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# Solid-state fermentation of cereal grains and sunflower seed hulls by *Grifola gargal* and *Grifola sordulenta*

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ABSTRACT

*Grifola gargal* and *Grifola sordulenta* are edible and medicinal mushrooms from Andino-Patagonian forests. There is a need to find an alternative source for these mushrooms other than gathering them due to increasing pressure on their habitats. Thus, in order to find an appropriate technological pathway to grow these mushrooms, solid-state fermentation (SSF) in different substrates was studied. Mycelia cultivation on grains exhibited the best results when using wheat grains at pH 5.3, 24 °C, in darkness. When using sunflower seed hulls (SSH) the protein content of the growth medium increased significantly after 45 days SSF and a good laccase activity was measured. Further mycelium growth optimization was achieved in the presence of 0.01 N $H_2SO_4$, 20 μg/g Mn(II) and 100 μg/g Zn(II) in *G. gargal* (50% SSH and 30% milled SSH, 15% residual substrate of *Pleurotus ostreatus*, and 5% wheat bran) and in *G. sordulenta* (80% SSH, 15% residual substrate of *P. ostreatus* and 5% wheat bran). Present preliminary studies on basidiome production showed that cultivation conditions should require at least a sterile substrate, 10–12 % inoculation rate, cold shock for primordia induction, and control from air borne contamination.



## Introduction

"Mushrooms" are the reproductive structures within a whole organism, with a special arrangement of the fungal multicellular web also present in the growing substrate, "the mycelium". In lignocellulolytic fungi, the mycelia growth in substrates takes place using different catabolic pathways which gives access to carbohydrates that are otherwise effectively protected by lignin. This process can be prolonged until nutrient depletion, when specific environmental cues trigger reproductive responses which may include the development of basidiomes (=mushrooms).

Medicinal and edible mushrooms are mostly found in the higher Basidiomycetes and they usually have a saprophytic and an aerobic growth habit which allow them to grow on different lignocellulosic materials (Chang, 2008). Species such as *Grifola gargal* and *Grifola sordulenta* have received increasing attention in the field of applied mushroom biotechnology. These species grow in natural forests in the Andino-Patagonic areas of Argentina and Chile, causing white rotting mainly in dead tissues of *Nothofagus obliqua* and *Nothofagus dombeyi*, respectively (Rajchenberg, 2006). *Grifola gargal* is consumed as food, and both species have an almond flavor which is an unusual trait among edible mushrooms that is derived from secondary metabolites. Regarding their functional properties, the antigenotoxic and antioxidant activities in their methanolic extracts were investigated (Postemsky et al., 2011, Postemsky and Curvetto, 2014a, Postemsky and Curvetto, 2015); also ergothioneine was claimed to be the main antioxidant molecule in hot water extracts of *G. gargal*, which also exhibited anti-inflammatory activity (Ito et al., 2011). Antioxidant activity due to phenolic compounds, including flavonoids, was also found in hydroalcoholic extracts of *G. gargal* (Schmeda-Hirschmann et al., 1999; De Brujin et al., 2010). Furthermore, wheat grains solid-state fermentation (SSF) in these species results in biotransformed grains with antioxidant and antigenotoxic properties, and thus a flour with an enhanced functional value can be obtained (Postemsky et al., 2011, Postemsky and Curvetto, 2014a). Nevertheless, at present grains carrying mycelium are commonly used for making spawn, i.e. the inoculum used in large-scale mushroom cultivation. Therefore, in the present study, SSF of cereal and oilseed grains by *G. gargal* and *G. sordulenta* was studied to find a technological method for cultivating hypothetically functional foods and to produce spawn for the mushroom industry. Previous studies on SSF with these *Grifola* species have not been reported for either the lignocellulolytic

*Abbreviations:* SSF, Solid-state fermentation; SSH, sunflower seed hulls.

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bioprocess or cultivation at a large scale. Strain selection has to be carried out in wild-strain (non-domesticated) isolates. So, in an effort to obtain domesticated strains for both *G. gargal* and *G. sordulenta*, mycelia from different isolates were periodically cultured (from 2006 to 2009 and to the present) in agar semi-solid medium enriched with milled sunflower (*Helianthus annuus*) seed hulls (SSH), according to Postemsky et al. (2006). The selection of sunflower seed hulls, an abundant residue of the oil industry, was also undertaken as a contribution to their profitability and ecologically friendly disposal into the environment. They represent 18–20% weight of the seed and the 2013 production of sunflower seed oil was estimated at ca. 680,000 tons of SSH in Argentina. It has already been found that these hulls are a suitable substrate for SSF with mushrooms, due to their convenient particle size and shape, which make it possible to obtain a substrate with high porosity and a good nutrient content, appropriate for sustaining mycelium growth under aerobic conditions (Curvetto et al., 2004).

Since *G. gargal* and *G. sordulenta* are only found in protected areas of Argentina and Chile, e.g. *Parque Nacional Lanín* (Argentina), it is strongly recommended that these mushrooms are not gathered until ecological studies can assert that they are not in a vulnerable condition. On the other hand, SSH have already been found as an outstanding lignocellulosic material for mushroom cultivation, on account of the previously mentioned properties. In fact, some optimized protocols of edible and medicinal mushroom species cultivated on a substrate based mainly on these hulls have been reported: *Ganoderma lucidum* (González Matute et al., 2002; Bidegain et al. unpublished results), *Pleurotus ostreatus* (Curvetto et al., 2004), *Lentinus edodes* (Curvetto et al., 2005), *Hericium erinaceus* (Figlas et al., 2007), *Agaricus blazei* (González Matute et al., 2010), and *Schizophyllum commune* (Figlas et al., 2014).

The main objectives of the present study were to optimize the formula for the SSF of grains and also of SSH based substrates, and to evaluate controlled environmental conditions for mushroom cultivation of *G. gargal* and *G. sordulenta* using SSH as the main substrate for SSF.

## Materials and methods

### *Fungal sources*

*Grifola gargal* Singer (strain: CIEFAP #191) and *G. sordulenta* Mont. (Singer) (strain: CIEFAP #154) were obtained from CIEFAP (*Centro de Investigación y Extensión Forestal Andino Patagónico*, Argentina). Agar cultures were prepared according to Postemsky et al. (2006).

### *Solid-state fermentation of cereal and oilseed grains*

Substrates, formulated with cereal or oilseed grains, were subjected to SSF by both *Grifola* species using the *linear growth test*, as described elsewhere (Postemsky et al., 2014). Briefly, soaked grains (10 g) were placed in a glass tube (16 mm diameter) to reach a density of 0.45–0.62 g $ml^{-1}$ and sterilized at 121 °C for 90 min. Inoculation was performed by placing a disk of nutrient agar carrying young mycelia on one end of the sterilized substrate. Mycelium was allowed to grow for 30 days at 18 ± 1 °C, 90% relative humidity and in darkness. Afterwards, the length of mycelium was used to calculate the mass of substrate (DW) colonized per day using the formula: *Substrate Colonization Rate* (mg $day^{-1}$) = (mg $cm^{-3}$ *density of substrate in dry basis* × cm *length of mycelial growth* × 2.01 $cm^2$ *area of the tube*)/days *of incubation*. This parameter was obtained in order to exclude the variation between substrate densities, which was considerable. Apparent mycelium density was also registered.

Substrate formulations (soaked grains) were prepared as follows: 600 g grains of either wheat (*Triticum durum*), millet (*Panicum miliaceum*), wheat: millet (4:1,w/w), corn (*Zea mays*), sunflower (*Helianthus annuus*) or corn: sunflower (1:1, w/w) were soaked for 16 h in 400 ml of calcium salts water dispersions adjusted to two pH conditions, "pH 6.5" (1.5% $CaCO_3$w/w and 0.8% $CaSO_4$w/w, pH values between 6.2 and 6.8) and "pH 5.3" (0.02% $H_2SO_4$ 98% w/w and 0.8% $CaSO_4$ w/w, pH values between 5.1 and 5.6).

