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BIOTECHNOLOGY OF WINERY AND VINE WASTES RECYCLING BY *IN VITRO* CULTIVATION OF EDIBLE AND MEDICINAL MUSHROOMS

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Abstract: Annually, huge amounts of wine and vine wastes cause serious environmental pollution effects by their accumulation in vineyards as well as at nearby winery factories. Much worse is their burning on the soil surface or incorporation into its structure. The optimal and efficient way to solve these problems is to utilize a biotechnology to recycle this wine and vine wastes. Hence the main aim of this study was to establish the best biotechnology for winery and vine wastes recycling by using them as suitable growth substrata for edible and medicinal mushrooms. For this purpose, two species of Basidiomycete mushroom, *Ganoderma lucidum* (Curtis) P. Karst (common name: Reishi) and *Pleurotus ostreatus* (Jacquin ex Fries) Kummer (common name: Oyster Mushroom) were used as pure mushroom cultures in experiments. Stock cultures were maintained on malt-extract agar (MEA) slants (20% malt extract, 2% yeast extract, 20% agar-agar). Slants were incubated at 25°C for 120–168 h and stored at 4°C. Then, the pure mushroom cultures were expanded by growing in 250-ml flasks containing 100 ml of liquid multigrain-extract medium at 23°C on rotary shaker incubators at 110 revs min^{-1} for 72–120 h. The experiments of inoculum preparation were set up under the following conditions: constant temperature, 23°C; agitation speed, 90–120 revs min^{-1}; pH level, 5.0–6.0. All mycelial mushroom cultures were incubated for 120–168 h. During the incubation time period, all the spawn cultures were maintained in special growth rooms, designed for optimal incubation at 23°C. In the next stage of the experiments, culture composts for mushroom growth were prepared from lingo-cellulose wastes as vineyard prunings and marc of grapes to be used as substrata for mycelia development and fruit body formation. Therefore three variants of culture composts were prepared from marc grapes and vineyard prunings in the following ratios: 1:1, 1:2, 1:4 (w/w). The effects of compost composition (carbon, nitrogen and mineral sources) as well as other physical and chemical factors (temperature, inoculum amount, pH level and incubation time, etc.) on mycelial growth and especially on fruit body formation were investigated. During the whole stage of fruit body formation and development the main culture parameters were set up and maintained continuously at the following levels, depending on each mushroom species: air temperature, 15–17°C; air flow volume, 5–6m^3/h; air flow speed, 0.2–0.3 m/s; relative moisture content, 80–85%, light intensity, 500–1000 luces for 8–10 h/d. The recorded results that could influence mycelial growth as well as fruit body formation in *P. ostreatus* and *G. lucidum* were compared with the same fungal cultures grown on poplar logs used as control samples.

Keywords: biotechnology, environmental pollution, fungal cultures, *in vitro* cultivation, edible and medicinal mushrooms, *Ganoderma lucidum*, *Pleurotus ostreatus*, recycling, vine wastes, winery wastes

Introduction

The agricultural processes as well as the industrial activities related to vine crops and wine processing have generally been matched by a huge formation of a wide range of wastes products. Many of these lingo-cellulose wastes cause serious environmental pollution effects, if they are allowed to accumulate in the vineyards or, much worse, burned on the soil [1, 2]. The solid substrate fermentation of plant wastes from the agro-food industry is one of the most challenging and technically demanding of all biotechnologies known to humankind [3-5]. The major group of fungi that degrade cellulose and **ligno**-cellulose materials are edible mushrooms of the Class Basidiomycetes [6-9]. The main aim of this study was to establish the best biotechnology for recycling winery and vineyard wastes by using them as a growth source for

edible mushrooms and, last but not least, to protect vineyard ecosystems [9-12]. Taking into consideration that most of the edible mushrooms species require a specific micro-environment including complex nutrients, the influence of all physical and chemical factors upon fungal biomass production and formation of mushroom fruit bodies has been studied by testing new biotechnological procedures [7-12].

