SUNFLOWER SEED-BASED MEDIUM FOR GROWTH OF *GANODERMA* SPP

N. Curvetto, R. González-Matute, D. Figlas and S. Delmastro

Departamento de Agronomía, Universidad Nacional del Sur, 8000 Bahía Blanca, Buenos Aires, Argentina.<micouns@criba.edu.ar>

ABSTRACT

Ganoderma lucidum and *Ganoderma oregonense* produce medicinal giant polysaccharides. We attempted to improve liquid culture to produce these polysaccharides in higher amounts. We added 65 $g.l^{-1}$ sunflower seeds or sunflower seed broth (equivalent to 16.3 $g.l^{-1}$ seeds) to basal media (malt extract, yeast extract, glucose: 20, 2 and 10 $g.l^{-1}$, respectively), pH 6.0. *G. lucidum* was incubated at 25°C and *G. oregonense* at 22°C, in darkness and shaken at 90 rpm. *G. lucidum* growth rate was maximal at day 4 and produced higher mycelium concentrations (9.0 $mg.ml^{-1}$) in both amended media at day 6, with the broth producing the maximum polysaccharide concentration (3.6 $mg.ml^{-1}$) at day 5. *G. oregonense* reached maximum growth rate by day 12, with a maximum of 10.3 $mg.ml^{-1}$ mycelium concentration in the broth medium; also polysaccharide production was much higher (3.4 $mg.ml^{-1}$) in this medium. Both amended media markedly improved the mycelial growth and polysaccharide production rates in *G. Lucidum* while only the broth medium produced a marked increase in both parameters in *G. oregonense*.

INTRODUCTION

Ganoderma spp are basidiomycetes in the family Polyporaceae. Known as reishi or mannentake in Japan and ling zhi in China, this mushroom has been used for medicinal purposes for hundreds of years, especially in China, Japan and Korea. It has been used in cancer treatment in conjunction with patient recuperation (Morigawa *et al*. 1986, Mizuno *et al*. 1995). Researchers have isolated biologically active substances with medicinal properties from *G. lucidum* (Mizuno *et al*. 1995) and these substances have shown anti-tumor effects (Sone *et al*. 1985). The main substance with anticarcinogenic

activity is a polysaccharide, 1,3-β-D-glucan, named "ganoderan", which was considered as a promising new type of carcinostatic agent for immunotherapy (Mizuno *et al*. 1995).

Ganoderma spp have traditionally been cultured over solid substrates like grain or straw. It usually takes some months for fruiting bodies to form. This method is too time-consuming to isolate biologically active substances, and for this reason, liquid cultures have also been tested (Tseng *et al*. 1984). The homogeneous distribution of mycelium in the liquid medium not only helps to shorten culture time, but also reduces the time required for the production of liquid inocula (Yang and Jong 1987).

It is generally accepted that the viscosity of the liquid medium determines the diameter of the mycelial spheres. To increase the number and uniformity of the spheres, different carbon and nitrogen additives have been proposed, among them corn flour, methylcellulose, carboximethylcellulose and alginic acid (Yang and Jong 1987). These additives also avoid the undesirable formation of anaerobic cores in large mycelium spheres that lead to autolysis.

Processing sunflower seeds by autoclaving produces some mineral and organic substances, including soluble and insoluble carbohydrates, lipids, amino acids, vitamins and pectin (Park *et al*. 1997). Some of these substances have energetic and nutritional value for mushroom culture. Sunflower seeds contain about 280-470 $g.kg^{-1}$ lipid, 170-270 $g.kg^{-1}$ raw protein and 320-360 $g.kg^{-1}$ acid detergent fiber (Cancalon 1971).

Sunflower pectin retains water, gels well, and has a high viscosity at pH values between three and seven (Park *et al*. 1997). Incorporating processed sunflower seeds and their residues into the medium may not only increase the nutritive value of the medium, but may also increase its viscosity, thereby yielding smaller and more uniform mycelial spheres. This enriched medium could also improve 1,3-β-D-glucan polysaccharide production and mycelium yield by removing possible nutritional deficiencies.