### *Solid-state fermentation using "One liter bottle-spawn technique"*

Wheat grains (250 g) were placed in a 1 l glass bottle containing 190 ml of calcium salts water dispersion (1.5% $CaCO_3$ w/w and 0.8% $CaSO_4$ w/w), soaked for 16 h and then sterilized at 121 °C for 90 min. A final weight of 470 g with 42–44% water content was obtained. Inoculation of sterilized grains was performed using mycelium grown in nutrient agar (4 wedges, 1.5% inoculation rate, by weight). Incubation of experimental units (n = 10) was performed at two temperatures (20 °C and 24 °C) and in darkness. Complete SSF was recorded at days 20, 25 and 30. After 30 days, the spawn was removed from the bottles and the number of clusters of colonized grains per treatment was counted.

### *Solid-state fermentation of sunflower seed hulls*

Table 1 shows both the initial composition and properties of SSH based substrates evaluated with the *linear growth test*. The substrate was prepared as follows: 440 g substrate mixtures (10–12% water content) were soaked in 560 ml of calcium salts water dispersion (0.5% $CaCO_3$ and 2% $CaSO_4$) with 60% final water content. Experimental tubes (n = 10) were filled with 10 g substrate, compressed and sterilized (121 °C, 2 h). Water content and pH were obtained at the time of substrate inoculation. Incubation (45 days) took place in darkness, at 18 ± 1 °C, 90% RH, in a culture chamber. The substrate colonization rate (mg $day^{-1}$) was calculated as previously indicated in 2.1.

Five experimental units were randomly selected to determine the protein content and laccase (EC 1.10.3.2) activity. The substrate (3 g) was extracted in 10 ml of 0.05 M $KH_2PO_4/K_2HPO_4$ buffer, pH 6.8 with 0.1% Triton-X, during two minutes, using a mortar and pestle, and the resulting suspension was filtered through a Whatman#4 filter paper and stored at 4 °C for 16 h. Proteins were determined by the method of Bradford (1976) with bovine serum albumin as a standard and the results were expressed in μg protein $g^{-1}$ DW substrate. Laccase activity was analyzed as described elsewhere (Postemsky et al., 2014), enzyme units were defined as the amount of enzyme oxidizing 1 mmol of syringaldazine [N,N'-bi(3,5-dimethoxy-4-hydroxybenzylidene hydrazine)] (λ525 nm extinction coefficient ε = 65,000 $M^{-1}$ $cm^{-1}$). The initial (basal) protein content and laccase activity were obtained in control samples before the inoculation step. The results were expressed as enzymatic units per unit dry mass (U $g^{-1}$ DW).

Colonized substrates coming from three experimental units were dried, ground (1 mm grid size) and pooled to determine the fiber fractions (lignin, cellulose and hemicellulose) by the Van Soest acid detergent fiber method as described by González Matute et al. (2010). The results were calculated from the difference in the content of each fiber fraction obtained before and after the running of mycelium in the different substrates.

### *Optimization of solid-state fermentation of sunflower seed hulls*

Further optimization of substrate formulation in SSH based substrates was evaluated with the linear growth test. Optimized basal substrates were formulated according to the best results of

**Table 1**
Solid-state fermentation of sunflower seed hulls based substrates by *Grifola gargal* and *G. sordulenta* analyzed through the *linear growth test*. Substrate components were: sunflower seed hulls (SSH), residual substrate from *Pleurotus ostreatus* cultivation made of sunflower seed hulls ($SSH_R$) which were also milled (SSH milled, $SSH_R$ milled). Wheat bran (WB) was used as a supplementary nutrient source. Substrate initial properties: density (D), protein content (P) and laccase activity (L) are presented. Substrate colonization rate (SCR), mycelium apparent density (AD), increments in protein content (P) and increments in laccase activity (L) were obtained after 45 days of SSF.