Materials and Methods

For the main purposes of this study, two species **belonging to** Basidiomycetes fungi, *Ganoderma lucidum* (Curtis) P. Karst (common name: Reishi) and *Pleurotus ostreatus* (Jacquin ex Fries) Kummer (common name: Oyster Mushroom) were used as pure mushroom cultures isolated by the authors from the natural environment and now being **preserved** in the local collection of the University of Pitesti. Stock cultures were maintained on malt-extract agar (MEA) slants (20% malt extract, 2% yeast extract, 20% agar-agar). Slants were incubated at 25°C for 120–168 h and stored at 4°C. The pure mushroom cultures were expanded by growing in 250-ml flasks containing 100 ml of liquid malt-extract medium at 23°C on rotary shaker incubators at 110 revs min^{-1} for 72–120 h. To prepare the inoculum for the spawn cultures of *G. lucidum* and *P. ostreatus* the pure mushroom cultures were inoculated into 100 ml of liquid malt-yeast extract culture medium with 3–5% (v/v) and then maintained at 23–25°C in 250 ml rotary shake flasks. The experiments of inoculum preparation were set up under the following conditions: constant temperature, 25°C; agitation speed, 90-120 revs min^{-1}; initial pH, 5.5–6.5. All the mushroom seed cultures were incubated for 120–168 h. After inoculum preparation, the experiments were focused on obtaining the spawn of *G. lucidum* and *P. ostreatus*. In this respect, the seed cultures of these mushroom species were inoculated in liquid culture media (20% malt extract, 10% wheat bran, 3% yeast extract, 1% peptone) at pH 6.5 previously distributed into rotary shake flasks of 1,000 ml. During the incubation time period, all the spawn cultures were maintained in special culture rooms, designed for optimal incubation at 25°C. In the next stage of the experiments, the culture composts were prepared from the **ligno**-cellulose wastes resulting from vineyard prunings and marc of grapes. In this respect, three variants of culture compost were prepared made of marc grapes and vineyard prunings in the following ratios: 1:1, 1:2, 1:4 (w/w). The vine and winery wastes were mechanically pre-treated with an electric grinding device to break down the lignin and cellulose structures in order to make them more susceptible to the actions of enzymes [10-12]. After that, specific sources of carbon, nitrogen and minerals were added in the mentioned amounts to each variant of culture composts. All the culture compost variants made of ground vineyard and winery wastes were transferred into 1,000-ml glass jars and disinfected by steam sterilization at 120° C for 60 min. When the jars filled with compost were chilled they were inoculated with liquid spawn already prepared.

Results and Discussion

Each variant of culture compost for mushroom growth was inoculated using liquid spawn with an age of 72–220 h and volume size 3–9% (v/w). During the period of 18–20 days after this inoculation, all the mushroom cultures had developed a significant mycelial biomass on the culture substrata of vineyard prunings and marc of grapes [10-12].

According to the recorded results of the experiments performed, the optimal laboratory-scale biotechnology for edible mushroom cultivation on composts made of marc of grapes and vineyard prunings was established (Fig. 1).

