Factors affecting physiology of mycelial growth and mushrooms aroma production in solid state fermentation

W. KABBAJ[1], M. BENSOUSSAN[2] AND S. ROUSSOS[1]

[1] Laboratoire de Biotechnologie, Centre ORSTOM. 911 avenue d'Agropolis, BP 5045, 34032 Montpellier, France.

[2] Université de Bourgogne, ENS.BANA, 21000 Dijon, France.

SUMMARY

Effects of different nitrogen sources and C/N ratios on mycelial growth and intensity of volatile aroma production, by three species of *Morchella* and one *Pleurotus* species, were studied in agar and solid support media. The results formed a base for selection of sodium nitrate as a nitrogen source, C/N ratio of 10 and *M.esculenta* as potential fungi for production of morel mushroom aroma by mycelial cells in solid state fermentation. Gas chromatographic analysis revealed wide differences in aroma compounds, produced by *M. esculenta* and *P. cornucopiae*. As the aroma compounds produced by mycelial cells of *M. esculenta* are identical to those formed by the fruiting bodies, the present results open up a never, simpler and economic strategy for production of highly priced morel mushroom aroma.

Keywords : Aroma, solid state fermentation, support solid medium, *Morchella esculenta, Pleurotus cornucopiae*, mycelial cells, nitrogen sources, C/N ratio, apical growth, aroma profile.

RESUME

Effets de la source d'azote sur la physiologie de croissance mycélienne et la production d'arôme de champignons cultivés sur support solide.

KABBAJ W., BENSOUSSAN M. ET ROUSSOS S.

L'effet de différentes sources d'azote et du rapport C/N sur la croissance mycélienne et sur l'intensité aromatique dégagée par trois espèces du genre *Morchella* et d'une espèce du genre *Pleurotus* a été étudié sur agar puis sur support solide. Les résultats ont été à la base du choix du nitrate de sodium comme source d'azote, du rapport C/N de 10 ainsi que du mycélium de *M. esculenta* comme source potentielle pour la production de l'arôme de morille. L'identification des molécules contribuant à l'arôme réalisée par CPG-SM a révélé de larges différences entre *M. esculenta* et *P. cornucopiae*. Dans la mesure où l'arôme produit par le mycélium rappelle celui du carpophore, ce résultat constitue une méthode simple, rapide et économique pour la production de l'arôme morille .

Mots clés : Arôme, fermentation en milieu solide, *Morchella esculenta, Pleurotus cornucopiae*, mycélium, sources d'azote, croissance apicale, rapport C/N, profil aromatique.

INTRODUCTION

Morchella species, a group of highly priced edible mushrooms, are well known for their ability to produce volatile metabolites of pleasant aroma (Litchfield, 1967). The produce is consumed as a delicacy. Moreover, *in vitro* cultivation of *Morchella* is difficult and, hence, recovery of aroma compound from morel mushrooms on industrial scale is not yet carried out.

Most edible fungi form aroma compounds during fruiting body development (Gross and Asther, 1989). However, morel mushrooms have a rare ability of forming similar aroma compounds both at mycelial and fruiting body stages (Gilbert, 1960). The biotechnological advances in easier cultivation of the mycelium biomass of edible fungi by solid state fermentation can, therefore, be exploited to produce these aroma compounds, without the necessity of waiting upto the fruiting body development stage. Such aroma will be of commercial value, especially due to paucity of good natural aroma for use in foods and food-products.

A number of reports on the mycelial cell production of *Morchella* in liquid culture are available (Fron, 1905; Brock, 1951; Willam et al., 1956; Gilbert, 1960; Litchefield *et al*, 1963, Le Duy *et al*.., 1974; Buscot, 1993; Bensoussan *et al*, 1995). However, only one report is available on mycelium production of *Morchella* by solid state fermentation (Launay, 1989).

