CULTIVATED BASIDIOMYCETES AS A SOURCE OF NEW PRODUCTS: - IN VITRO CULTIVATION DEVELOPMENT - SELECTION OF STRAINS RESISTANT TO *TRICHODERMA VIRIDE* - SEARCH FOR NEW ACTIVE COMPOUNDS - FACTORS INFLUENCING PLASTICITY IN *GRIFOLA FRONDOSA*

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IMPRIMATUR POUR LA THESE

Cultivated Basidiomycetes as a source or new products: - in vitro cultivation development, - selection of strains resistant to *Trichoderma viride*, - search for new active compounds, - factors influencing plasticity in *Grifola frondosa*

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To my loved ones:

Chléa and Loan, my children

Nicolas, my husband

my mother Nancy

my sisters Rebecca, Christine and Laura

my father Pier Luigi left too early

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KEY WORDS

Mushroom, Basidiomycete, cultivation, culture, fructification, substrate, *Trichoderma viride*, selection, strain, resistance, biological test, *Grifola frondosa*, metabolite profile.

ABSTRACT

Higher mushrooms are a source of still little explored and exploited for new products. They draw ever more interest of scientific and industrial spheres. In this context we wanted to fructify in vitro chosen species to have a better understanding of factors influencing carpogenesis and to test fruit bodies' extracts on different targets, among them *Trichoderma viride* against which we had to develop a test. We had also planned to develop a selection method of resistant strains to *T. viride* which represents the major problem in our cultivation process and in industrial cultures in general. We wanted to study in a model species factors influencing metabolites profile in the fruit body.

Fourteen species have been successfully cultivated according to techniques previously developed in the laboratory. Moreover 37 new strains from 27 species have been tested for their fruiting ability on different substrates in industrial bags or in polypropylene pots. In most experiments only the four substrates A, B, C and D varying in their C:N ratio were used. A few species were tested on other substrates, like *A. blazei* which was the only secondary saprophytic species studied. Successful fruiting was achived for 59.5% of the strains and 63% of the species. Some species had a high specificity of substrate for fruiting, while others could fruit on all the tested substrates. There was a positive correlation between C:N ratio and fructifying success: 56% of the species fructified on the A substrate with higher nitrogen content (C:N= 75), but percentage decreased with nitrogen to a 34.8% success on the D substrate with lower Nitrogen content (C:N=265). Intraspecific adaptation to different substrates was also observed. Thus a substrate designed for a strain would not necessarily be suitable for another strain. In conclusion primordia initiation, fruiting, yield, but also time before collection depended of the substrate type, but also from the strain used.

Trichoderma viride is the worst pest encountered in many mushroom growers' farms. Thus, to our point of view, aptitude to compete this pest was a characteristic to select beside fruiting capacity in new strains to work with. Testing the strains in Teflon® tubes showed to be advantageous to select resistant strains to that mould at low cost and in a relatively short time. Moreover it allows to bring information on the behavior of each strains tested on various substrates which composition has clearly shown to influence substrate invasion rate and resistance. To develop the tests five strain of each the two species *G. frondosa* and *F. pinicola* were used. Results show that there are indeed great variations between strains and for a given strain on different substrate like observed during culture development. Substrate quantities included in the tubes, as well as the size of the colony when introducing the fungal pest, have shown to influence final results of *T. viride* progression in the substrate. This test seems adequate for our aim however with a few adaptations to mushroom species are some are naturally much more resistant than others.

An autobiographic bioassay against *T. viride* was adapted from existing protocols. Biological tests against *Bacillus subtilis*, *Cladosporium cucumerinum, Candida albicans*, *Biomphalaria glabrata* and *Aedes aegyptii*, as well as against the radical DPPH were performed for some species. Only a few activities mainly due to fatty acids

were detected. Linoleic acid 9-cis,12-cis-octadecadienoique revealed to be the molecule responsible for the snail *B. glabrata* death at least in *G. frondosa* extract.