| Treat-ments | Substrate composition (%) | | | | | Initial properties[a] | | | *Grifola gargal*[b] | | | | *Grifola sordulenta*[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SSH | SSH milled | $SSH_R$ | $SSH_R$ milled | WB | D g $ml^{-1}$ | P μg g $DW^{-1}$ | L U g $DW^{-1}$ | SCR mg DW $day^{-1}$ | AD | ΔP μg g $DW^{-1}$ | ΔL U g $DW^{-1}$ | SCR mg DW $day^{-1}$ | AD | ΔP μg g $DW^{-1}$ | ΔL U g $DW^{-1}$ |
| T1 | 100 | – | – | – | – | 0.45 | 0.8 ± 0.4 fg | 0.17 ± 0.08 cdef | 111 ± 1.0 a | +++ | 56 ± 2.1 a | 0.51 ± 0.14 abcd | 98 ± 1.2 a | +++ | 40 ± 3 bc | 0.14 ± 0.07 hi |
| T2 | – | – | 100 | – | – | 0.45 | 6.7 ± 0.5 ab | 0.35 ± 0.12 a | 83 ± 1.0 d | +++ | 14 ± 1.4 cdefg | 0.26 ± 0.13 efg | 28 ± 1.2 efg | + | 5 ± 3 d | 0.38 ± 0.09 cdefg |
| T3 | – | 100 | – | – | – | 0.50 | 1.6 ± 0.4 defg | 0.23 ± 0.09 abcde | 106 ± 1.0 ab | +++ | 29 ± 1.9 abcde | 0.71 ± 0.18 ab | 87 ± 1.2 ab | ++ | 16 ± 3 cd | 0.68 ± 0.17 abc |
| T4 | – | – | – | 100 | – | 0.50 | 2.2 ± 0.4 cdef | 0.20 ± 0.08 bcdef | 53 ± 1.0 h | + | 10 ± 1.6 efg | 0.46 ± 0.12 bcdefg | 1 | 0 | 3 ± 3 d | 0.41 ± 0.08 bcdef |
| T5 | 50 | | 50 | – | – | 0.45 | 3.3 ± 0.5 abcd | 0.28 ± 0.09 abc | 93 ± 1.0 c | +++ | 38 ± 2.1 abc | 0.57 ± 0.15 abc | 97 ± 1.2 a | +++ | 43 ± 3 bc | 0.38 ± 0.17 cdefgh |
| T6 | 50 | – | – | 50 | – | 0.45 | 3.6 ± 0.4 abcd | 0.23 ± 0.08 abcde | 90 ± 1.0 c | +++ | 32 ± 1.5 abcd | 0.21 ± 0.11 g | 83 ± 1.2 abc | +++ | 21 ± 3 bcd | 0.45 ± 0.10 abcdef |
| T7 | – | 50 | 50 | – | – | 0.50 | 2.7 ± 0.5 bcde | 0.22 ± 0.11 abcde | 102 ± 1.0 b | +++ | 15 ± 1.2 cdefg | 0.49 ± 0.14 bcde | 86 ± 1.2 ab | ++ | 41 ± 3 bc | 0.34 ± 0.11 efgh |
| T8 | – | 50 | – | 50 | – | 0.55 | 1.8 ± 0.4 defg | 0.11 ± 0.07 ef | 86 ± 1.0 cd | ++ | 13 ± 1.4 defg | 0.36 ± 0.12 defg | 2 | 0 | 2 ± 4 d | 0.45 ± 0.09 abcdef |
| T9 | 80 | – | – | – | 20 | 0.50 | 2.6 ± 0.4 bcde | 0.07 ± 0.06 f | 103 ± 1.0 ab | +++. | 16 ± 2.0 bcdefg | 0.46 ± 0.15 bcdefg | 107 ± 1.2 a | +++ | 96 ± 4 a | 0.36 ± 0.11 defgh |
| T10 | – | – | 80 | – | 20 | 0.50 | 7.6 ± 0.7 a | 0.23 ± 0.08 abcde | 86 ± 1.0 cd | ++ | 8 ± 1.2 fg | 0.40 ± 0.16 cdefg | 43 ± 1.2 cdef | + | 7 ± 3 d | 0.52 ± 0.14 abcde |
| T11 | – | – | – | 80 | 20 | 0.62 | 5.2 ± 0.9 abc | 0.14 ± 0.06 ef | 51 ± 1.0 h | ++ | 21 ± 1.6 abcdef | 0.63 ± 0.18 abc | 5 | 0 | 6 ± 3 d | 0.57 ± 0.12 abcde |
| T12 | – | 80 | – | – | 20 | 0.62 | 3.1 ± 0.4 abcd | 0.16 ± 0.06 def | 90 ± 1.0 c | +++ | 36 ± 1.7 abc | 1.02 ± 0.18 a | 69 ± 1.2 bcd | +++ | 27 ± 3 bcd | 0.28 ± 0.12 fghi |
| T13 | 25 | 75 | – | – | – | 0.55 | 1.4 ± 0.4 efg | 0.28 ± 0.07 abc | 91 ± 1.0 c | +++ | 47 ± 2.1 ab | 0.61 ± 0.18 abc | 68 ± 1.2 bcd | ++ | 4 ± 3 cd | 0.09 ± 0.08 i |
| T14 | – | – | 25 | 75 | – | 0.50 | 2.8 ± 0.8 abcde | 0.33 ± 0.09 ab | 63 ± 1.0 fg | + | 12 ± 1.6 defg | 0.23 ± 0.12 g | 4 | 0 | 2 ± 3 cd | 0.27 ± 0.10 fghi |
| T15 | – | 75 | – | 25 | – | 0.62 | 0.8 ± 0.5 g | 0.22 ± 0.06 abcde | 70 ± 1.0 e | ++ | 6 ± 1.8 g | 1.08 ± 0.18 a | 45 ± 1.2 cde | + | 4 ± 3 cd | 0.19 ± 0.11 ghi |
| T16 | – | 25 | – | 75 | – | 0.55 | 3.3 ± 0.5 abcd | 0.28 ± 0.08 abc | 68 ± 1.0 ef | ++ | 18 ± 1.6 bcdefg | 0.50 ± 0.13 abcd | 1 | 0 | 3 ± 3 cd | 0.21 ± 0.12 ghi |
| T17 | 15 | 65 | – | – | 20 | 0.53 | 3.1 ± 0.9 abcd | 0.12 ± 0.06 ef | 81 ± 1.0 d | +++ | 33 ± 1.7 abc | 1.18 ± 0.18 a | 81 ± 1.2 abc | +++ | 25 ± 4 bcd | 0.92 ± 0.14 ab |
| T18 | – | – | 15 | 65 | 20 | 0.53 | 5.7 ± 1.0 abc | 0.27 ± 0.06 abcd | 54 ± 1.0 h | ++ | 23 ± 1.7 abcdef | 0.52 ± 0.10 abcd | 8 | 0 | 2 ± 4 cd | 1.46 ± 0.21 a |
| T19 | – | 65 | – | 15 | 20 | 0.59 | 0.8 ± 0.4 fg | 0.24 ± 0.08 abcde | 68 ± 1.0 ef | +++ | 9 ± 1.1 efg | 0.47 ± 0.17 bcde | 61 ± 1.2 bcd | ++ | 10 ± 3 cd | 0.24 ± 0.13 fghi |
| T20 | – | 15 | – | 65 | 20 | 0.55 | 1.5 ± 0.5 defg | 0.34 ± 0.10 ab | 60 ± 1.0 g | ++ | 24 ± 2.1 bcdefg | 0.23 ± 0.13 fg | 31 ± 1.2 def | + | 3 ± 3 cd | 0.63 ± 0.23 abcd |

[a] Initial substrate properties: pH values and water content were of 5.7–6.2 and 64–70%, respectively. Mean values ± $SE_{MEAN}$ ($n = 5$, $SE_{MEAN} = (S^2_{Ti}/n_{Ti})^{½}$) for protein content and laccase activity are given, Kruskal Wallis test ($\alpha = 0.05$) was used to separate mean values of analyzed parameters.

[b] Mean values ± $SE_{ANOVA}$ of SCR ($n = 10$, $SE_{ANOVA} = (CM_{error}/n_i)^{½}$), treatments exhibiting a SCR less than 10 mg DW $day^{-1}$ were excluded from analysis because of negligible mycelial growth. Data were ln-transformed, different letters indicate significant differences determined with Tukey's test ($\alpha = 0.05$). Mean values ± $SE_{MEAN}$ ($n = 5$, $SE_{MEAN} = (S^2_{Ti}/n_{Ti})^{½}$) for increments in protein content and laccase activity are given, Kruskal Wallis test ($\alpha = 0.05$) was used to separate mean values of analyzed parameters. Apparent mycelial density (AD) was classified as +++ (=*dense*), ++ (=*soft*), + (=*faint*) or 0 (=*no-growth*).

**Table 2**
Culture ambient conditions at different phases in *Grifola gargal* and *G. sordulenta* basidiome development. Assay procedure is described in "Solid-state fermentation of sunflower seed hulls for mushroom cultivation". Primordial induction was done when yellowish exudates appeared. Additional induction for basidiome development was provided following pileus formation.

| Experiment# (n=) | Substrate; weight per bag; and spawn | Ambient conditions[a] | | |
|---|---|---|---|---|
| | | Mycelium running | Primordial induction | Basidiome development |
| *E1* (15) | S1, S2; 600 g; wheat | *Controlled chamber*<br>18 °C; 60% HR; 40/60 days;<br>0.8 air changes; no light | *Controlled chamber*<br>10/12 °C; 60% HR; 30 days;<br>0.8 air changes; no light | *Controlled chamber*<br>10/12 °C; 90% HR; 120–210 days;<br>4 air changes; 500 lux |
| *E2* (20) | S1; 600 g; wheat | *Controlled chamber*<br>18 °C; 60% HR; 40/60 days;<br>0.8 air changes; no light | *Mushroom greenhouse*<br>−1/−5 °C; >95% HR; 30 days;<br>>8 air changes; no light | *Mushroom greenhouse*<br>−1/-5 °C; >95% HR; 30 days;<br>>8 air changes; <500 lux |
| *E3* (15) | S1, S3; 600 g; wheat | *Controlled chamber*<br>18 °C; 60% HR; 40/60 days;<br>0.8 air changes; no light | *Cold storage*<br>5 °C; 55/65% HR; 30 days;<br>1 air change; no light | *Controlled chamber*<br>5 °C (night)/8 °C (light); 90% HR;<br>15–30 days; 4 air changes; 500 lux |
| *E4* (20) | S1; 900 g; wheat | *Controlled chamber*<br>18 °C; 60% HR; 40/60 days;<br>0.8 air changes; no light | *Cold storage*<br>5 °C; 55/65% HR; 0 days;<br>1 air change; no light | *Cold storage*<br>5 °C; 70/95% HR; 15–30 days;<br>1 air change; 500 lux |
| *E5* (10) | S1; 900 g; wheat/wheat: millet/corn/sunflower seeds/corn: sunflower seeds. | *Controlled chamber*<br>18 °C; 60% HR; 40/60 days;<br>0.8 air changes; no light | *Cold storage*<br>5 °C; 55/65% HR; 30 days;<br>1 air change; no light | *Controlled chamber*<br>5 °C (night)/8 °C (light); 95% HR;<br>15–30 days; 8 air changes; 500 lux |

[a] *Controlled chamber* was of 420 l, (Mo. CCC-20 manufactured by SECELEC – CONICET, Bahía Blanca, Argentina); *mushroom greenhouse* area was 60 $m^2$ (5 m height); *cold storage* was of 20 $m^2$ (2.5 m height).

mycelium performance described in 2.4. Optimized basal substrate for *G. gargal* was 50% SSH (12 × 5 mm mean size) and 30% milled SSH (2 × 1 mm mean particle size), 15% biotransformed SSH residual from *P. ostreatus* cultivation and 5% wheat bran (*T. aestivum*). Optimized basal substrate for *G. sordulenta* consisted of 80% SSH, 15% biotransformed SSH residual from *P. ostreatus* cultivation and 5% wheat bran. Dry components were mixed and then soaked for 16 h in salt water dispersion (0.5% $CaCO_3$ and 2% $CaSO_4$) up to 60% water content.

