_0 and LT_{50} did not differ significantly between treatments for strain CNRCB1 (Table 2). This indicates that the aerial conidia, produced in each of the mixtures, initiate the infective cycle at a similar time (between 5.9 and 6.9 days), and killed 50 % of the population between 7.9 and 8.2 days; although values for *S* (asymptotic survival level) differed between mixtures, conidia obtained in the PR:WH 100:0 and 90:10 mixtures reached a higher mortality, significantly different from those of other

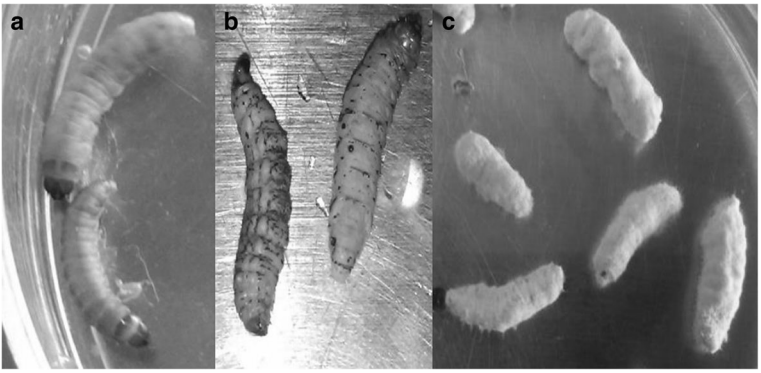

Fig. 4 Typical stages of infection of *Galleria mellonella* larvae by *Isaria fumosorosea* CNRCB1: **a)** uninfected larvae; **b)** melanised larvae, **c)** larvae with mycosis. Infectivity parameters are described in the text

treatments ($p<0.05$). The fact that ARSEF3302 strain was less lethal to *G. mellonella* ($S\leq$ 50 %) than CNRCB1 strain, made irrelevant the evaluation of LT_{50}. However, no significant difference ($p>0.05$) in t_0 and S values was found with such strain. Thus, the mixture of water hyacinth with rice grains had no detrimental effect on infectivity parameters of those strains of *I. fumosorosea* when tested with *G. mellonella*, since the main effect was on the conidiation level.

Analysis of the infectivity parameters showed that conidia of the CNRCB1 strain achieved an S value of between 12 and 24 %, with an LT_{50} between 7.9 and 8.6 days. These values were similar to that reported recently by Carrillo-Pérez et al. [1] for aerial conidia produced in a surface culture of *I. fumosorosea* P43A in bioassays on *Galleria mellonella* larvae (8.3± 1.45 days). On the other hand, the conidia of strain ARSEF3302 produced an average final survival (S) of 55 %, and the t_0 was similar among all mixtures of PR:WH (6 days), similar to that of the CNRCB1 strain (t_0=6.5 days); nonetheless, ARSEF3302 strain proved to be less infective than CNRCB1 strain (Table 2). This observation is supported by another recent study showing that the ARSEF3302 strain has lower infectivity toward *Galleria mellonella* compared to strain CNRCB1 [19]. In addition, the conditions for optimal conidia production were not necessarily the optimal for obtaining high quality conidia [17, 34].

Conclusion

As concluding remarks, water hyacinth is a suitable texturizer in solid-state culture, which in turn improves the production of conidia by *I. fumosorosea* by increasing the ε to an optimal levels. The infectivity of conidia was not altered substantially in those treatments with high conidial yields, which is an important factor to consider during the production of conidia of entomopathogenic fungi at larger scales.

Table 2 Infectivity parameters of the conidia of the CNRCB1 and ARSEF3302 strains of *Isaria fumosorosea* with *G. mellonella* as the host

Strain	ε[a] (dimensionless)	t_0[b] (days)	LT_{50}[c] (days)	S[d] (%)
I. fumosorosea CNRCB1	0.24	6.92±0.52[e]	8.28±0.47[e]	12±4.47[g]
	0.34	6.67±1.11[e]	8.59±0.48[e]	14±5.48[f, g]
	0.36	6.46±0.83[e]	8.09±0.69[e]	22±4.47[e, f]
	0.40	5.92±0.80[e]	7.93±0.23[e]	24±5.48[e]
I. fumosorosea ARSEF3302	0.24	5.55±0.64[e]	*NR*[h]	42±8.37[f]
	0.34	6.53±1.20[e]	*NR*[h]	50±10.0[e, f]
	0.36	6.55±0.69[e]	*NR*[h]	56±5.48[e]
	0.40	5.10±0.81[e]	*NR*[h]	58±4.47[e]

[a] Porosity

[b] Delay time, the time when first dead larva appears

[c] The median lethal time, when 50 % mortality is reached

[d] The percentage survival

[e, f, g] Means in the same column with different letters indicate a significant difference ($p<0.05$)

[h] Not reached

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