Solid state fermentation (SSF) is well known to offer many advantages over the conventional submerged fermentation, especially in the case of fungi (Hesseltine, 1972; Mudgett, 1986). The conditions of solid state fermentation are very similar to those involved in the fungal growth that occurs in nature (Roussos, 1985). Moreover, the porosity of the substrate facilitates easier aeration and oxygen transfer (Raimbault and Alazard, 1980). The technique is simpler and the liquid effluents formed are minimal (Lambert and Meers, 1983). In particular, the oxidation of lipids and fats is stimulated in solid state fermentation, due to the limited use of water in the media (Loncin, 1976). Consequently, it is possible to obtain 1-octen,3-ol, which represent 80% of the aroma fraction in fungi, through the enzymic oxidation of linoleic acid in solid state fermentation (Wurzenberger and Grosch, 1982, 1984 a,b).

The present study focuses on the aroma production and apical growth of three species of *Morchella* (*M. esculenta, M. crassipes and M. hortensis*) and one species of *Pleurotus* (*P. cornucopiae*) in solid state fermentation in response to different nitrogen sources and C/N ratios. The volatile aroma compounds formed by *M. esculenta* have also been characterized and compared to those formed by *P. cornucopiae.*

It is emphasized that *M. esculenta* was never grown earlier on sugarcane pith bagasse in solid support fermentation, nor its aroma compounds were characterized.

MATERIALS AND METHODS

FUNGAL CULTURE

Three species of the genus *Morchella* were used, along with one species of *Pleurotus* for comparative purposes. *M. esculenta* 91.9 (isolated from Danish forests), *M. hortensis* MH 88.7 (isolated from Provence region), *M. crassipes* MCR 92.24 (No. 28963, CBS, Baarns) and *P. cornucopiae* AVPL Corrèze (isolated from Provence region) were obtained from the collection of station d'Agronomie et de Mycologie, INRA, Clermont Ferrand, France. These cultures were maintained on Potato Dextrose Agar (DIFCO, Detroit USA) slants at 4°C and subcultured every 3 months.

CULTURING IN PETRI DISHES

Media with five different nitrogen sources (asparagine, glycine, tryptophane, sodium nitrate and ammonium sulphate) were used to select the best nitrogen source, based on the elongation of apical growth of mycelial cells. All these media had a constant C/N ratio of 10 and contained nitrogen at a level of 2 g/L. Consequently, the concentration of glucose used in the media ranged from 6.03 to 10.50 g/L. The other common components of the media included (g/L) KH_2PO_4 1, $MgSO_4$ 0.5, agar 15 and distilled water 1000. The pH of the media was adjusted to 6.9 before autoclaving at 121°C for 20 min. Inoculum used was 1 cm^2 piece of mycelial growth from PDA medium grown at 20°C for 7 days. The plates were incubated (static) at 20°C for 80 h and the elongation of the apical growth of the cells was measured.

CULTURING IN BOTTLES

Round bottles of 250 ml capacity, provided with autoclavable caps, were used and each bottle was charged with 40 g sugarcane pith bagasse (as inert support), after impregnating with nutrients. It occupied about one third of the total volume of the bottle. The nutrient medium (C/N ratio of 10 and 20), absorbed on a pith bagasse, contained (g/L) glucose 30, sodium nitrate 4 or 8 (as per desired C/N ratio), KH_2PO_4 4, Na_2HPO_4 1.6, $MgSO_4$ 4, $ZnSO_4$ 0.04, $MnSO_4$ 0.04, and distilled water 1000.

Inoculation was done, using 3 pieces of 1 cm^2 size of the mycelial growth from PDA medium, by placing these at an equal distance in the medium, along the walls of the bottle and at 1.5 cm height from the bottom of the flask. All these operations were carried out under aseptic conditions. Other details are as described above for culturing in Petri dishes. Samples were taken for determining apical growth of the mycelium at different time intervals. The sniffing for aroma notes was done at 216 h.

PARAMETERS MEASUREMENTS

The apical growth of the fungi was estimated in terms of the elongation of mycelial cells was expressed in mm. The changes in the pH of medium were determined, as with the method used by Raimbault and Alazard, 1980. Qualitative measurements of the aroma produced by the culture were taken by sniffing and were classified into four categories, i.e., absent -, just perceptible +, strong ++, and very strong +++.