The aim of our work was to optimize mycelial production of *G. lucidum* and *G. oregonense* in liquid culture for later use as liquid inoculants or for polysaccharide production for use with medicinal purposes. It is expected that an optimized protocol could provide a basis for its eventual utilization on a commercial scale.

MATERIALS AND METHODS

Microorganisms and culture medium

Ganoderma lucidum (Curt.:Fr.) P. Karst. and *G. oregonense* (Pers.) Patt strains were obtained from Fungi Perfecti (Olympia, WA, USA). Cultures were kept in agar nutritive medium consisting of ($g.l^{-1}$): malt extract (20), yeast extract (2) and agar (20) at pH 6.0. Petri dishes inoculated with *G. lucidum* were incubated in darkness at 25°C for 5-7 days and maintained at 4°C until used. *G. oregonense* was incubated at 22°C for 12-15 days and maintained at 4°C until used. Growth cultures of *G. lucidum* and *G. oregonense* were used for 10 and 20 days, respectively.

Liquid culture

A 200-ml sample of MYG medium (liquid nutritive medium of malt extract, yeast extract and glucose), pH 6.0 (control) was placed in 1-l Erlenmeyer flask to obtain an air-medium ratio of 5:1 (Yang and Jong 1987).

Sunflower seeds (65 $g.l^{-1}$) were added to basal medium (control+seeds). To test the effect of broth, sunflower seeds (65 $g.l^{-1}$) were added to 1 l distilled water and autoclaved at 120°C for 40 min. A 50-ml sample of this autoclaved mixture was added to 150 ml concentrated MYG medium in order to preserve the basal formulation of the medium (control+broth). Media were autoclaved for 30 minutes at 120°C. The final pH after autoclaving was ca. 5.0 for all formulations.

Two triangular sections of young mycelium from each strain, equal to 5 cm^2 area, were inoculated in each Erlenmeyer flask and placed on an orbital shaker at 90 rpm. This shaker speed was chosen to avoid deleterious effects on polysaccharide production that higher speeds ones could cause (Yang and Liau 1988a).

Ganoderma lucidum and *G. oregonense* cultures were incubated in the dark at 25°C and 22°C, respectively. Samples from *G. lucidum* liquid cultures were obtained from day 2 to day 6 and from *G. oregonense* from day 5 to day 13. Viscosity determination
A Cannon Fenske viscosimeter (Analis- ASTM D 445) was used to measure viscosity of the basal and broth-supplemented media at 38°C.

***G. lucidum* and *G. oregonense* growth and total polysaccharides production in liquid culture**

Mycelial samples were obtained daily by centrifuging each medium at 650 x g for 20 min and washing the mycelial pellets in water. Mycelia were dried at 80°C to a constant weight. The supernatants were collected; crude polysaccharide was precipitated by addition of four volumes of 95% ethanol and centrifuged at 650 x g for 10 min. The precipitate was dried at 60°C (Yang and Liau 1988a). Total polysaccharide in the culture medium was determined by the phenol-sulphuric acid assay (Dubois *et al*. 1956). In order to separate polysaccharides from the mycelium and liberate endoglucans by cellular lysis (Yang and Liau 1988a), liquid cultures were treated for 30 minutes with a Branson 220 ultrasonicator (50 kcycles.s^{-1}). Data were analysed by ANOVA and means separated according to Tukey´s test.

RESULTS AND DISCUSSION

Mycelial growth in liquid medium

Mycelial growth of *G. lucidum* in liquid medium amended with sunflower seeds or a broth from sunflower seeds showed a typical growth curve, with a 2- to 3-day lag phase, followed by exponential growth to a stationary phase after 6 days (Figure 1).