In order to obtain different substrate formulations the optimized basal substrate (n = 6) was pretreated with 0.01 N $H_2SO_4$ (with no $CaCO_3$ added); supplemented with sulfates of either $NH_4$(I) (200 μg $g^{-1}$), or Mn(II) (20 μg $g^{-1}$), or Cu(II) (100 μg $g^{-1}$), or Zn(II) (100 μg $g^{-1}$); supplemented with either 20% *N. obliqua* or *Populus nigra* chips (1 cm length), or 20% wheat straw (*T. aestivum*, 1 cm length) or 5% sunflower oil, on a wet weight basis. In order to perform SSF with different substrate formulae tubes were filled with 10 g substrate to achieve a density of 0.45–0.48 g $cm^{-3}$. The SSF was performed as indicated in 2.4, but the incubation time was 35 days.

### *Solid-state fermentation of sunflower seed hulls for mushroom cultivation*

The effects of environmental conditions on primordial production and basidiome development under SSF conditions were evaluated by running five separate experiments (*E1–E5*, using 10–20 experimental units as detailed in Table 2). Substrate formulations used were 100% SSH (S1), 78% SSH, 19% wheat straw and 3% wheat bran (S2) and 50% SSH, 50% milled SSH (S3), all including 0.5% $CaCO_3$, 2% $CaSO_4$ and containing 60% water. Substrate was soaked for 16 h, sterilized (121 °C, 2 h, twice), and packed in 100 μm polyethylene bags at *c.a.* 0.48 g $cm^{-3}$ (S1, S2) or 0.63 g $cm^{-3}$ (S3). Bag sizes were 12 × 20 cm and 15 × 30 cm (600 g and 900 g, respectively). Spawning rate was 10–12% (fresh weight). Table 2 shows the experimental conditions used in each growing phase during basidiome development.

Analyses of fiber fraction in substrates were performed as described in Section 2.4, including substrates from mushroom production of *P. ostreatus*, *G. lucidum* and *Grifola frondosa* cultivated by SSF in SSH based substrates as positive controls.

### *Data analysis*

Data from the lineal growth test were subjected to one way-ANOVA. Differences detected by ANOVA were analyzed using Tukey's test. Data were examined for normality (modified Shapiro–Wilks test, $\alpha = 0.05$) and for homoscedasticity (Levene's test, $\alpha = 0.05$), and when necessary the ln transformation of data was performed in order to satisfy these assumptions. Protein content and laccase activity were analyzed using the non-parametric test of

**Table 3**
Grain SSF performance by *Grifola gargal* and *G. sordulenta*. The *linear growth test* was used to study the substrate colonization rate (SCR) and apparent density (AD) at two pHs' for different grain composition after 30 days of fermentation.

| Grains | *Grifola gargal*[a] | | | | *Grifola sordulenta*[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | pH 6.5 | | pH 5.3 | | pH 6.5 | | pH 5.3 | |
| | SRC mg DW $day^{-1}$ | AD | SRC mg DW $day^{-1}$ | AD | SRC mg DW $day^{-1}$ | AD | SRC mg DW $day^{-1}$ | AD |
| Wheat | 159.6 a | +++ | 161.0 a | +++ | 105.3 a | ++ | 104.0 a | +++ |
| Wheat: millet (4:1) | 140.8 ab | +++ | 117.3 bcd | ++ | 106.7 a | ++ | 94.8 ab | +++ |
| Corn | 99.3 d | +++ | 111.1 cd | +++ | 80.6 b | +++ | 78.5 b | ++ |
| Sunflower seeds | 124.9 bcd | ++ | 139.3 ab | +++ | 88.9 ab | ++ | 105.7 a | ++ |
| Corn: sunflower seeds (1:1) | 140.6 ab | +++ | 135.3 abc | +++ | 77.7 b | ++ | 75.3 b | +++ |

[a] Mean values ± $SE_{ANOVA}$ of SCR (n = 6, $SE_{ANOVA} = (CM_{error}/n_i)^{½}$). Different letters indicate significant differences determined with Tukey's test ($\alpha = 0.05$). Apparent mycelial density (AD) was classified as +++ (=*dense*), ++ (=*soft*), + (=*faint*) or 0 (=*no-growth*).