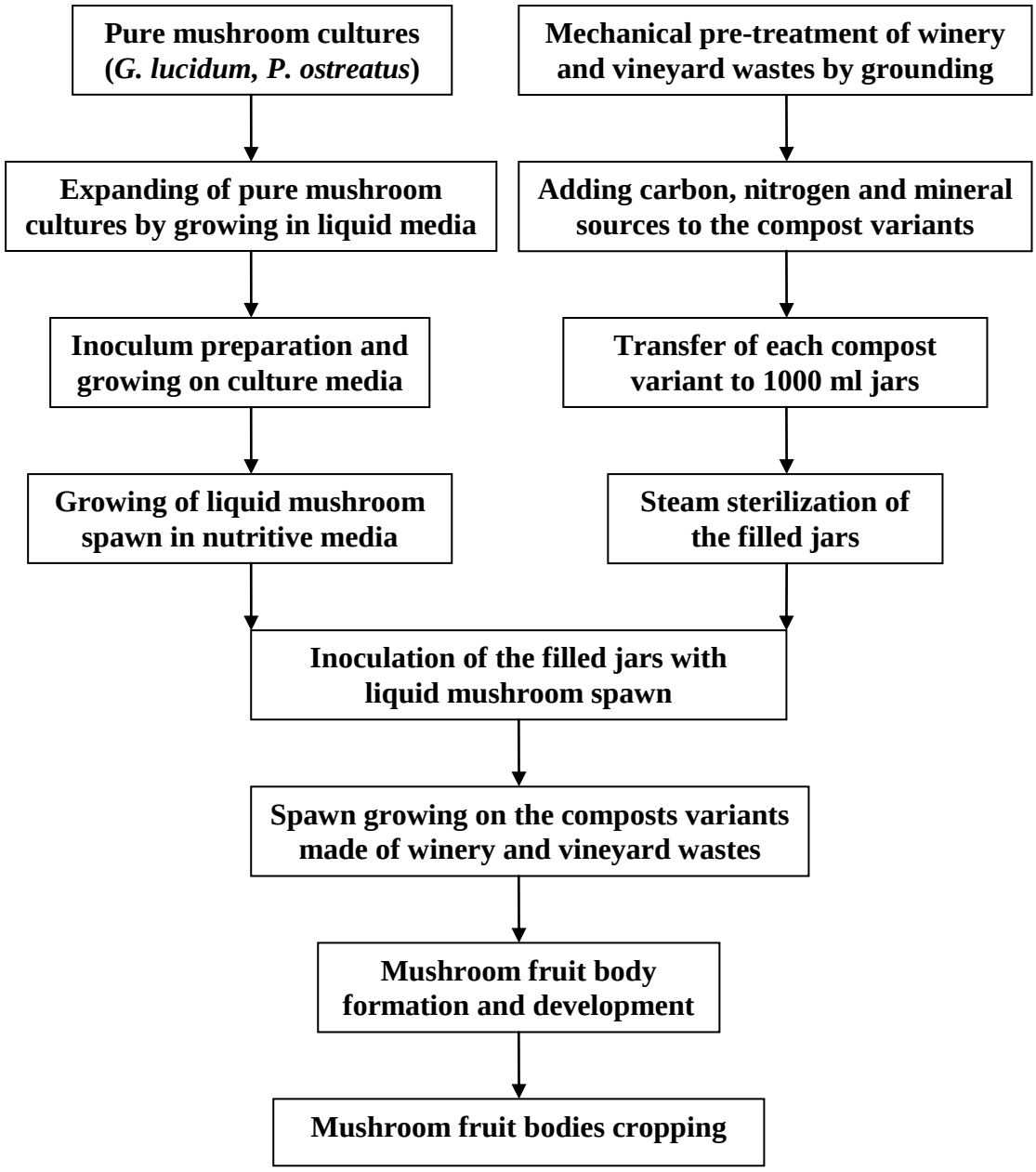

Fig. 1: Scheme of laboratory-scale biotechnology for edible mushroom production by recycling winery and vineyard wastes

The effects induced by some additional ingredients as carbon sources upon the mycelial growth during the incubation were investigated, as shown in Fig. 2.

Each carbon source was added to the basal composts at a concentration level of 5% (w/w) and the incubation time period lasted 168–288 h [12-14].

Maltose, among all the carbon sources tested, showed the highest influence upon mycelial growth and fresh fungal biomass production, about 28–35g%. The effects of nitrogen sources were recorded as shown in Fig. 3.

