Micología Aplicada International
ISSN: 1534-2581
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Colegio de Postgraduados
México

Postemsky, P.; González Matute, R.; Figlas, D.; Curvetto, N.
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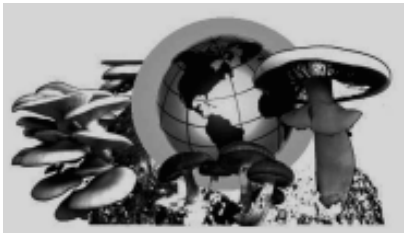
Micología Aplicada International, vol. 18, núm. 1, january, 2006, pp. 7-12
Colegio de Postgraduados
Puebla, México



MICOLOGIA APLICADA INTERNATIONAL, 18(1), 2006, pp. 7-12

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OPTIMIZING *GRIFOLA SORDULENTA* AND *GRIFOLA GARGAL* GROWTH IN AGAR AND LIQUID NUTRIENT MEDIA

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Accepted for publication December 16, 2005

ABSTRACT

***Grifola sordulenta* and *Grifola gargal* are native edible mushroom species of Argentina. The study of their growth is useful as a first step for the optimized production of those constituents with nutritional, nutriceutical, and pharmacological value. The effect of temperature, pH, and supplements (millet and sunflower seed hulls) on agar medium and the effect of temperature and sunflower seed broth on liquid medium, were evaluated. On malt-yeast-peptone-agar medium, the highest rate of mycelial growth and biomass production for both *Grifola* species was at pH 4. After 20 days culture, both biomass production and colony diameter were significantly higher at 18 C than at 24 C in the case of *G. sordulenta*, while *G. gargal* did not grow at 24 C. For both species, addition of milled sunflower seed hulls (0.4%) to the medium significantly increased mycelial growth diameter and biomass production. In the case of liquid culture of *G. sordulenta*, the addition of 26% or 39% sunflower seed broth to the medium significantly increased the mycelial biomass (by almost 3 times) compared to the control. There were no differences in the mycelial biomass production at 24 C, irrespective of the sunflower seed broth content, and also the mycelial biomass was much lower than the one obtained at 20 C. *G. gargal* showed a significant mycelial mass increase (*ca*. 100%) at both 26% and 39% sunflower seed broth at 20 C, while at 24 C there were no significant differences in relation to sunflower seed broth rates compared to control. The best culture conditions for agar and liquid culture of these *Grifola* species are discussed.**

Key words: *Grifola gargal, Grigola sordulenta,* mycelium culture, mycelium biomass, agar culture, liquid culture.

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INTRODUCTION

The medicinal properties of several mushroom species have been widely recognized in China, Korea and Japan for thousands of years. Recently, the interest in products derived from mushrooms for medicinal purposes has resurged. *Grifola frondosa* has important medicinal attributes[4,14]. As a result, the annual production of *G. frondosa* is strongly increasing not only because of its excellent taste, but also its nutritional, nutriceutical and medicinal values[1]. *G. frondosa*, also known as maitake, is a basidiomycete that belongs to the *Polyporaceae* family; it exhibits multipileate, flabellate and imbricate massive fructifications, and grows in the base or in the roots of broad leaf trees. It can invade the center of the tree causing an extensive degradation (white rot).

At present, most maitake is marketed as food. Dried fruit bodies are also used in the production of many healthy foods, such as, maitake tea, whole powder, granules, drinks, and tablets[9].

Grifola sordulenta and *Grifola gargal* are species first described by Singer[10]. The stipes of *G. sordulenta* rise from a large common trunk-like base from which secondary stipes branch off. Its spores vary from subglobose to short ellipsoid or ellipsoid shapes. Rajchenberg and Greslebin[8] found a *Grifola* species, which they identified as *G. sordulenta*, appreciated as a food among native populations, which decayed standing and/or fallen trees of several *Nothofagus* species, the main native forest resource in Patagonia, southern Argentina. However, the nutraceutical properties and pharmacological potential of *G. sordulenta* have not yet been studied. Conventional taxonomic studies[3,13] showed that *G. sordulenta* is a very closely related species to *G. frondosa* (Dicks.: Fr.)S.F. Gray. According to Nobles[6], *G. frondosa* differs from *G. sordulenta* in culture by a faster growth rate, the formation of chlamydospores, and by ready sporulation in culture.