EXTRACTION OF AROMA

The fermented support solid medium (10 g) was mixed with 40 ml distilled water and subjected to ultrasonic treatment at ambient temperature (20° C) for 20 min, with a view to break the cell wall. The resulting mass was subjected to extraction-distillation of the aroma compounds in a Likens-Nickerson apparatus, modified by Godefroot et al (1981). Samples were simultaneously distilled with hexane over a period of 60 min.

IDENTIFICATION OF AROMA COMPOUNDS

The volatile compounds present in the distillates from solid state culture of *M. esculenta* and *P. cornucopiae* were individually subjected to gas chromatography (Fison-Trio 1000), equipped with a flame ionization detector. The column used was DB5 of 30 m length. The oven temperature was raised from 50 to 300°C at a rate of 2°C/min. The injector temperature was 250°C in a splitless mode. The retention time was compared with the reference compounds for their identification.

RESULTS AND DISCUSSION

NITROGEN SOURCES VS GROWTH

Data on the effect of three organic and two inorganic nitrogen sources, with respect to the elongation of mycelial cells of three species of *Morchella* and one *Pleurotus* species, in the medium with C/N ratio of 10, showed different responses to the sources studied, at 80 hours (Table 1). However, the maximum increase in the apical growth of all the cultures was observed in sodium nitrate medium, though the efficiency of asparagine was equal to that of sodium nitrate in the case of *M. hortensis*. Ammonium sulphate promoted negligible or very slight increase in the elongation of the mycelial cells of all the cultures, except for a definitive increase in the case of *P. cornucopiae*. The negative response to ammonium sulphate by *Morchella* species might be due to formation of H_2SO_4 and the consequent reduction in the pH of the medium. This reason is supported by the fact that the optimum pH for the growth of *Morchella* is between 6.93 to 8.30 (Brock, 1951).

Table 1. Effect of different nitrogen sources on the apical elongation of the mycelial cells (mm) at 80 h in Petri dishes culturing on agar medium.

Nitrogen sources	Apical elongation of mycelial cells (mm)			
	M. esculenta	*M. crassipes*	*M. hortensis*	*P. cornucopiae*
Asparagine	22	20	40	5
Glycine	17	21	22	11
Tryptophane	12	14	23	0
Sodium-nitrate	25	22	40	13
Ammonium-sulphate	+/-	+/-	+/-	11

The results indicate that the utilization of different amino acids, as nitrogen sources, by all *Morchella* species, was slower than that of sodium nitrate, except for a more or less similar results in the case of *M.crassipes* growing on asparagine and glycine. Among the amino acids, the efficiency of tryptophane was lowest in the case of all the cultures, except for its equal efficiency with glycine in the case of *M. hortensis*. However, the growth of *P.cornucopiae* was absent in medium containing tryptophane.

Based on the above results, sodium nitrate was selected as the best nitrogen sources for all the cultures. Its use was also advantageous, as it was much cheaper than the amino acids and did not drastically lower the pH of the medium, as happed with ammonium sulfate.

EFFECT OF C/N RATIO ON GROWTH AND AROMA PRODUCTION

The grading of the aroma intensity, as determined by sniffing, is presented in Table 2, with respect to two different C/N ratios and the cultivation of the fungi on support media in the culture bottles. *M.hortensis* was not capable of producing aroma, even at a C/N ratio of 20, while the aroma production was equal at both ratios in the case of *M. crassipes* and *P. cornucopiae*. In the case of *M. esculenta* , the aroma production was lower at a C/N ratio of 20, as compared to that of 10.

Table 2. Effect of different C/N ratios on the intensity of aroma production at 216 h in bottle culture under solid state fermentation involving use of sugarcane pith bagasse as inert solid for absorbing nutrient.

C/N ratio	Intensity of aroma production			
	M. esculenta	*M. crassipes*	*M. hortensis*	*P. cornucopiae*
10	+++	+++	-	++
20	++	+++	-	++

- : Absent, + : perceptible, ++ : strong, +++ : Very strong.