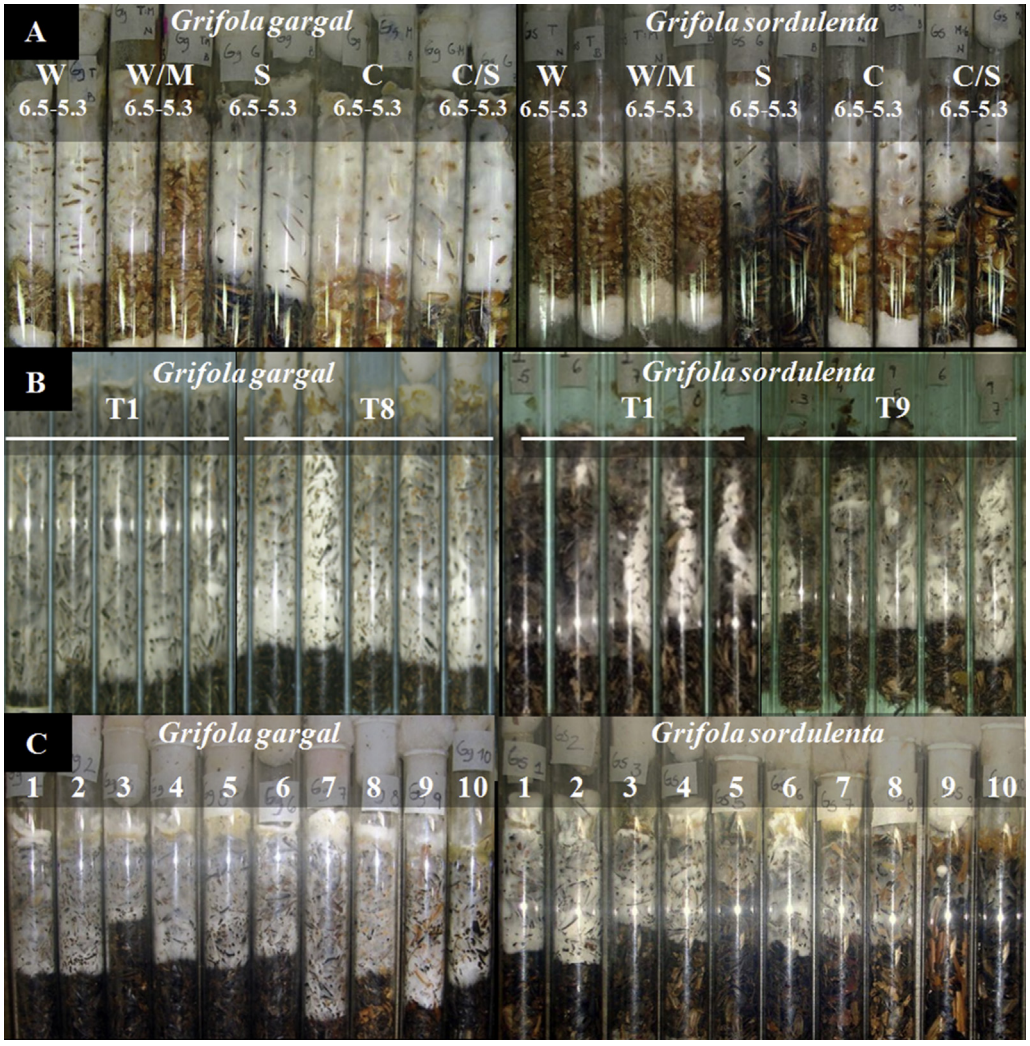


**Fig. 1.** Solid-state fermentation of grains and sunflower seed hulls based substrates by *Grifola gargal* and *G. sordulenta*. **A**: Mycelium growth in grains after 30 days of cultivation. Grain-substrates are: wheat (W), wheat: millet (W/M), sunflower seeds (S), corn (C) and corn: sunflower seeds (C/S), at pH values of 6.5 and 5.3. **B:** SSF of SSH. Images of *G. gargal* and *G. sordulenta* showing the treatments which exhibited excellent mycelium growth (T1, T8 and T1, T9, respectively) after 45 days of cultivation. **C:** Optimization of SSF of SSH based substrates. Mycelia from *Grifola gargal* and *G. sordulenta* after 35 days of cultivation. Optimized basal substrates (1) were pretreated with 0.01N $H_2SO_4$ (2) or supplemented with either 200 μg $g^{-1}$ $NH_4$(I) (3), 20 μg $g^{-1}$ Mn(II) (4), 100 μg $g^{-1}$ Cu(II) (5), 100 μg $g^{-1}$ Zn(II) (6), 20% *N. obliqua* wood chips (7), 20%, *P. nigra* wood chips (8), 20% wheat straw (9) or 5% sunflower oil (10).

Kruskal Wallis ($\alpha = 0.05$). These analyses were performed using the Infostat software (Di Rienzo et al., 2010).

The number of bottles containing fully colonized grains was analyzed by the Fisher's exact test ($\alpha = 0.1$), software: Vassar Stats: Website for Statistical Computation (http://vassarstats.net/ Accessed 2014).

## Results and discussion

### *Solid-state fermentation of cereal and oilseed grains*

A higher substrate colonization rate and better apparent density of *G. gargal* were found in SSF with wheat (both pHs), wheat: millet (pH 6.5), corn: sunflower (both pHs) and corn (pH 5.3); whereas in *G. sordulenta* they were found in wheat (pH 5.3) (Table 3 and Fig. 1A). In spite of the extensive use of millet for spawn production and substrate supplementation (Shen and Royce, 2001; Akavia et al., 2009), in our experiments millet combined with wheat did not improve the substrate colonization rate that was found using wheat alone, but on the contrary, a decrease in the apparent density of mycelium was observed at pH 5.3. Concerning corn grains, observations of a good mycelium quality in *G. gargal* were consistent with previous evaluations of these grains for spawn production of *G. frondosa* (Montoya-Barreto et al., 2008). Based on these results, the wheat-based substrate formulation (pH 5.3) is proposed as a convenient choice for mass culture of mycelium, which can also be a convenient source for obtaining a functional flour, i.e. by milling biotransformed wheat grains with medicinal properties (Postemsky et al. 2010; Postemsky et al. 2014).

### *Solid-state fermentation using "One liter bottle-spawn technique"*

The number of fully colonized bottles of wheat-grain spawn were studied at different times at two incubation temperatures. In both species, the higher number of fully-colonized bottles were obtained at 24 °C (Fisher's exact test, $p = 0.07$), and therefore this temperature was recommended for incubation rather than 20 °C, which was otherwise better for the vegetative culture in semisolid and liquid media (Postemsky et al., 2006). The values for the colonization rate were lower than the ones found in *G. lucidum* (10

days, González Matute et al., 2002) or in *P. ostreatus* (15 days, Curvetto et al., 2004) cultured in a wheat grain based substrate under similar environmental conditions.

Inoculum efficiency, estimated as the number of cluster units per weight of spawn (Akavia et al., 2009), was considerably higher in wheat grains and wheat: millet (4:1) combination (18 and 27 units/gram, respectively) than in corn and sunflower seed and/or their combinations (5–8 units/gram, data not shown). Preliminary observations indicated that temperatures over 28 °C were harmful to *G. gargal* and *G. sordulenta* SSF in grains (data not shown) and that an almond aroma was only observed in healthy colonies by day 25–30.

### *Solid-state fermentation of sunflower seed hulls*

The results of SSF of sunflower based substrates by *G. gargal* and *G. sordulenta* using the *linear growth test* are shown in Table 1 and the fiber content in substrates before and after SSF are presented in Table 4. Sunflower seed hull fermentation treatments with *G. gargal* and *G. sordulenta* (45 days of SSF) showing the best performance were those which exhibited a substrate colonization rate of 102–111 mg DW $day^{-1}$ and 97–107 mg DW $day^{-1}$, respectively, and a dense apparent density and protein content increments (related to the control) of 15–56 μg $g^{-1}$ DW and 40–96 μg $g^{-1}$ DW, respectively (Fig. 1B).

Sunflower seed hulls (obtained from an oil factory) are an excellent material to formulate substrates for SSF by both *Grifola* species and the milled form of SSH was good for SSF with *G. gargal*. On the other hand, it was found that the residual substrate of *P. ostreatus* cultivation can also be used to get the same quality of mycelium growth, but only when used in proportions lower than 50%. Wheat bran supplementation (20%) on SSH substrate improved the protein content in the case of *G. sordulenta* (T9: 96 μg $g^{-1}$ DW) as indicated by a significant increase in the soluble protein content. An increase in the protein content is used as an indication of the mycelium growth rate over a given time. In this sense, the improved growth rate obtained in SSF by *G. sordulenta* was consistent with previous results found in SSF by *G. frondosa*, when substrate supplementation with 20% wheat bran produced a reduction in the time to reach the phase of complete mature mushrooms (Shen and Royse, 2001). Treatments corresponding to low density substrate packing (0.45–0.50 g $ml^{-1}$) produced excellent mycelium growth. Hence, in order to favor the gas exchange and thus mycelium growth, higher substrate packaging density should be avoided. Moreover, the addition of 50% (or higher) of low size particulate substrate, such as milled substrate or wheat bran, would increase the substrate density packing and thereby its use should be carefully observed to avoid compaction if SSF on these substrates is made at a large scale. Indeed, detrimental effects of high substrate density can be observed with both species in treatments with similar nutrient quality but different density values, *e.g.* T1, T9 (low density) vs. T13, T12 (high density).

When analyzing protein contents in substrate extracts, it was found that *G. gargal* and *G. sordulenta* increased the protein content in SSH to 56 μg $g^{-1}$ and 40 μg $g^{-1}$, respectively (T1 45 days after colonization, Table 1), whereas *P. ostreatus* presented a lower protein content of *c.a.* 7 μg $g^{-1}$ (T4 before colonization, Table 3). These results should be considered relevant if the SSF process is aimed at animal feed production (Van Soest, 2006).

Laccase activity in residual substrates from mushroom cultivation showed higher values in substrate treatments exhibiting good to very good mycelium growth (*G. gargal* and *G. sordulenta,* respectively). Those substrate treatments that showed an excellent mycelium growth resulted in lower laccase activity, which was expected since the enzyme activity is known to be high when substrate is being actively degraded by rapidly growing mycelium.