Finally comparison of *G. frondosa* DCM and MeOH fruit bodies extracts by TLC and HPLC showed mainly a great variation in the metabolites profiles in function of the technique used (freezing or not, drying or lyophilisation,), but also in function of the strains but not of the substrate composition for the studied strain. This tends to prove that we need to be very careful in the treatment of fungal material to obtain stable results.

In conclusion the aims of this work were fulfilled. This work is encouraging to continue in this line of research. We can hope to find new flavours or active compounds in the future, when considering the number of species which could be tested. It would be particularly interesting to focus on compounds against *T. viride* as there is a lack of research in this domain and an urgent need for new products to fight this pathogen.

MOTS CLES

Champignon, Basidiomycète, culture, fructification, susbstrate, *Trichoderma viride*, sélection, souche, résistance, test biologique, *Grifola frondosa*, profil de métabolites.

RESUME

Les champignons supérieurs sont une source encore très peu explorée et exploitée de nouveaux produits naturels. Ils attirent toujours plus l'intérêt des milieux scientifiques et industriels. Dans ce contexte, nous voulions faire fructifier in vitro des espèces choisies afin de mieux comprendre les facteurs influençant la carpogénèse et de tester les extraits de fructification sur différentes cibles biologiques, parmi lesquelles *Trichoderma viride* pour lequel nous devions développer un test. Nous avions également prévu de développer une méthode de sélection de souches résistantes à *T. viride* qui représente le problème majeur dans nos procédés de culture et dans les cultures industrielles en général. Enfin nous voulions étudier chez une espèce modèle les facteurs influençant le profil de métabolites dans la fructification.

Quatorze espèces de Basidiomycètes ont été cultivées selon des techniques développées auparavant dans le laboratoire. De plus, 37 nouvelles souches de 27 espèces ont été testées pour leur capacité à fructifier sur différents substrats en sacs industriels ou en pots de polypropylène. Dans la plupart des expériences seuls les 4 substrats A, B, C et D variant principalement dans leur rapport C/N ont été utilisés. Quelques espèces ont été testées sur d'autres substrats, comme *Agaricus blazei* qui est le seul saprophyte secondaire étudié durant ce travail. Le processus de fructification a abouti pour 59.5% des souches et 63% des espèces. Certaines espèces avaient une grande spécificité de substrat pour fructifier alors que d'autres pouvaient fructifier sur tous les substrats testés. Il y avait une corrélation positive entre le rapport C :N et le succès à la fructification : 56% des espèces ont fructifié sur le substrat A qui a la plus grande teneur en azote (C :N= 75), mais le pourcentage diminue en même temps que la teneur en azote jusqu'à un succès de 34.8% sur le substrat D (C :N=265). Une adaptation intra spécifique à différents substrats a également été observée. C'est pourquoi un substrat composé pour une souche donnée ne conviendrait pas nécessairement à une autre souche de la même espèce. En conclusion l'initiation des primordia, la fructification, le rendement, mais aussi le temps jusqu'à la récolte dépendent du type de substrat, mais aussi de la souche utilisée.

Le problème majeur durant la culture de champignons est le mycoparasite *Trichoderma viride*. A notre avis, l'aptitude à la compétition contre ce micromycète est une caractéristique à sélectionner parallèlement à la capacité à fructifier, lors du choix d'une nouvelle souche. Nous avons démontré que les tests en tubes de Téflon® étaient adéquats pour sélectionner des souches résistantes à un coût moindre et en un temps relativement court. De plus cela permet d'apporter des informations sur le comportement de chaque souche testée sur les différents substrats dont la composition a clairement montré l'influence sur la rapidité de colonisation et sur la résistance. Pour développer la méthode de sélection 5 souches de chacune des espèces *Grifola frondosa* et *Fomitopsis pinicola* ont été testées. Les résultats ont montré qu'il y avait une grande variation entre souche et pour une même souche inoculée sur différents substrats comme observé durant les expériences de culture. La quantité de substrat dans les tubes, la taille

de la colonie à l'introduction de *T. viride* ont eu une influence sur les résultats finaux de la progression de *T. viride* dans le substrat. Cette méthode semble adéquate pour atteindre l'objectif fixé.