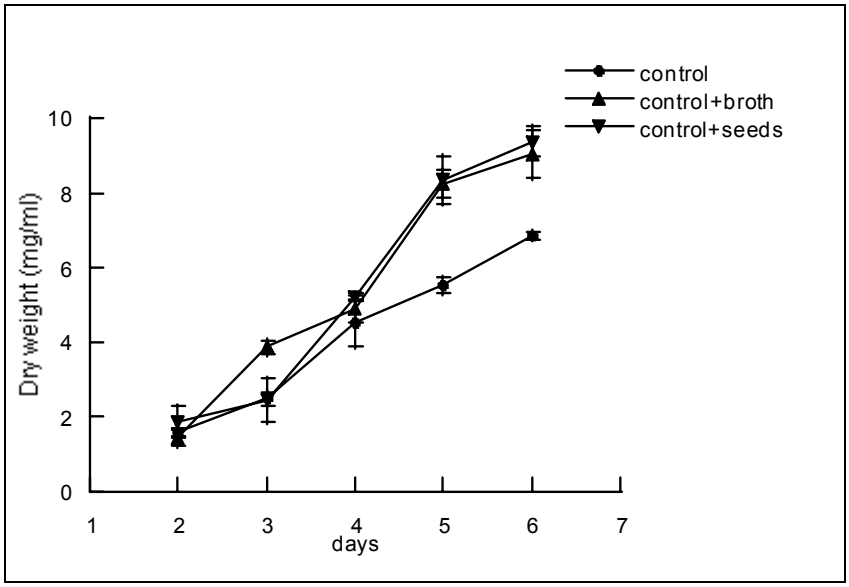

Figure 1. Mycelial growth of *Ganoderma lucidum* strain in liquid MYG medium with and without sunflower seeds (65 g.l^{-1} / 1:4 dilution) sunflower seed broth.

Maximum mycelium concentration in supplemented media was approximately 30% higher compared to the control. By day 6, mycelial growth was 9.4, 9.0 and 6.9 mg.ml^{-1}

for sunflower seed, broth supplemented and basal medium, respectively. The data suggest that the effect of the media on mycelium production was significant, $P\angle 0.001$. The maximum mycelial concentration for *G. lucidum* CCRC 36123 by Yang and Liau (1998b) was 3.5 $mg.ml^{-1}$ at pH 4.9 in a glucose-ammonium chloride medium and a temperature between 30-35°C.

In the case of *G. oregonense*, only the broth-supplemented medium showed a sigmoid-like growth, i.e. lag phase growth during days 5-9, exponential growth between days 9-12 to reach a maximum at day 12 (Figure 2). In the control medium and the sunflower seed medium, there were not marked changes in the growth rate. A maximum mycelium concentration, produced at day 12, was 10.3 $mg.ml^{-1}$ with broth supplemented medium while sunflower seed-supplemented and control media reached values of 5.5 and 2.2 $mg.ml^{-1}$, respectively for the same day. Data analyzed showed that the effect of the media is considered significant ($P\angle 0.001$). Thus, adding sunflower broth to MYG medium markedly improved *G. oregonense* growth.

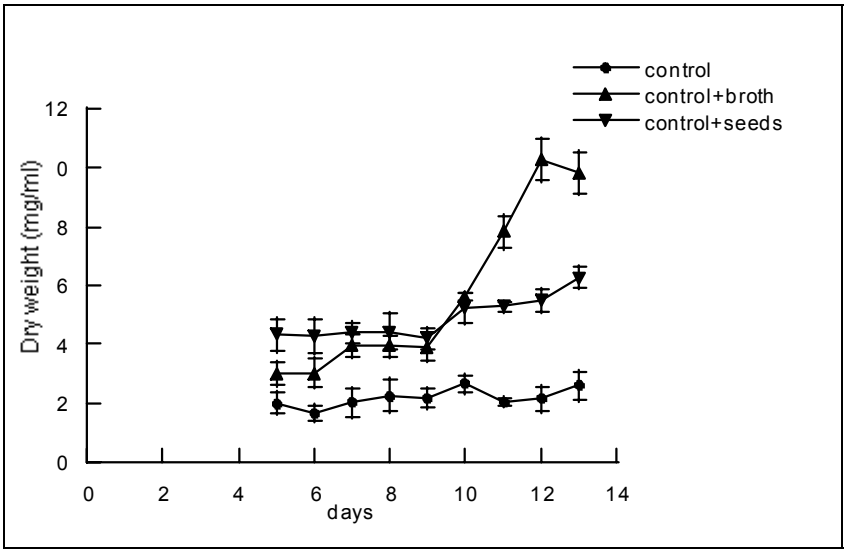

Figure 2. Mycelial growth of *Ganoderma oregonense* strain in liquid MYG medium with and without sunflower seeds (65 $g.l^{-1}$ / 1:4 dilution) of sunflower seed broth.