In terms of growth of the mycelial cells, the results gave excellent insight on the effect of C/N ratios (Fig. 1). In the case of all cultures, the use of C/N ratio of 20 resulted in a longer lag phase and lower growth. In the case of *M.esculenta* the difference was dramatic at a C/N ratio of 20, since the lag phase was very long and the growth was less than 50%, even at 125 hours as compared with that at 75 hour, having a C/N ratio of 10. The colonization of the support by *M. esculenta* was rapid and complete at C/N ratio of 10.

Such rapid growth is of particular importance, when the cultivation is carried out at a larger scale, due to the possibility of development of contaminant microorganisms. No contamination was observed in lab scale column fermentors, due to the care taken during the entire fermentation process. No such contamination problem will be encountered in bottle cultures, because of easier maintenance of aseptic conditions, but this will be difficult in the case of larger column fermenters.

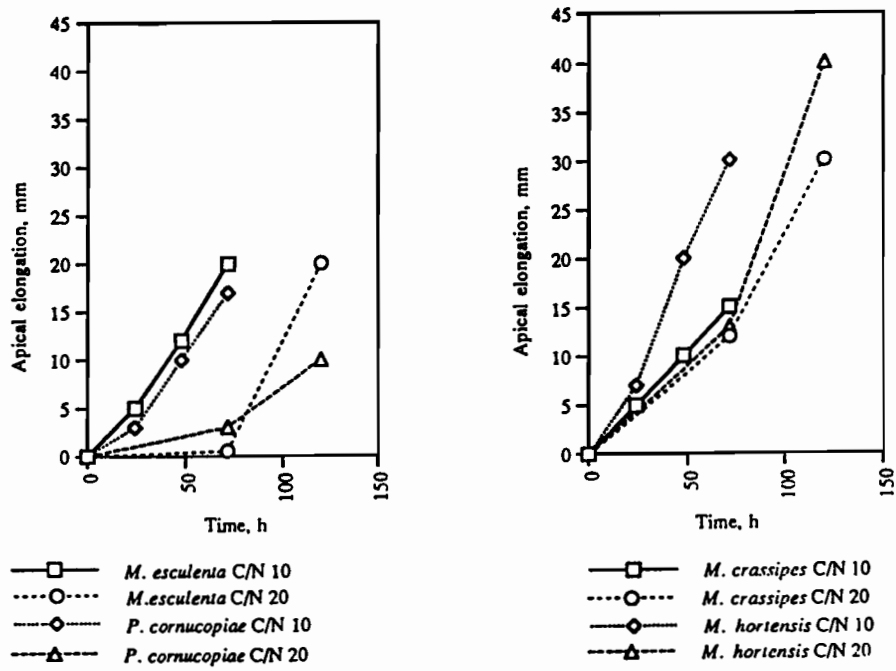

Fig. 1 : Elongation (mm) of apical mycelial cells of the cultures in media with two different C/N ratios under solid state culture on support in the cultures bottles.

It is of interest to point out that the mushroom aroma is the secondary metabolite (Grosch, 1987). Its production, however is not affected by the C/N ratio, except , for a slight reduction at a higher ratio in the case of *M. esculenta.*

Based on the above results, *M. esculenta* was selected for further studies, using a C/N ratio of 10 and sodium nitrate as the nitrogen source.

IDENTIFICATION OF AROMA COMPOUNDS

The aroma was produced under optimized conditions using *M. esculenta*. *P. cornucopiae* was also studied for comparative purposes. The chromatograms obtained by CG-MS are presented in Fig. 2.