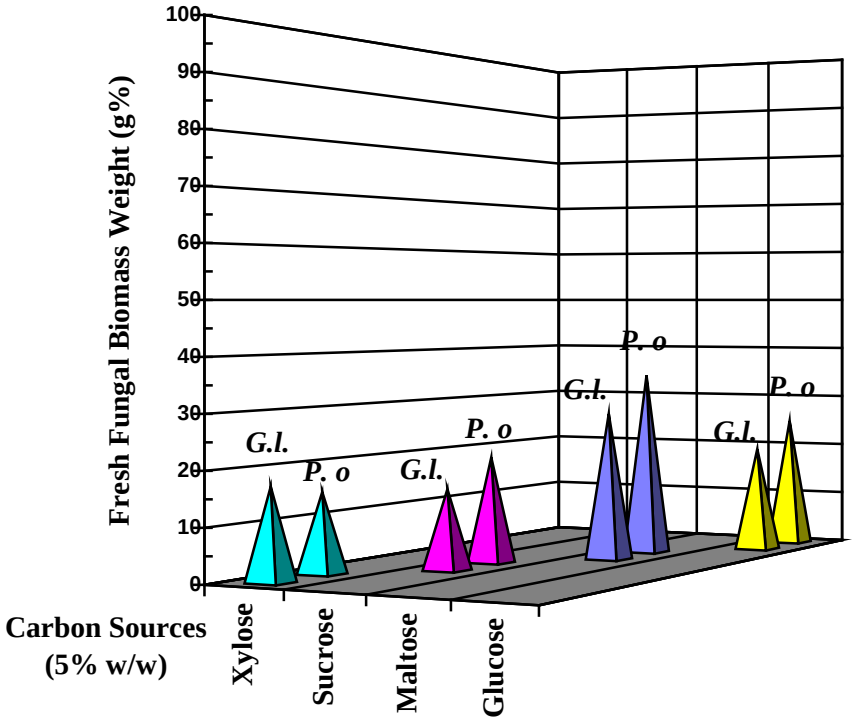

Fig. 2: Comparative effects of carbon sources upon mycelial growth of *P. ostreatus* (*P.o.*) and *G. lucidum* (*G.l.*)

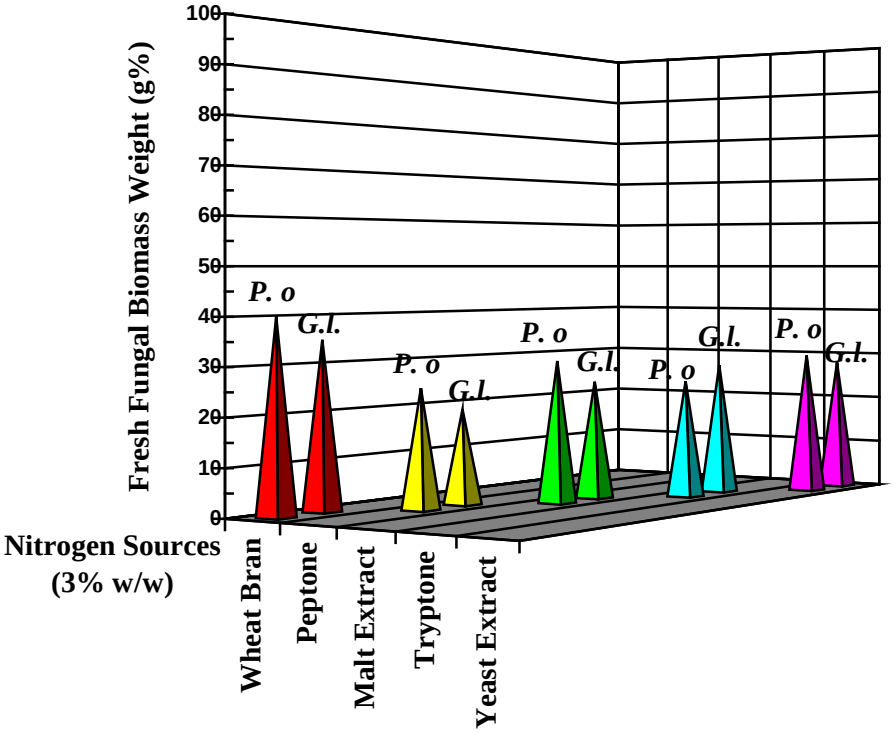

Fig. 3: Comparative effects of nitrogen sources upon mycelial growth of *P. ostreatus* (*P.o.*) and *G. lucidum* (*G.l.*)

Among the five nitrogen sources examined, wheat bran was the most effective for mycelial growth and fungal biomass, at 35–40 g% fresh fungal biomass weight, closely followed by malt extract at 25–30 g% fresh fungal biomass weight. Peptone, tryptone and yeast extract are also well-known nitrogen sources for fungal biomass synthesis but their efficiency in these experiments was relatively lower than the mycelial growth and fungal biomass production induced by wheat bran added as natural organic nitrogen sources [15-17]. All the experiments were carried out for 288 h at 25°C with the initial pH 6.5 and all data are the means of triple determinations carried out on the variants of composts made of vineyard prunings and marc of grapes in the ratio 1:4.