A related species, *Grifola gargal* has an annual basidiocarp, multipileate, formed by numerous pilei rossette-like, imbricated, and growing from a common point[7]. Its substrates are the living trunks, stumps or fallen trunks of *Nothofagus* species in forests of southern Argentina and south-central Chile. This fungus is tasty and fleshy, most appealing for its aromatic scent.

Determining the optimal conditions for mycelial production of *G. sordulenta* and *G. gargal* is a fundamental step for the optimized production of some of their constituents with potential nutritional, nutriceutical, and pharmacological value. Also, as it has been the case with other mushrooms[12], the use of liquid inoculum can markedly accelerate mushroom spawn production for use in mushroom growth in solid state, and also for continuous liquid cultivation to produce mycelium and to obtain fungal substances. In order to optimize the agar and liquid culture medium for *G. sordulenta* and *G. gargal*, we studied their mycelial growth response to different nutrient medium composition, pH, and temperature.

MATERIALS AND METHODS

Mushroom strains. *Grifola sordulenta* (Mont.) Singer and *Grifola gargal* Singer were obtained from CIEFAP (Centro de Investigación y Extensión Forestal Andino Patagónico, Chubut, Argentina). *G. sordulenta* strain was CIEFAP 154, from the Parque Nacional Los Alerces, Chubut,

Table 1. Influence of the pH of the MYPA modified medium on mycelial growth rate (mm day^{-1}) and biomass production (mg day^{-1}) of *Grifola sordulenta* and *Grifola gargal*.

pH	*Grifola sordulenta*		*Grifola gargal*	
	Mycelial growth mm day^{-1}	Biomass mg day^{-1}	Mycelial growth mm day^{-1}	Biomass mg day^{-1}
4	3.4 (0.20) a*	3.8 (0.07) a	4.3 (0.36) c*	5.2 (0.26) c
5	2.9 (0.20) b	2.6 (0.07) ab	2.7 (0.28) d	4.4 (0.21) cd
6	2.7 (0.17) b	1.1 (0.11) b	2.3 (0.30) de	4.2 (0.20) cd
7	2.6 (0.34) b	1.1 (0.16) b	1.8 (0.32) e	3.7 (0.36) d

* Different letters represent highly significant differences ($p < 0.01$) within each species. Mean values were separated by the Tukey test. Values in brackets represent standard error.

Argentina, and *G. gargal* strain was CIEFAP 191 from Neuquén, Argentina.

Effect of pH and temperature. Mycelium of both *Grifola* species were inoculated into Petri dishes containing modified MYPA medium (20 g L^{-1} malt extract, 5 g L^{-1} yeast extract, 2.5 g L^{-1} peptone, 10 g L^{-1} glucose, 20 g L^{-1} agar), at different starting pH conditions: 4, 5, 6, or 7. They were incubated at 18±1 C in darkness for 20 days. The mycelial growth rate was determined by measuring the diameter of colonization of the medium as a function of time (n=10) and the biomass of dried mycelium (n=5), obtained by weight after separation from the agar by heat in a microwave (BGH, Mod 16600, 1,250 watts of output) during 1 min, followed by water evaporation in an oven until constant weight. The best growth responses were obtained at pH 4. This pH was chosen to further evaluate the effect of temperature (18±1 C and 24±1 C) both on mycelial growth rates and biomass of dried mycelium.

Effect of adding millet or sunflower seed hulls (SSH) powder to the agar medium. Either 0.4% (w/v) millet or SSH powder was incorporated into the modified MYPA medium, and incubated at 18±1 C. The mycelium growth of *G. sordulenta* and *G. gargal* was determined by measuring the diameter of the mycelium (n=10), and the biomass of dried mycelium (n=5) as above.

Effect of adding sunflower seed broth (SSB) to the liquid medium. A basal MYP medium composed by 20 g L^{-1} malt extract, 2 g L^{-1} yeast extract, 1 g L^{-1} peptone, 0.5 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.2 g L^{-1} $MnSO_4 \cdot 5H_2O$, 30 g L^{-1} glucose, and 65 g L^{-1} of whole sunflower seeds, at pH 4, was used. The addition of 1.63%; 3.25%; 6.5%; 13%; 26% and 39% (v/v) SSB to this medium incubated at either 20±1 C or 24±1 C was evaluated. To obtain seed broth, a suspension of each concentration of sunflower seeds was autoclaved in distilled water at 120 C for 40 min and filtered[2].