Un test autobiographique contre *T. viride* a été adapté de protocoles existants. Les tests biologiques contre *Bacillus subtilis*, *Cladosporium cucumerinum*, *Candida albicans*, *Biomphalaria glabrata* et *Aedes aegyptii*, ainsi que contre le radical DPPH ont été réalisés sur 8 espèces à l'Institut de Pharmacognosie et Phytochimie de l'Université de Lausanne. Seul un petit nombre d'activités ont été décelées principalement dues à des acides gras. L'acide linoléique 9-cis,12-cis-octadécadiènoique était la molécule responsable de la mort de *B. glabrata* dans une extrait de *G. frondosa*.

Finalement la comparaison d'extraits au DCM et au MeOH de fructification de *G. frondosa* par TLC et HPLC a montré une grande variation dans les profils de métabolites en fonction des techniques utilisées (congélation ou non, séchage à l'air ou lyophilisation) et de la souche, mais non de la composition du substrat sur lequel le champignon avait poussé. Cela tend à prouver qu'il faut être très attentif au traitement du matériel fongique afin d'obtenir des résultats stables.

En conclusion, les buts de ce travail ont été accomplis. Ce travail est encourageant pour continuer dans cette ligne de recherche. Nous pouvons espérer un jour trouver de nouveaux arômes ou composés actifs étant donné le nombre d'espèces qui pourraient être testées. Il serait particulièrement intéressant de se focaliser sur des composés contre *T. viride* étant donné le manque de recherche actuellement réalisés sur ce sujet et le besoin urgent de nouveaux produits pour lutter contre ce pathogène.

ABBREVIATIONS

AM	Agar-Malt
BE	Biological efficiency
CC	Column Chromatography
CO_2	Carbon dioxide
DCM	dichloromethane
DMSO	dimethylsulfoxyde
DPPH	1,1-diphenyl-2-picrylhydrazyle radical
EtOAc	Ethyl Acetate
HPLC	High pressure liquid chromatography
H_2SO_4	Sulphuric acid
i.v.	Intravenous
MeOH	methanol
Lig	Ligroin
p.o.	Per oral
RF	retention factor
s.c.	Subcutaneous
TCM	traditional Chinese medicine
TLC	thin layer chromatography
UV	Ultra violet
$ZnSO_4$	Zinc sulfate

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1 CONTEXT AND AIMS OF THE PRESENT WORK

The mycology group in which this work was realised has evolved from the work on fungal lignocellulositic material degradation by primary saprophytic Basidiomycetes to the use of this knowledge for applications like pulp paper biobleaching, biodefibrillation and for the development of edible mushroom cultures. This work was planned to explore new potentials for cultivated mushrooms.

Mushrooms are attracting an ever-growing interest in scientific and industrial fields. They represent a wide pool of still unexplored and unexploited sources of new products like pharmaceuticals, flavourings, cosmetic components or nutraceuticals. Even if hundreds of works have been published on active metabolites found in mushrooms, the physiology of the species studied is often little known.

In this context, this work's objectives were wide:

- First, selected Basidiomycetes species should have their in vitro culture developed in view of obtaining well formed and contaminant-free fruit bodies (chapter 4.1). This section should allow a better understanding of factors influencing carpogenesis.
- As one of the major problems in the cultivation process is damage induced by the mycoparasite *Trichoderma viride,* it was planned to develop a method to select resistant strains (chapter 4.2). Not only could we have more success in further cultures, but it could also improve chances of finding active compounds, as resistance is known to be bound to the production of active compounds in some mushroom species.
- Fruit body extracts should be tested against different biological targets (chapter 4.3). A bioassay against *T. viride* should be developed to detect if resistance is due to active compounds or to overall competition ability. Thus, the importance of the collaboration with Professor Kurt Hostettmann of the Laboratory of Pharmacognosy and Phytochemestry of the University of Lausanne (of the University of Geneva at the time of redaction), Switzerland, specialised in the search for new active compounds in plants.
- Moreover, as mushrooms are very modulable organisms, we planned to prospect the factors influencing metabolites profile in the fruit body of a model species (chapter 4.4). Understanding this phenomenon could lead to enhancing active metabolites production in a given mushroom species in the future. Collaboration with the Laboratory of Organic Chemistry of the Universtity of Neuchâtel led by Professor Tabacchi was elaborated for chemical analysis.