These results suggest that inclusion of sunflower seed broth in the formulation of a liquid culture for *G. lucidum* and *G. oregonense* could markedly improve mycelium production. We recommend sunflower seed broth because its handling is easier than seeds. In an inoculum, a high concentration of mycelium could speed up the colonization of seeds for spawn or substrate, and improve economic profits.

Polysaccharide production

Total polysaccharide production by *G. lucidum* as endo- and exo-glucans was low (0.5 $mg.ml^{-1}$) during the first 3 days, but increased markedly to a maximum concentration of 3.1 and 3.6 $mg.ml^{-1}$ in the seed and seed broth supplemented media, respectively at day 5 (Figure. 3).

These values show significant differences ($P \angle 0.001$). A decrease in total polysaccharides was observed at day 6, probably from decomposition due to the development of adverse conditions (Yang and Liau 1998a). Under their conditions of a temperature between 30 and 35°C and a pH between 4.0 to 4.5, *G. lucidum* CCRC 36123 produced 1.6 $mg.ml^{-1}$ total polysaccharides in a glucose-ammonium chloride medium.

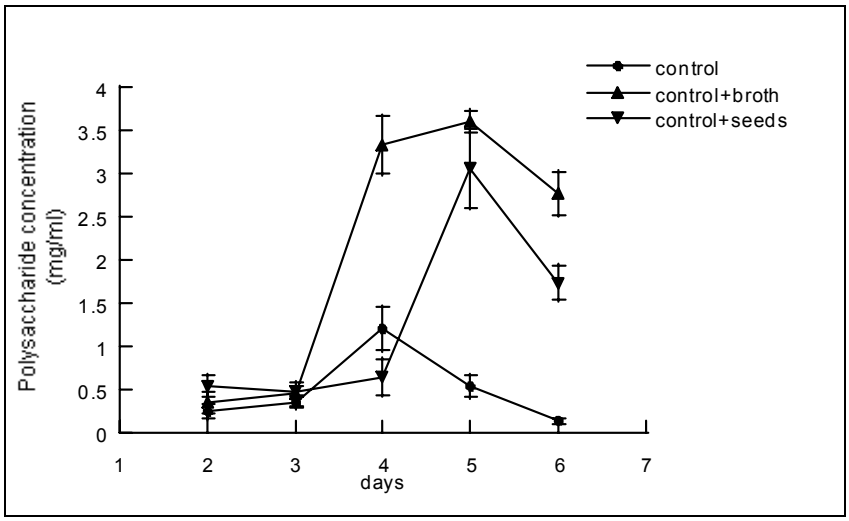

Figure 3. Polysaccharide production by *Ganoderma lucidum* in liquid MYG medium with and without sunflower seeds (65 $g.l^{-1}$) or a 1:4 dilution of sunflower seed broth.

With regards to *G. oregonense*, only broth-supplemented medium produced a marked increase in exo- and endo-polysaccharides, reaching a maximum of 3.4 $mg.ml^{-1}$ at day 12. In the control medium, polysaccharide concentration was 0.2 $mg.ml^{-1}$, while in the seed-amended media, it was 0.7 $mg.ml^{-1}$ at day 12 (Figure. 4). Statistical data show significant differences, $P \angle 0.001$). Sunflower seed-supplemented medium did not show polysaccharide increases during the culture period, possibly due to nutritional limitation.