For each treatment, 50 mL of medium was added to six 250 mL Erlenmeyer flasks to obtain an air to medium relationship of 5:1 [12]. Each flask was inoculated with 6 circles of 6 mm diameter of young mycelium of *G. sordulenta* or *G. gargal* grown on agar medium. The flasks were incubated in darkness at 20±1 C or 24±1 C with horizontal orbital shaking at 90 rpm,

for 20 days. To measure the biomass, the mycelium was recovered from the liquid medium by centrifugation at 3,000 rpm for 30 minutes and dried in an oven at 80 C until constant weight.

Statistical analysis. The results were analyzed by simple ANOVA and the mean multiple comparisons test of Tukey.

RESULTS

For both *Grifola* species, mycelial growth rate, *i.e*. the size colony change with time, and biomass production rate in agar medium, at 18 C, were highest at pH 4 (**Table 1**). For *G. sordulenta*, no significant differences were observed in the mycelial growth rate in the pH range 5-7. Hence, for subsequent evaluation of the effect of temperature or additives on the mycelial growth rate, pH 4 was chosen for both strains.

An increase in temperature from 18 C to 24 C in the culture conditions for *G. sordulenta*, significantly decreased ($p < 0.01$) either the size colony change rate (from 3.4 mm day^{-1} to 0.8 mm day^{-1}) or the biomass production rate (3.8 mg day^{-1} to 1.4 mg day^{-1}). At 18 C *G. gargal* grew at a rate of 4.3 mm day^{-1} and 5.2 mg day^{-1} of size colony increase and mycelium mass production, respectively, while did not grow at all at 24 C.

With the aim of increasing the *G. sordulenta* and *G. gargal* mycelial mass, and thus its production rate during the cultivation period, 0.4% (w/v) powder of either SSH or millet was incorporated into the agar culture medium, under the same incubation conditions (darkness, 18 C). With the addition of SSH powder, the mycelial growth rate and biomass production rate of *G. sordulenta* significantly increased ($p<0.01$) (3.3 mm day^{-1} and 5.5 mg day^{-1}, respectively) when compared to control (2.9 mm day^{-1} and 3.3 mg day^{-1}). Addition of millet did not

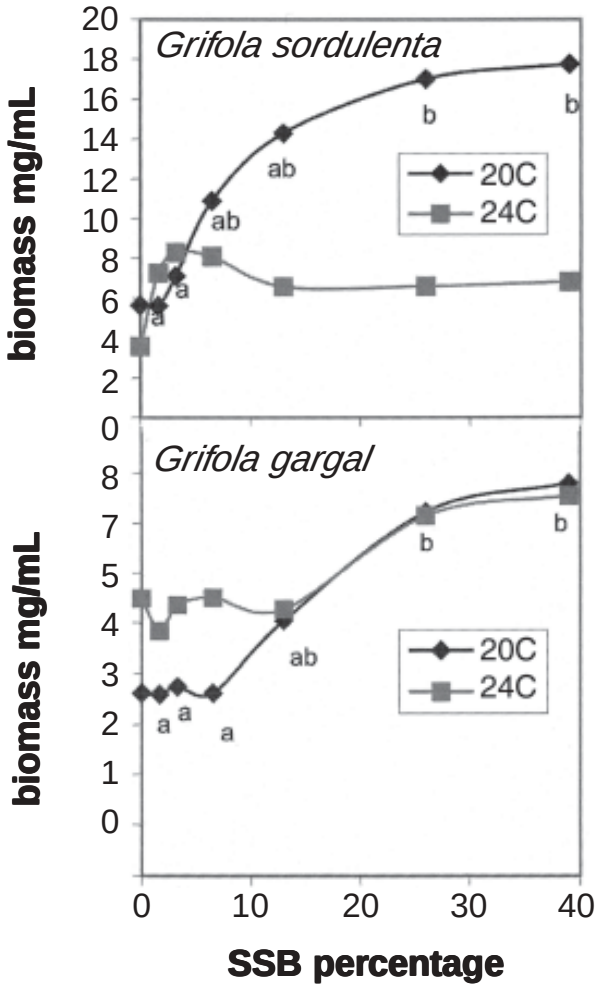

Fig. 1. Biomass production of *Grifola sordulenta* and *Grifola gargal* in modified MYP liquid culture media containing sunflower seed broth (SSB). Mycelium was cultivated in liquid modified MYP medium with the addition of different SSB concentrations (1.63%, 3.25%, 6.50%,13%, 26%, and 39%), at 20 C or 24 C, for 20 days. Values following by the same letter are not significantly different, $p>0.05$.

significantly increase any of those parameters (2.7 mm day^{-1} and 3.6 mg day^{-1}).