Fiber analysis revealed that treatments with excellent mycelium growth of both *G. gargal* and *G. sordulenta* caused mineralization (higher ash content) by consuming hemicelluloses, cellulose and readily available nutrients (cellular content) (Table 6). In treatments in which *G. gargal* mycelium showed an excellent growth, a reduction in the hemicellulose fraction was observed principally with a proportional reduction in the lignin fraction (treatments: T1, T3, and T9). Other treatments showed a reduction in either the cellulose fraction (T7, T9) or the cellular content fraction (T3). With regard to *G. sordulenta*, cellulose was more biodegraded in T1,

**Table 4**
Changes in substrate fiber composition following SSF by *Grifola gargal* and *G. sordulenta*. Cellular content (CC), hemicellulose (HC), cellulose (C), lignin (L) and ashes (A) are given for T1–T20 substrate/treatments obtained before and after SSF. Initial values are expressed on dry weight basis. After 45 days SSF results of variations greater than ±10% for each fiber fraction of *G. gargal* and *G. sordulenta* solid-state fermented substrate are presented and also the variations in the fiber components. A quali/quantitative stimation of mycelium growth performance (MGP) was defined considering the SCR, AD and P (see "Solid-state fermentation of sunflower seed hulls" and Table 1) and it was named as *excellent* (Exc.), *good*, *fair* or *low*.

| Treat-ments | Initial fiber content (%) | | | | | *Grifola gargal* | | | | | | *Grifola sordulenta* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CC | HC | C | L | A | ΔCC | ΔHC | ΔC | ΔL | ΔA | MGP | ΔCC | ΔHC | ΔC | ΔL | ΔA | MGP |
| T1 | 33 | 8 | 36 | 15 | 8 | +13 | −33 | | −15 | +20 | Exc. | | | −20 | | +22 | Exc. |
| T2 | 41 | 5 | 28 | 14 | 12 | | −150 | +15 | | | Fair | 12 | −11 | −12 | | −17 | Low |
| T3 | 41 | 7 | 31 | 12 | 9 | | −40 | | | +18 | Exc. | −12 | | | +29 | +13 | Good |
| T4 | 37 | 5 | 32 | 14 | 12 | | | | +13 | | Low | | 23 | −14 | +26 | | Low |
| T5 | 41 | 6 | 31 | 13 | 9 | | −100 | | +13 | +18 | Good | | | | +28 | +13 | Exc. |
| T6 | 33 | 6 | 34 | 17 | 10 | | | | | | Good | | 20 | −10 | | | Good |
| T7 | 34 | 5 | 34 | 17 | 10 | | +38 | −10 | −55 | +38 | Exc. | | | −10 | | | Good |
| T8 | 32 | 6 | 34 | 18 | 10 | | | | −13 | | Good | 12 | | | | | Low |
| T9 | 33 | 12 | 32 | 16 | 7 | +21 | −100 | −10 | −33 | +36 | Exc. | +14 | −41 | | −33 | +38 | Exc. |
| T10 | 40 | 7 | 25 | 17 | 11 | +25 | −250 | | −70 | | Good | +14 | −27 | | −31 | | Low |
| T11 | 42 | 4 | 26 | 17 | 11 | | +43 | +10 | −31 | | Low | | +38 | | +21 | | Low |
| T12 | 32 | 12 | 34 | 15 | 7 | +20 | −20 | −13 | −25 | +13 | Good | +21 | −41 | −13 | −15 | +16 | Fair |
| T13 | 32 | 8 | 36 | 18 | 6 | | −14 | −29 | +28 | +14 | Good | | | | | +28 | Fair |
| T14 | 34 | 6 | 31 | 16 | 13 | | | | +11 | | Low | +12 | | | | −15 | Low |
| T15 | 32 | 6 | 34 | 18 | 10 | | +14 | | −20 | | Fair | +19 | | −13 | −20 | | Low |
| T16 | 32 | 7 | 34 | 17 | 10 | +11 | −40 | | | | Fair | +15 | −27 | −13 | | | Low |
| T17 | 34 | 9 | 31 | 16 | 10 | | −13 | | −33 | | Fair | | | | −14 | +12 | Good |
| T18 | 36 | 6 | 29 | 17 | 12 | | +14 | | | | Low | +11 | | | −21 | | Low |
| T19 | 32 | 10 | 32 | 15 | 11 | | −25 | | | | Fair | 15 | −18 | | | | Fair |
| T20 | 37 | 8 | 29 | 16 | 12 | | −14 | | −23 | | Low | 11 | −23 | | −14 | −17 | Low |

**Table 5**
Solid-state fermentation improvement of an *optimized basal substrate*. Substrate colonization rate (SCR) values and mycelial apparent density (AD) were obtained after 35 days of incubation. The sign "!" indicates the presence of mycelial aggregations.

| Treatments[a] | *Grifola gargal*[b] | | *Grifola sordulenta*[b] | |
|---|---|---|---|---|
| | SCR mg DW $day^{-1}$ | AD | SCR mg DW $day^{-1}$ | AD |
| Basal | 95 ± 4.1 de | +++ | 117 ± 4.7 a | +++ |
| $H_2SO_4$ 0.01 N | 100 ± 4.1 cde | +++ ! | 118 ± 4.7 a | +++ ! |
| $NH_4$(I) 200 μg $g^{-1}$ | 88 ± 4.1 e | ++ | 109 ± 4.7 ab | +++ |
| Mn(II) 20 μg $g^{-1}$ | 114 ± 4.1 bc | +++ ! | 111 ± 4.7 ab | +++ ! |
| Cu(II) 100 μg $g^{-1}$ | 99 ± 4.1 cde | +++ | 64 ± 4.7 de | + |
| Zn(II) 100 μg $g^{-1}$ | 107 ± 4.1 bcde | +++ ! | 118 ± 4.7 a | +++ ! |
| *N. obliqua* 20% | 136 ± 4.1 a | +++ ! | 79 ± 4.7 cd | ++ |
| *P. nigra* 20% | 123 ± 4.1 ab | +++ | 89 ± 4.7 bc | + |
| Wheat straw 20% | 112 ± 4.1 bcd | +++ ! | 64 ± 4.7 de | + |
| Sunflower oil 5% | 93 ± 4.1 de | +++ | 50 ± 4.7 e | + |

[a] *Optimized basal substrate* for *G. gargal* was: 50% SSH and 30% milled SSH, 15% residual sunflower seed hulls from *P. ostreatus* cultivation and 5% wheat bran. *Optimized basal substrate* for *G. sordulenta* was: 80% SSH, 15% residual SSH from *P. ostreatus* cultivation and 5% wheat bran. Experimental treatments were: control (Basal), pretreatment with 0.01 N $H_2SO_4$ (final pH 4.8), supplementation with mineral salts added as sulfates, lignocellulosic sources (1 cm length) or sunflower oil by % fresh weight.

[b] Mean values of substrate colonization rate (SCR) ± standard error (n = 6): $SE_{ANOVA} = (CM_{error}/n_i)^{½}$. Data were ln-transformed, different letters indicate significant differences determined with Tukey's test ($\alpha = 0.05$).

whereas hemicellulose was in T9; in this latter treatment the lignin fraction was also degraded. All fiber fractions were degraded equally in treatment T5. Lignin degradation in the residual substrate (made of SSH) was reported as depending on the nutrient balance of the medium (González Matute et al., 2013). This study also revealed that certain treatments presenting excellent mycelium growth also presented a reduction in the lignin fraction. This fact may be of consideration when the aim of SSF is to obtain laccase or other ligninolytic enzymes from residual substrate.

Nevertheless, when comparing substrate colonization rates with previous results obtained with other efficient white rot fungi it can be observed that the *G. gargal* and *G. sordulenta* colonization rate values were ca. 25 and 12 times lower than those of *G. lucidum* and *H. erinaceus,* studied under same conditions (González Matute et al., 2002; Figlas et al., 2007), which clearly shows the lower lignocellulolytic activity of those mushrooms. Based on these results, a second experiment was conducted to further optimize mycelium growth by substrate supplementation (see 3.4).