2 INTRODUCTION

2.1 Mushroom culture

2.1.1 Culinary and medicinal mushrooms culture

Mushrooms have been used by man for thousands of years, not only for food, but also for medicinal purposes, as tools, tinder, dyes, perfumes, soap, decoration, magic (Thoen, 1982) . First intentional cultivation goes back to 600 AD for the edible and medicinal mushroom *Auricularia auricula-judae* (Chang and Miles, 2004). Historical records from that time up to 1991 mention that 38 species of mushroom were cultivated from which only 2 were not for alimentary use and 8 had been cultivated prior to the 20th century, moreover, 29 species have been cultivated only since 1950 (Chang and Mile, 2004). The increase of the number of cultivated species corresponds to the rapid augmentation of mushroom production in the world.

In Europe few species (for instance, *Agrocybe aegerita*) have been empirically cultivated since ancient time, 1st century A.D according to Plinus the ancient and to Dioscoride. The first precise description of culture was that of *Agaricus bisporus*, the famous button mushroom, by the French botanist Tournefort in 1707 (Delmas, 1989).

To mimic the conditions occuring in nature when mushrooms fruit was the first approach to ecological studies. However, since these beginnings, much has evolved in mushroom culture: from the first empirical trials to the now highly technical modern way of production, the knowledge of this field has grown. Strains selection, sprawn type, substrates, structures, methods for harvesting and marketing have contributed to the great increase of mushroom production, especially in the last decades. However next to the high technology used to produce certain species, others are still primitive. Culture techniques indeed, vary in function of the species, the country and its environmental politics. In a number of countries, primary mushrooms are cultivated on logs. An enormous quantity of trees is cut for this purpose, which is incompatible with a concept of sustainable development. Other types of culture are made of compost or lignocellulositic wastes using various materials such as sawdust, straw, cotton, rice or sugar cane wastes.

This culture improvement parallels the great increase of mushroom consumption in the world. Beside this gastronomic use, *Ganoderma lucidum*, *Schyzophyllum commune* or *Trametes versicolor* are examples of species appreciated for their medicinal properties. It is probable that in the next decades the quantity of mushrooms cultivated for medicinal use will largely increase due to the growing interest in the use of these organisms for conserving and improving health. Already we can observe ever more internet sites praising the almost miraculous properties of some mushrooms. Behind this popular and often exaggerating window on the therapeutic values of the fungi world there is a much more serious and laborious work done by scientists to find new activities, to confirm old uses, to study modes of action, toxicity and other aspects of their active molecules.

2.1.2 The problem of fruiting

If the nutritional requirements of mycelium are quite simple, the complexity of fruiting processes in Macromycetes limits the success of mushroom domestication. It is often difficult to induce culture to produce fruiting structures in vitro (Croan and Kim, 1997). Knowledge of these mechanisms are indeed limited. Mycologists have long recognised the importance of environmental factors in fungal reproduction (Croan and Kim, 1997; Croan and Highley, 1991; Leatham and Stahmann, 1987, Niederpruem, 1963), while some work on the influence of substrate composition has also been done. (Job and Schiff Giovannini, 2004, Shen and Royse, 2001; Kalberer 2000; Kirshoff, 1996; Royse, 1996; Sing and Verma, 1996; Bjurmann, 1984, 1988; Gold and Chang, 1979) and of genotype (Shen and Royse, 2002; Kirshoff, 1996) However, work on in vitro carpogenesis by some wood-decay Basidiomycetes demonstrated variability in the process (Croan and Kim, 1997; Croan and Highley, 1991; Bjurman, 1988; Gold and Chang, 1979).