For *G. gargal*, the addition of SSH powder significantly increased both the mycelial growth rate (4.9 mm day^{-1}) and the biomass production rate (7.3 mg day^{-1}) with respect to the control (4.3 mm day^{-1} and 5.2 mg day^{-1}, respectively). The addition of 0.4% millet did not have a positive effect but decreased those parameters to 3.9 mm day^{-1} and 2.3 mg day^{-1}.

Thus, for both strains, the best culture conditions were pH 4, at 18 C and a MYPA medium with the addition of 0.4% SSH powder.

When *G. sordulenta* was incubated for 20 days at 20 C in liquid modified MYP medium with addition of 39% SSB, the mycelial growth almost tripled the control value of *ca*. 6 mg L^{-1}, with more than 17 mg L^{-1}, reaching a plateau at 26% SSB (**Fig. 1**). When incubation proceeded at 24 C, an increase in the SSB content did not produce a significant change in the mycelial biomass production, but remained almost constant around 7 mg L^{-1} which was clearly lower than the higher mycelium mass obtained at 20 C.

G. gargal, cultured under the same conditions as above, at 20 C showed a significant increase ($p<0.05$) in mycelial biomass at both 26% and 39% SSB content, *ca*.100% increase in relation to the control treatment. At 24 C, the mycelial mass increase was only 50% at either broth content, in relation to the control treatment (**Fig. 1**). At this temperature there were not significant differences in mycelial mass production in relation to SSB rates, neither were found to be significant the mycelial biomass differences between temperatures (20 C and 24 C) at any SSB rate.

DISCUSSION

At 18 C, the highest mycelial growth rate of *G. sordulenta* and *G. gargal* in agar culture medium was obtained at pH 4. This result closely agrees with the previously reported for *G. frondosa* by Miyauchi *et al.*[5], who found the best mycelial growth at pH 4 and 5. They also reported poor growth at pH 6 or higher; however, in the case of *G. sordulenta* we did not find significant differences within pH 5-7.

We also evaluated the effect of a temperature change on mycelial growing in agar nutrient media for both *Grifola* species. When the temperature increased from 18 C to 24 C, there was a significant decrease in both mycelial growth rate and biomass production for *G. sordulenta*. *G. gargal* did not grow at all at 24 C. These results differ from those reported for *G. frondosa* by Stott and Mohammed[11], who found an optimal growth temperature of 25 C, and a good but slow growth at 20 C.

The addition of sunflower seed hull powder to MYPA medium at pH 4, notably increased the mycelial growth of *G. sordulenta* and *G. gargal* evaluated by the colony size through diameter measurement, reaching 6.6 and 9.8 cm, respectively, in 20 days at pH 4 and 18 C. These values were superior to those reported by Rajchenberg[7] for these strains: 5.5-8.5 cm and 3 cm in 6 weeks, respectively. Thus, it was possible to effectively diminish the time for obtaining a high quality inoculum of *G. sordulenta* and *G. gargal* in agar nutrient medium in a Petri dish, the first step in the productive cycle of the mushroom.

When cultivation was performed in modified MYP liquid medium, the best incubation temperature was 20 C for *G. sordulenta* and there were no significant differences between temperatures for *G.*

gargal. Adding 26% sunflower seed broth increased the mycelial growth of *G. sordulenta* and *G. gargal* at 20 C, with significantly higher mushroom biomass values. Higher concentrations of broth did not significantly increase the mycelium production. Whole seeds contain nutrients in which the basal medium could be deficient. Then, it is expected that at least part of those nutritional components would be released when boiling the whole sunflower seeds in water by autoclaving and hence contributing to an improved nutrient medium when the broth is incorporated to the liquid MYP medium at appropriate rates.

Our work suggests that the best culture conditions for these two species were: 1) Modified MYPA medium, *i.e*. MYPA (20 g L^{-1} malt extract, 5 g L^{-1} yeast extract, 2.5 g L^{-1} peptone, 10 g L^{-1} glucose, 20 g L^{-1} agar), with addition of 0.4% milled sunflower hulls at pH 4 and 18 C, for cultivation on agar nutrient medium; and 2) Modified MYP medium, *i.e*. MYP (20 g L^{-1} malt extract, 2 g L^{-1} yeast extract, 1 g L^{-1} peptone, 0.5 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.2 g L^{-1} $MnSO_4 \cdot 5H_2O$ and 30 g L^{-1} glucose) with addition of 6.5% (w/v) sunflower seed and 26 % sunflower seed broth at pH 4 and 20 C, for liquid culture.

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