### *Optimization of solid-state fermentation of sunflower seed hulls*

The results obtained in both mushroom species after 35 days of SSF indicate that the substrate colonization rate in optimized basal substrate treatment was high, which also exhibited a higher apparent mycelium density (Table 5; Fig. 1C). Substrate colonization rate in *G. gargal* was improved by 1.2 fold with 20 μg $g^{-1}$ Mn(II) and by 1.4 or 1.3 times, respectively, when 20% *N. obliqua* or *P. nigra* chips were included as part of the substrate. In addition, cultures from some other treatments exhibited aggregations of mycelium masses (a phenomenon not seen in the previous experiment) which were considered to be an improvement in the colonization (Fig. 1C; Table 5). The results from this experiment also revealed that SSF by *G. gargal* can still be upgraded by supplementing the basal substrate with both minerals and other lignocellulosic sources. Substrate colonization rate increments, due to lignocellulosic supplementation, were expected to occur in the case of *N. obliqua* chips since this tree species is its natural nutrient source. More interestingly, these results were corroborated with *P. nigra*, as the ability of *G. gargal* to grow on a tree of this species has recently been reported (Pozzi et al., 2009); therefore residues of this wood from the forest industry can be used as a lignocellulosic source to sustain *G. gargal* cultivation. Moreover, improvements observed with wheat straw supplementation justify a more profound study aimed at making profitable use of this lignocellulosic material.

**Table 6**
Flow chart showing an optimized protocol for growing *Grifola gargal* and *G. sordulenta* in substrates formulated with sunflower seed hulls as emerged from present and previous studies.

| Materials and methods | *Grifola gargal* | *Grifola sordulenta* |
|---|---|---|
| Semisolid medium (20–25 days)[a]<br>Growing at 18–21 °C, in darkness. | Modified MYPA: 20 g $l^{-1}$ malt extract, 5 g $l^{-1}$ yeast extract, 2.5 g $l^{-1}$ meat peptone, 10 g $l^{-1}$ glucose or sacarose, 20 g $l^{-1}$ agar, 0.4% (p/v) milled sunflower seed hulls, at pH 4. | |
| Spawn (25–30 days)<br>Growing at 21–24 °C, in darkness<br>Use mycelium with active growth. | Wheat; wheat: millet (4:1); corn:sunflower seeds (1:1). | Wheat; corn; corn: sunflower seeds (1:1). |
| Substrate formulation<br>>16 h soak in water with 2% $CaSO_4$ and 0.5% $CaCO_3$. | 50% sunflower seed hulls, 30% milled sunflower seed hulls, 15% residual substrate[b], 5% wheat bran. | 80% sunflower seed hulls, 15% residual substrate[b], 5% wheat bran. |
| Supplements<br>Dilute acid and mineral supplements are added in soaking dispersion; 20% of lignocellulosic are mixed before soaking. | 0.01 $H_2SO_4$; 20 μg $g^{-1}$ Mn(II); 100 μg $g^{-1}$ Zn(II); *N. obliqua* chips, *P. nigra* chips, wheat straw. | 0.01 $H_2SO_4$; 20 μg $g^{-1}$ Mn(II); 100 μg $g^{-1}$ Zn(II); wheat straw. |
| Culture method<br>Axenic conditions (121 °C, 2 h) and "synthetic logs" system are recommended. | Density of substrate may be 0.45–0.55 g $cm^{-3}$; for aeration use cotton plugs or similar. | |
| Substrate inoculation<br>Thoroughly mixed in the substrate. | Generous inoculum of 10–15% (by fresh weight); aseptic conditions. | |
| Mycelium running (40–60 days)<br>18 °C, 60% HR, ≥1 air change per day of the room, in darkness. | Almond aroma indicates a satisfactory growth; ensure the drainage of fluid from the bag. | |
| Induction (≤30 days)<br>5 °C, 60%HR., ≥1 air change per day of the room, in darkness. | Avoid overproduction of primordia produced by air accumulation between the bag and the substrate; move to environmental controlled chamber when primordia are detected. | |
| Mushroom production (15–30 days)<br>Temperature cycles of 5 °C and 8 °C; 8 h photoperiod (300–500 lux); 85–95% HR; ≥8 air change per day of the room, aseptic air is recommended. | Progression of basidiomes can be followed by their morphological phases: *brain*, *cauliflower* and *cluster* phases; cultivation period should not be longer than 30 days. | |

[a] Results obtained from Postemsky et al. (2006).
[b] Residual substrate of *Pleurotus ostreatus* grown in sunflower seed hulls (if absent use sunflower seed hulls).

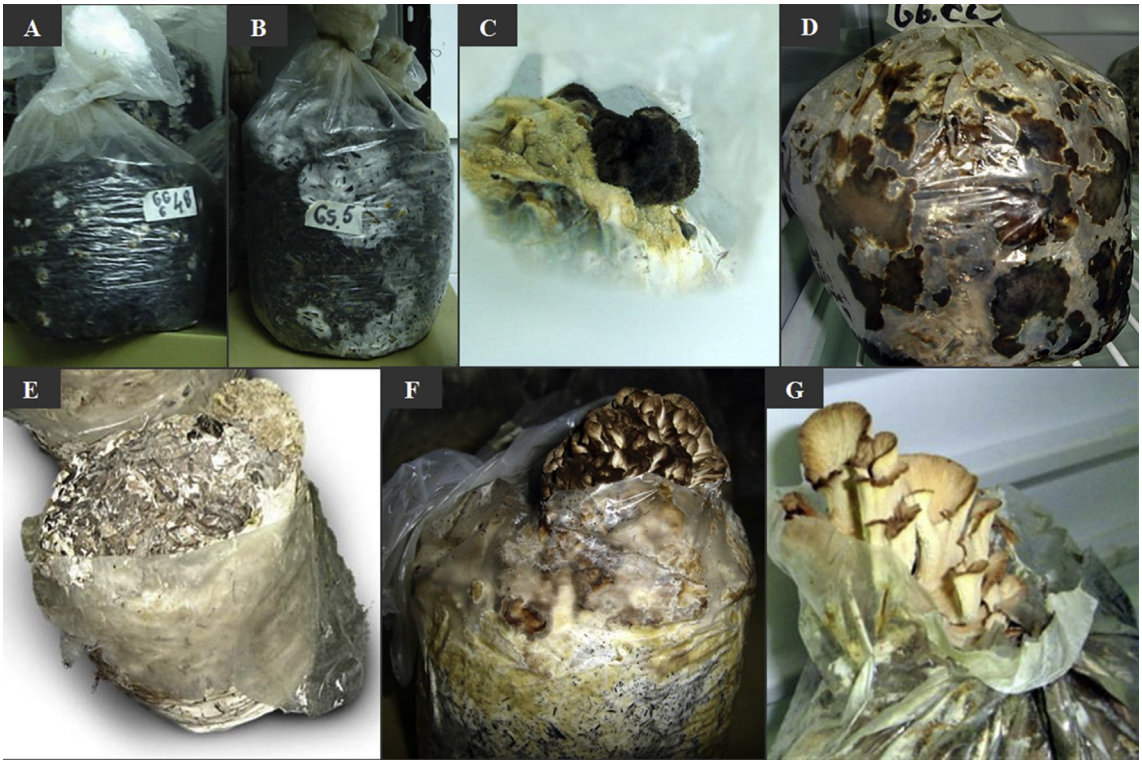


**Fig. 2.** Basidiome development in *Grifola gargal* and *G. sordulenta*. **A:** *G. gargal* (5 days); **B:** *G. sordulenta* (15 days); **C:** primordia after induction; **D:** hyperprimodial production; **E:** basidiome of *G. sordulenta* at "branch" phase; **F:** basidiome of *G. gargal* at "branch" phase; **G:** basidiome of "*G. gargal*" at branch phase, ready to harvest.