2.1.3 Interest for new cultivated species

Industries are interested in new products; pharmaceuticals, cosmetics with antiradicalar activities, flavours or dietary supplements. Mushrooms represent an interesting source for new discoveries. Cultivating them allows an independence on seasonal fruiting and also to reproduce the culture of a given strain under standard trophic and environmental conditions. Pharmacological and industrial research often requires a steady metabolite profile which was shown to be influenced by strains and culture conditions (Scherba et al., 1999; Müller et al., 1994; Jang and Birmingham, 1993; Gallois et al., 1990; Nishitob et al., 1986; Adam et al., 1967), and even by damage to the fruit body (Pang et al. 1992; Pang and Sterner, 1991). If collected in the wild, it is influenced by the age of the carpophores, the time and place of harvest (Guillot and Konska, 1997). The influence of certain factors on the metabolite profiles will be studied in chapter 4.4 of the present work.

2.1.4 Mushroom culture in our laboratory

Our laboratory used its knowledge of wood degradation by fungi (Job, 2003; Job et al., 1996; Job et al., 1991; Job and Rjchenberg, 1988; Job and Wright, 1986) to develop the following edible mushrooms cultivation on lignocellulositic substrates: *Agrocybe aegerita, Fistulina hepatica, Flammulina velutipes, Ganoderma lucidum, Grifola frondosa, Hericium erinaceum, Hypsizigus marmoreus, Kuehneromyces mutabilis, Lentinus edodes, Lyophillum decastes, Lyophillum ulmarium, Pholiota nameko, Pleurotus citrinopileatus, Pleurotus eous, Pleurotus eryngii, Pleurotus ostreatus*. This allowed increased knowledge on the fruiting process of primary saprophytic mushrooms. Culture development was and still is possible, partly because of the participation of an industrial partner in the realisation of larger scale experiments: Fermenta SA in Payerne, Switzerland.

In the present work, it is planned to increase the number of cultivated species that are not chosen for their organoleptic qualities, mainly primary saprophytic Aphyllophorales. This should furnish material for activity screening, and also lead to a better understanding of nutritional and environmental factors influencing carpogenesis.

2.2 Selection of strains resistant to Trichoderma viride

2.2.1 The problem of *Trichoderma* spp. in mushroom cultures

In the world of mushroom culture, many pests and diseases can affect production, problems depending on the species, the country and the infrastructures. Among the pathogens in some cultures, hyphomycetous fungi including *Trichoderma* are most common (Goltapeh and Danesh, 2000; Komatsu, 1976).

In our laboratory, the worst mushroom antagonist encountered during culture development research is a fungus of the genus *Trichoderma* that was determined as *Trichoderma viride* by optic microscope. In our cultures this fungus is very virulent and is capable of attacking and killing the mycelium of certain cultured species in just a few days. It generally appears as a white patch on the substrate then rapidly changes to a green color (conidia formation) together with a rapid extension of the colony. *Trichoderma viride* is indeed considered as one of the most virulent competitors in certain mushroom cultures (Delmas, 1989). It can appear on improperly prepared substrates and can even resist certain types of sterilisation (Delmas, 1989).

To our point of view, aptitude to competition is therefore a parameter to select in screening new strains to be cultivated. Resistant strains would be more productive during culture and furnish a better chance to find antibiotic substances like those demonstrated in the resistant strains of *Lentinus edodes* (Shiitake) (Tokimoto and Komatsu, 1995). Indeed, secondary metabolites might act as chemical weapons to defend a substrate against competitors (Sterner et al., 1985) and also bring interesting new lead compounds for further studies on antimycotic agents. It is particularly important in the case of the genus *Trichoderma* as there are only two existing fungicides against this pathogen in mushroom culture, Benlate® and Thiophanate-methyl (Senator 70WP) and the first is no longer available.