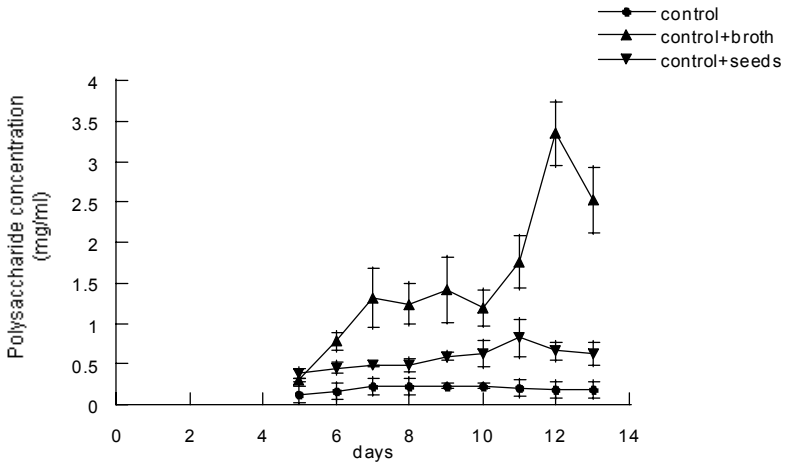

Figure 4. Polysaccharide production by *Ganoderma oregonense* in liquid MYG medium with and without sunflower seeds (65 $g.l^{-1}$) or a 1:4 dilution of sunflower seed broth.

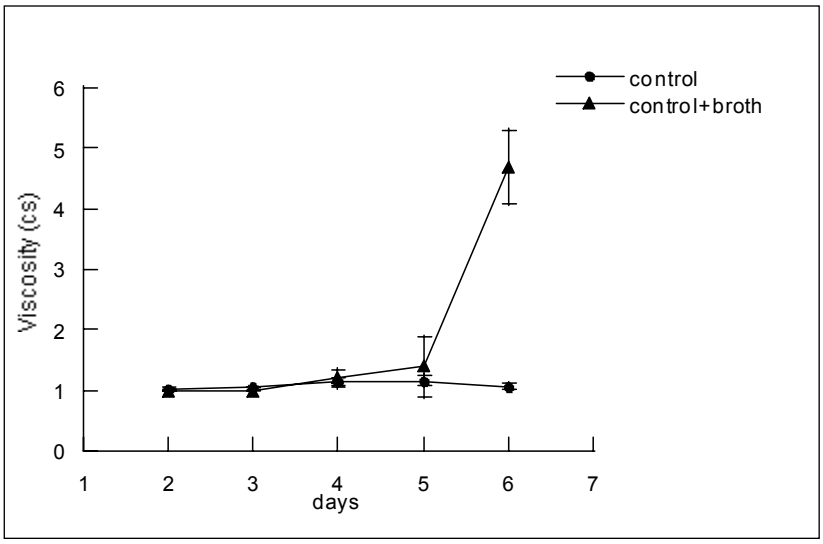

Figure 5. Culture viscosity of *Ganoderma lucidum* in liquid MYG medium with and without a 1:4 dilution of sunflower seed broth.