*Solid-state fermentation of sunflower seed hulls for basidiome production*

After spawning, the time to achieve complete substrate colonization was between 40 and 60 days (Fig. 2A–B) for the SSH based substrates with a high percentage of inoculation (10–12%) with *G. gargal* and *G. sordulenta* spawn. After such prolonged periods, the base content of the bags became more compact and showed fluid accumulation. This phenomenon, and also opportunistic aerobic microorganisms, may have favored the high rate of contamination recorded at the end of that phase (70–80%). Hence there were no significant differences at the time needed to complete colonization of the different substrates, due to the reduction of experimental units.

However some observations may still be of interest. Firstly, the mycelium running phase was easily perceived by the intensity of almond aroma which became more pronounced by the end of this phase. Secondly, any mycelium growth inhibition by thermogenesis was observed with the provision of moist air (18–24 °C; 60% RH) and adequate aeration of synthetic logs through cotton plugs, therefore the ventilation by micro-holes was considered unnecessary, as happens when cultivating other mushrooms with higher substrate colonization rates (Postemsky et al., 2014).

After mycelium running, primordia were induced by decreasing the ambient temperature (−5 °C–12 °C). Developmental events were the appearance of yellowish exudates (0–10 days), followed by primordia development (20–30 days) and a decrease in the almond aroma was also noted. After different thermal shock treatments, *ca*. 80% of the experimental units showed developed primordia (Fig. 2C) while the rest did not complete this phase due to contamination. It was interesting to note that hyper production of primordia took place in low density substrates (with a plenum of free air between the substrate and the bag) (Fig. 2D). By affecting the nutritional relationship source-sink, these phenomena could eventually affect mushroom production by producing basidiomes of smaller size.

Basidiome development was studied under different controlled environmental conditions of temperature, light, humidity and ventilation and it was achieved at 5–8 °C, 85–95 % RH (mist type), 500 lux (fluorescent lights), with 8 daily changes of fresh air (free of contaminants). Fructifications were fully developed in 3–4 weeks under these conditions; contamination appeared on basidiomes if a longer incubation time was allowed. Though smaller in size, mature fructifications were morphologically similar to those found in nature, reaching a size of 10–12 cm × 8.5 cm (Fig. 2E–G). Mushroom development also showed phases similar to those mentioned for *G. frondosa*, i.e. brain, cauliflower and cluster (Stott and Mohammed, 2004). *Grifola gargal* basidiomes were obtained in 20–40% of the experimental units, of 10–15 g in weight, containing 40–50% water (due to dehydration of the basidiomes) or 80–85% (in normal, fresh basidiomes) and they yielded a biological efficiency of 3–10% as percentage of mushrooms (FW) obtained per substrate mass (DW). *Grifola sordulenta* basidiomes were obtained in 17% of the experimental units, of 8–14 g in weight, with a similar water content of 70–85% in normal, fresh basidiomes and they yielded a 2–6% biological efficiency. While the possibility of obtaining acceptable mushrooms of *G. gargal* and *G. sordulenta* grown on synthetic-logs with axenic substrate was thus demonstrated, more SSF studies are needed in controlled ambient conditions, which should include a strict aseptic environment to obtain higher mushroom yields.

A new insight of the substrate biotransformation after SSF was revealed from the fiber analysis of colonized substrates (Fig. 3). Ash content was incremented from 8% to 13 % by *G. gargal* (day 210) and *G. sordulenta* (day 150) while the control species (*G. frondosa*, *G. lucidum* and *P. ostreatus*) increased in the ash content by 22–23%

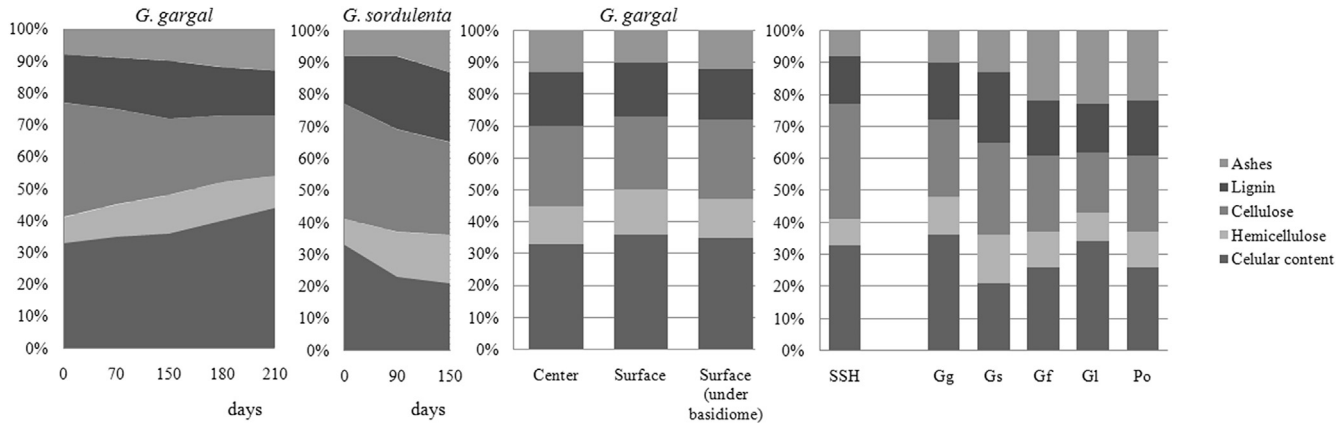


**Fig. 3.** Fiber analysis showing the composition profile of sunflower seeds hulls based substrates after solid-state fermentation with *Grifola* spp., *Ganoderma lucidum* or *Pleurotus ostreatus*. Composition was expressed as percentage of the dry weight (%). From left to right: results of SSF of SSH by *G. gargal* and *G. sordulenta* from day 0 to 210 and 150 days, respectively; results of SSF of SSF by *G. gargal* at different substrate portions (center, surface, surface under basidiomes); results of SSF of SSH by *G. gargal* (Gg, day 210), *G. sordulenta* (Gs, day 150), *G. frondosa* (Gf, day 150), *G. lucidum* (Gl, day 150) and *P. ostreatus* (Po, day 150).

(150 days). *Grifola gargal* proportionally reduced the cellulose fraction homogeneously throughout the whole substrate. In the case of *G. sordulenta*, a higher proportional reduction of fiber fraction was determined for cellulose and cellular content. With regard to the positive control species (*G. frondosa*, *G. lucidum* and *P. ostreatus*) the lignin, cellular content and cellulose fractions (*G. frondosa* and *P. ostreatus*), or the cellulose fraction only (*G. lucidum*), were proportionally reduced. Based on these results, it is suggested that *G. gargal* and *G. sordulenta* would need 3–4 times the time required for similar substrate biodegradation if soluble nutrients were still available, but it would be difficult to avoid contamination under these conditions. A prospective question would be whether the SSF could be improved by shortening the mycelium running phase by using higher spawning rates. However higher spawning rates would change the substrate composition considerably.

## Conclusions

Solid state fermentation by *G. gargal* or *G. sordulenta* works better with wheat grains at 24 °C. The bottle culture method is acceptable for obtaining spawn and biotransformed grains of these species. Sunflower seed hulls are lignocellulosic residues that can be subjected to SSF by *G. gargal* and *G. sordulenta* to obtain a residual substrate enriched in protein, with a good laccase activity and with lower lignin content than the original substrate. Sunflower seed hulls pretreated with dilute $H_2SO_4$ or supplemented with Mn(II) and Zn(II) salts were found to improve the mycelial colonization process. Artificial cultivation of *Grifola gargal* and *G. sordulenta* was achieved using SSH as the main substrate, but more studies are necessary to obtain an optimized protocol for their cultivation.

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