The influence of various mineral sources upon fungal biomass production was examined at a standard concentration of 1% (w/w). Among the various mineral sources examined ($CaCO_3$, $CaSO_4$, $MgSO_4 \cdot 5H_2O$, K_2HPO_4 and KH_2PO_4), $CaCO_3$ yielded the best mycelial growth as well as fungal biomass production at 28–32 g% and for this reason it was recorded as the most favourable mineral source (Fig. 4).

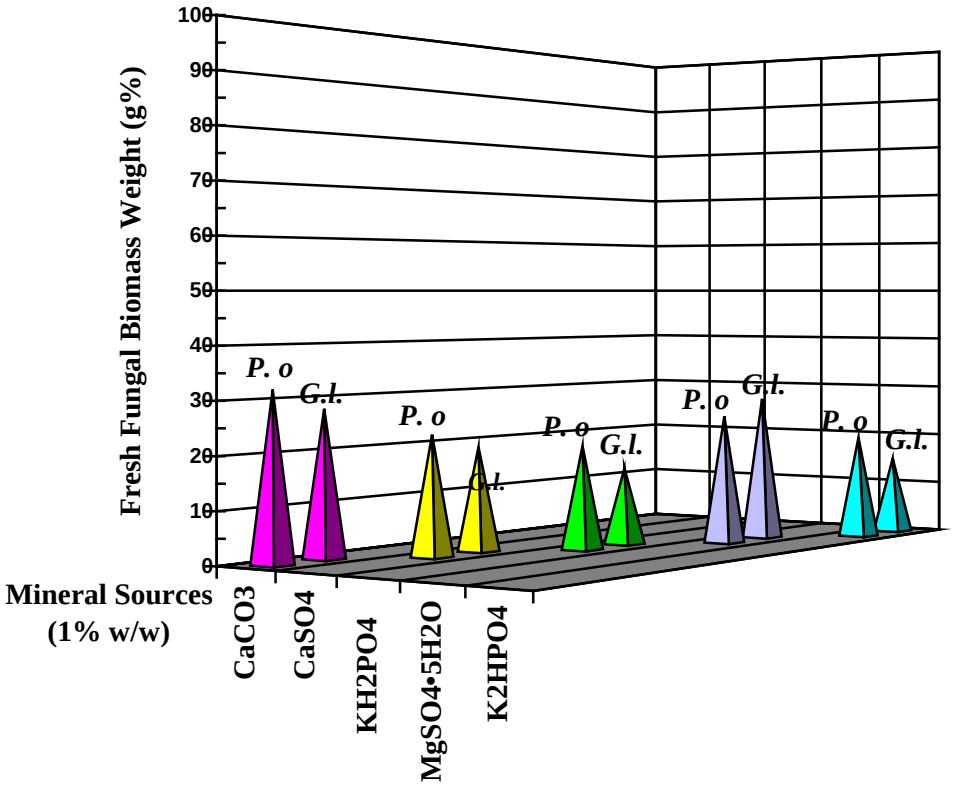

Fig. 4: Comparative effects of mineral sources upon mycelial growth of *P. ostreatus* (*P. o.*) and *G. lucidum* (*G. l.*)

Similar observations were recorded by Stamets (1993), during experiments concerning other techniques of mushroom cultivation, as well as by other researchers [15-17]. Also, other mineral sources tested, such as $MgSO_4 \cdot 5H_2O$ have shown an optimal influence upon fungal biomass growth [17-18]. At the same time, the mineral sources K_2HPO_4 and KH_2PO_4 as essential phosphates could improve the pH level through their buffering action, but they were less favourable for mycelial growth in submerged as well as in surface cultures of mushrooms. The experiments were carried out for 144 h at 25°C with the initial pH 6.5. Data are the means of

triple determinations carried out on the variants of composts made of vineyard prunings and marc of grapes in the ratio 1:4.