2.2.2 The genus *Trichoderma*

The genus *Trichoderma* belongs to the family Moniliaceae, order Moniliales, class Hyphomycetes, subdivision Deuteromycotina. This genus is one of the most ubiquitous of soil fungi and has been extensively studied because of its' antagonism and parasitism of other fungi.

The morphology of *Trichoderma* spp. is complex and unstable; historically the taxonomy of this genus has been confused and is still not clarified. The perfect forms of *Trichoderma* (imperfect form) are generally attached to the Hypocreales, but occasionally to the Eurotiales, Clavicipales or Sphaeriales. A given species of Hypocrea could have several *Trichoderma* anamorphs and on the contrary, a same "group of species" of *Trichoderma* can be attached to different Hypocrea species.

2.2.3 Virulence factors of *Trichoderma* spp.

Trichoderma spp. mycoparasitism is a complex process. First it's growth is directed toward the host hyphae, probably by chemotropism, at host contact it coils around the hyphae and penetrates the cellular wall by secreting lytic enzymes, principally chitinases and β-1-3-glucanases but also cellulases and protease (Mumpuni et al., 1997).

Trichoderma spp. have shown to produce more than 70 inhibitory substances including antibiotics (Sivasithamparam and Ghisalberti, 1998) with antimicrobial and antifungal activity: i.e. trichodermin (Godfredsen and Vangedal, 1965), Suzukacillin (Ooka et al. 1966), alamethicine (Meyer and Reusser, 1967), Dermadine (=U-21,963) (Pyke and Diets, 1966), Trichopolyns I and II produced by *T. polysporum* and which inhibit the respiration and growth of *L. edodes* (Tokimoto 1985). The specific chemical nature and presumed mode of action of the antibiotics render it difficult for antagonists to resist *Trichoderma* (Tang et al., 2001). Greater antibiotic production was observed on rich media (Wright, 1952) showing the importance of trophic factors.

Trichoderma spp. have fast growth rates, high productive capacities and produce a range of polysaccharidases allowing the use of the major nutritive resources (Savoie et al., 2000) which gives this species a strong competitive capacity.

This virulence of *Trichoderma* spp. makes this genus an important one among bio control agents in plant disease management and has even been modified to improve their efficacy by mutation, protoplast fusion and genetic manipulations (Baker, 1991; Lewis et al., 1991). However, they also seem to become more aggressive toward cultured mushrooms.

2.2.4 Resistance of cultured mushrooms to *Trichoderma* spp.

Mechanisms involved during competition between two fungi can be the competition for nutrients, chemical interference by production of antibiotics and extra cellular enzymes (Savoie et al., 2000) or mycoparasitism (Elad et al., 1982).

Experiments in resistance enhancement were mainly realised on widely cultivated mushrooms like *Lentinus edodes* or *Agaricus bisporus* by strain selection and medium formulation (Savoie et al., 2000; Goltapeh and Danesh, 2000; Mata et al., 1998; Tokimoto et al., 1994; Badham, 1991; Tokimoto and Komatsu, 1979).

Fast growing mushroom strains are important to compete *Trichoderma* spp. as this genus has a fast invasion rate with good capacities for nutrient use, together with a high reproductive rate. In the antagonism between *Lentinula edodes* and *Trichoderma* spp. for instance, it is in the first days that the competition takes place (Savoie et al., 2000)

In a study to determine the nature of the interaction between *Trichoderma* species and *Agaricus bisporus* mycelium, it was demonstrated that only a change of concentration in a MEA medium could influence the interaction (Goltapeh and Danesh, 2000). Badham (1991) also noticed that variations in the medium formulation and treatment could influence resistance between *L. edodes* and *T. herzianum*. In vitro studies have shown that C rich medium tends to favorise *L. edodes* while N rich medium favourites *Trichoderma* spp. (Tokimoto and Komatsu, 1979).