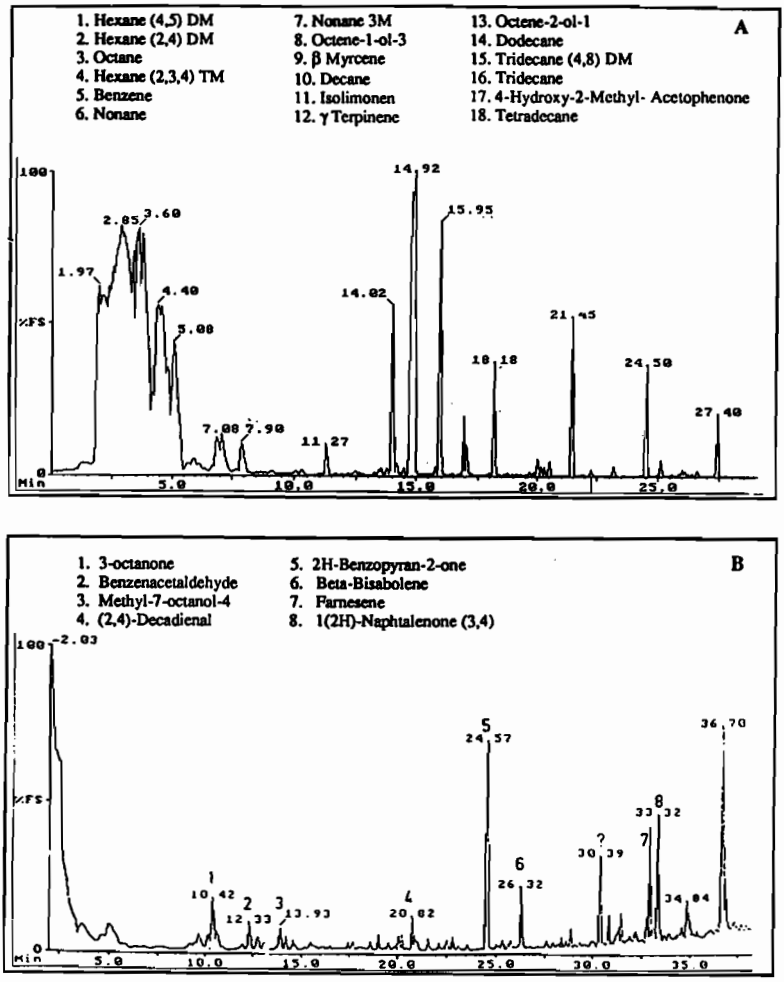

Fig. 2 : Chromatograms of aroma compounds produced by *M. esculenta* and *P. cornucopiae* at mycelial cell stage of the growth. A : *M. esculenta*, B: *P. cornucopiae*. The peaks are numbered and their identification indicated.

In the case of *M. esculenta*, a total of 18 fractions have beeen identified, with only 6 of them being aromatic in nature and with possible contribution to the aroma of *M. esculenta*. For example, 1-octen,3-ol confers the typical mushroom like aroma and this compound has been identified in the extract of several species of edible fungi, such as *Cantharellus cibarius, Boletus edulis, Lactarius trivialis, Lactarius torminosus, Lactarius rufus, Gyromitra esculenta , Agaricus bisporus* (Pyysalo and Suihko, 1976; Grove, 1980).

ß-myrcene and isolimonen, the compounds which impart pleasant and lemon-like odours, respectively, have also been identified in the extract of *M. conica* at the fruiting stage (Audoin et al, 1989). In fact, Isolimonen is the main aroma compound produced by *M. esculenta*. 2-octen,1-ol another compound produced by *M. esculenta,* is common for many different mushrooms and has a medicinal oily odour.

4-H -2- methyl acetophenone, the compound produced by *M. esculenta*, has a odour like that of orange-blossom. It is also produced by *M. esculenta* at the mycelial stage in liquid culture (Bensoussan *et al.*, 1995).

A total of eight compounds have been identified. Four of them are aromatic in nature (Gallois *et al*, 1990). None of these compounds are identical to those identified in the extract of *M. esculenta*.

The 2-H- benzopyrane-2-one, the major aroma compound in the extract of *P. cornucopiae*, smells like flower at a lower concentration, but smells like rubber at a higher concentration. The other three aroma are present in minor quantities and include 3-octanone (flower or lavender like odor), benzenacetaldehyde (almond like odor) and 2,4 Decadienal (fresh fruity like odor).

CONCLUSION

Data show the potential for growing *Morchella esculenta* mycelial cells on a support in solid state fermentation for aroma production. The source of nitrogen has a significant effect on the production of aroma by mycelial cells. Nitrate seems to be the most appropriate for the mycelial growth of *M. esculenta* and *P. cornucopiae*. In the case of the use of ammonium salts as a nitrogen source, the drop in the pH of the medium affects the growth of the culture.

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