Also, the influence of initial pH upon mushroom fruit body formation and development was observed and recorded (Fig. 5).

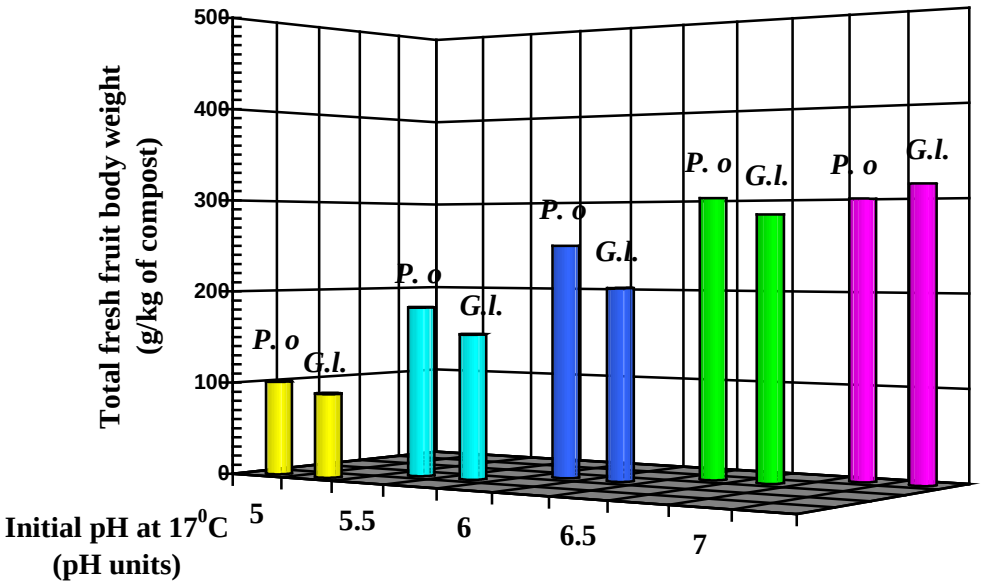

Fig. 5: The influence of initial pH upon fruit body formation of *P. ostreatus* (*P. o.*) and *G. lucidum* (*G. l.*)

The optimal pH levels for mushroom fruit body production were 6.5–7.0 for both mushroom species, recorded at the temperature level of 17°C.

The whole period of mushroom growth from inoculation to fruit body formation lasted 30–60 days, depending on each fungal species used in experiments and for this purpose special culture rooms were used. During the whole processes of fruit body formation and development, the culture parameters were set up and maintained at the following levels, depending on each mushroom species: air temperature, 15–17^{0}C; air flow volume, 5–6m^3/h; air flow speed, 0.2–0.3 m/s; relative moisture content, 80–85%, light intensity, 500–1,000 luces for 8–10 h/d.

The final fruit body production of these mushroom species used in experiments was recorded between 1.5–2.8 kg relative to 10 kg of composts made of vineyard and winery wastes.

Conclusions

1. The data recorded revealed that by applying this biotechnology, winery and vineyard wastes could be recycled as useful raw materials for the preparation of culture compost in order to obtain edible mushrooms

2. Studying the comparative effects of physical and chemical factors that could influence the mycelial growth as well as fruit body formation and development of *P. ostreatus* and *G. lucidum*, the following representative results were recorded:

– maltose, among all the carbon sources tested, showed the highest influence upon the mycelial growth and fresh fungal biomass production of about 28–35g%;

– among the five nitrogen sources examined, wheat bran was the most effective for the mycelial growth and fungal biomass production of *G. lucidum* and *P. ostreatus*, at 35-40 g% fresh fungal biomass weight, closely followed by the malt extract at 25–30 g%

– $CaCO_3$ yielded the best mycelial growth as well as fungal biomass production at 28–32g% and was recorded as the best mineral source;

– optimal pH levels for mushroom fruit body production were 6.5–7.0 for both mushroom species, recorded at the temperature level of 17°C;

3. The final fruit body production of these two mushroom species was recorded as 1.5–2.8 kg relative to 10 kg of compost made of vineyard and winery wastes.