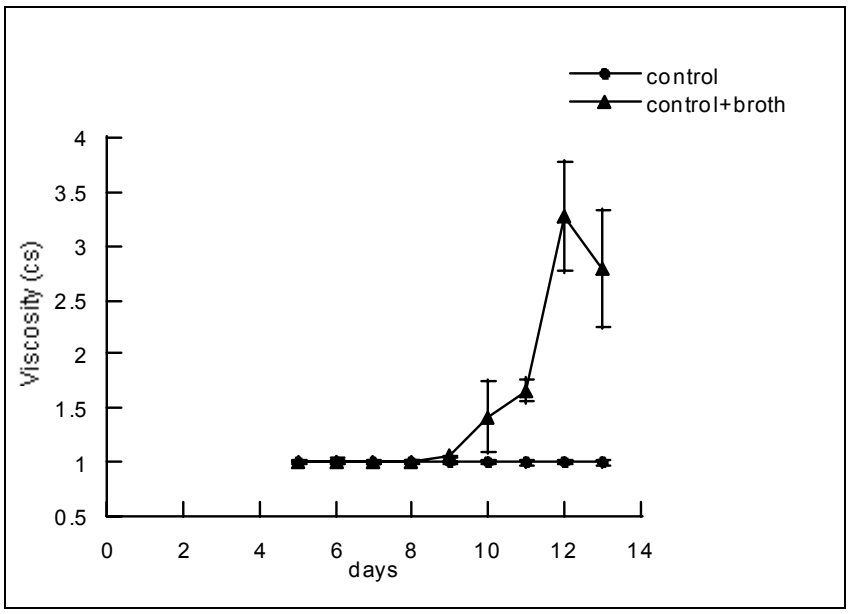

Figure 6. Culture viscosity of *Ganoderma oregonense* in liquid MYG medium with and without a 1:4 dilution of sunflower seed broth.

We suggest both amending MYG medium with sunflower seed broth and shaking the liquid culture as improved protocols for the production of immune modulating and antitumoral polysaccharides in both *Ganoderma* species.

Viscosity

G. lucidum liquid culture viscosity markedly increased between days 5 and 6 in sunflower broth-supplemented medium (Figure 5).

For *G. oregonense* viscosity increased at day 12 with sunflower broth-supplemented medium (Figure 6).

There is a positive relationship between mycelium production, polysaccharide production and viscosity. The incorporation of sunflower seed broth to basal medium did not increase the initial viscosity, probably due to its relatively low concentration in the medium.

CONCLUSIONS

Adding sunflower seeds or sunflower seed broth to MYG liquid medium increased *G. lucidum* mycelial growth and high molecular weight polysaccharide production in a shorter period, compared to basal MYG medium. However, sunflower seeds added to MYG medium did not improve *G. oregonense* performance; in this case, addition of sunflower seed broth markedly increased both mycelium and polysaccharide production.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Universidad Nacional del Sur, Argentina. We wish to thank R. Devalis for helpful laboratory assistance.

REFERENCES

Cancalon, P. 1971. Sunflower seed hulls chemical composition. J. *Am. Oil Chem. Soc*. *48*: 629-632.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem. 28*: 350-356.

Mizuno, T., G. Wang, J. Zhang, H. Kawagishi, T.Nishitoba and J. Li. 1995. Reishi, *Ganoderma lucidum* and *Ganoderma tsugae*: Bioactive substances and medicinal effects. *Food Rev.Intl. 11*: 151-166.

Morigawa, A., K. Kitabatake, Y. Fujimoto and N. Ikekawa. 1986. Angiotensin converting enzyme-inhibitory triterpenes from *Ganoderma lucidum. Chem. Pharm. Bull. 34*: 3025-3028.

Park, C.S., G.D. Marx, Y.S. Moon, D. Wiesenborn, K.C. Chang and V. Hofman. 1997. Alternative uses of sunflower: *In:* A.A. Scheiner (ed). *Sunflower Technology and Production*, Agronomy 35. ASA. CSSA. SSSA. 765-807.

Sone, Y., R. Okuda, N. Wada, E. Kishida and A. Misaki. 1985. Structure and antitumor activities of the polysaccharide isolated from fruiting body and the growing culture of mycelium of *Ganoderma lucidum*. *Agric.Biol. Chem. 49*: 2641-2653.

Tseng, T.C., M.S. Shiao, Y.S. Shieh and Y.Y. Hao. 1984. Study on *Ganoderma lucidum* 1. Liquid culture and chemical composition of mycelium. *Bot. Bull. Acad. Sinica 25*: 149-157.

Yang, F.C. and C.B. Liau. 1998a. The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. *Process Biochem. 33*: 547-553.

Yang, F.C. and C.B. Liau. 1998b. Effects of cultivating conditions on the mycelia growth of *Ganoderma lucidum* in submerged flask cultures. *Bioprocess Eng. 19*: 233-236.

Yang, Q.Y. and S.C. Jong. 1987. A quick and efficient method of making mushroom spawn. *Mush*. *Sc. 12*: 317-324.