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BIOTEHNOLOGIE DE RECICLARE A DEŞEURILOR VINICOLE ŞI VITICOLE PRIN CULTIVAREA *IN VITRO* A CIUPERCILOR COMESTIBILE ŞI MEDICINALE

(Rezumat)

Anual, cantităţi uriaşe formate din deşeuri vinicole şi viticole generează grave efecte de poluare ambientală prin acumularea acestora atât în podgorii, cât şi vecinătatea combinatelor de vinificaţie. Efecte mult mai negative se

produc prin arderea acestor deşeuri la suprafaţa solului sau prin încorporarea lor în structura sa. Modalitatea optimă şi eficientă de a soluţiona aceste probleme este aceea de a utiliza biotehnologia de reciclare a acestor deşeuri vinicole şi viticole. În acest context, principalul scop al acestei lucrări a fost cel de a stabili cea mai bună variantă de biotehnologie pentru reciclarea deşeurilor vinicole şi viticole prin utilizarea lor drept substraturi corespunzătoare de creştere a ciupercilor comestibile şi medicinale. În concordanţă cu acest scop, două specii de ciuperci din Clasa Basidiomycetes, respectiv, *Ganoderma lucidum* (Curtis) P. Karst (denumire populară: Reishi), precum şi *Pleurotus ostreatus* (Jacquin ex Fries) Kummer (denumire populară: ciuperca scoică) au fost utilizate sub formă de culturi pure în cursul experimentelor. Culturile de depozit au fost menţinute pe medii agarizate înclinate, compuse din malţ extract (20% extract de malţ, 2% extract de drojdii, 20% agar-agar). Mediile înclinate agarizate au fost menţinute la 25°C, timp de 120-168 h şi depozitate la 4°C. Apoi, culturile pure de ciuperci au fost multiplicate prin creşterea miceliilor în baloane cu o capacitate de 250 ml, conţinând 100 ml de mediu lichid compus din extracte ale unor seminţe de cereale, la 23°C, în incubatoare cu agitare rotativă la 110 rev min^{-1} timp de 72–120 h. Experimentele privind prepararea de inoculum au fost organizate prin asigurarea următoarelor condiţii: temperatură constantă la valoarea de 23°C; viteza de agitare, 90–120 rev. min^{-1}; nivelul de pH, 5.0–6.0. Toate culturile miceliene au fost incubate timp de 120–168 h. În cursul perioadei de incubare, aceste culturi miceliene au fost menţinute în camere speciale de creştere, proiectate pentru o incubare optimă la 23°C. În stadiul următor al experimentelor, composturile de cultivare destinate creşterii ciupercilor au fost preparate din deşeuri lignocelulozice cum sunt fragmente rezultate din tăierea coardelor de viţă de vie şi tescovina de struguri. În acest context, au fost utilizate trei variante de composturi de cultivare compuse din tescovină şi coarde de viţă de vie, în următoarele proporţii de greutate: 1:1, 1:2, 1:4. Au fost atent studiate efectele induse de compoziţia composturilor (carbon, azot, elemente minerale), precum şi cele ale factorilor fizici şi chimici (temperatura, cantitatea de inoculum, nivelul pH, durata de incubare etc.) asupra creşterii miceliului şi, în special, asupra formării corpurilor de fructificare. Pe parcursul întregii perioade de formare şi dezvoltare a corpurilor de fructificare ale ciupercilor cultivate au fost stabiliţi principalii parametri de cultivare şi au fost menţinuţi permanent la următoarele niveluri, în funcţie de fiecare dintre speciile de ciuperci utilizate: temperatura aerului, 15–17°C; debitul de aer, 5–6m^3/h; viteza fluxului de aer, 0.2–0.3 m/s; conţinutul de umiditate relativă, 80–85%, intensitatea luminii, 500–1000 lucşi, timp de 8–10 h/d. Toate rezultatele obţinute ce pot avea o influenţă asupra creşterii miceliene, precum şi asupra formarea corpurilor de fructificare aparţinând speciilor *P. ostreatus* şi *G. lucidum* au fost comparate cu probele martor reprezentate de aceleaşi culturi fungice, crescute pe substraturi constituite din butuci de lemn de plop.

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