Resistance mechanisms to *Trichoderma* spp were studied in detail with *L. edodes*. The rejection of *Trichoderma* by *L. edodes* coincided with a brownish pigmentation (formed by enzymatic oxidation of polyphenols) of the mycelium at the contact zone (Tokimoto, 1980). According to Savoie et al. (2000), when a brown line was formed in the cultivation of *L. edodes* on wheat straw, the progression of *Trichoderma* spp was dependent of the area size colonized by *L. edodes*. The authors thus postulate that the

chemical and physical barrier constituted by the polymerized phenolic compounds in the brown line is only temporarily effective, and that the antibiosis mechanisms dependent of the level of biomass present are then important in the chemical interference between the two antagonists. Indeed Tokimoto (1985) had previously shown that the antifungal fraction of *Lentinus* culture obtained before *Trichoderma* attack differed slightly from the fraction obtained after *Trichoderma* attack. In addition, toxicity of the antifungal fraction after *Trichoderma* attack was higher than before the attack. At least six antifungal substances were detected in the antifungal fraction prepared after contact between *T. polysporum* and *L. edodes*, but only three or four before, with variations in quantity in function of carbon sources (Tokimoto et al., 1987).

The *in vitro* strains and substrate formulation selections were realised whether in Petri plates (Goltapeh and Danesh, 2000; Tokimoto and Komatsu, 1995; Tokimoto, 1980), in glass tubes (Tokimoto and Komatsu, 1995), in drinking straw (Badham, 1991) or directly in culture bags (Tokimoto and Komatsu, 1995).

Agressivity was estimated in different manners: for instance Savoie et al. (2000) estimated the level of *Trichoderma* contamination of the surface covered by mold 15 days after inoculation. Badham (1991) indicated competition by the distance between the contact line of the two antagonistic fungi and the front mycelium of *L. edodes* (mm/day).Goltapeh and Danesh. (2000) compared the growth of the host mycelium with a control.

2.2.5 Developing a new selection method

To our point or view, competition aptitude should be a parameter to select in addition to fruiting capacity when screening for new strains to be cultivated. Thus we have chosen two species -*Grifola frondosa* and *Fomitopsis pinicola*- to evaluate different strains' ability to colonise the substrates and to compete with the damaging pest *T. viride.* These two species were chosen because in both cases mycelium is sensitive to *Trichoderma viride* in some conditions. The tests should be realized in Teflon® tubes on different substrates varying by their C/N coefficients in order to select fast and resistant strains in a relatively short time.

2.3 Active metabolites in mushrooms

2.3.1 Interest for mushrooms in the search of new active metabolites

Widespread infectious diseases have marked humanity throughout history. In 1928, Alexander Fleming discovered an antibiotic substance later known as penicillin produced by a fungus. This was a real revolution in medical sciences. After that discovery, an entire family of penicillin-based antibiotics was born. Later antimicrobials discoveries include streptomycin against tuberculosis, tetracycline, quinolones, antifungals, antiparasitics and more recently, antivirals (e.g. acyclovir). Nowadays more than 150 compounds compose the arsenal of antimicrobial weapons. Thus, since the discovery of the antibiotics, they have totally transformed the approach to infectious diseases. But another problem arises: antibiotic resistance. Now there is multi-drug resistant tuberculosis, penicillin-resistant pneumococci, resistant malaria (1.1 millons death in 1998), resistant strains of gonorrhoea, dysentery-causing shigella (2.2 millions

death in 1998) and salmonella. Unfortunately, since 1970, no new class of antibacterials has been developed to fight infectious diseases (WHO, 2000). Thus it is essential to find new compounds to fight ever-evolving resistant microbes.

Another important compound discovered in a fungus is cyclosporine (ciclosporin) in 1971. It was isolated from the fungus *Hypocladium inflatum gams (Tolypocladium inflatum)*. J. F. Borel discovered its immunosuppressive activity in 1976. However Stähelin refutes the commonly held view that Borel discovered its activity. It was approved for use in 1983 to reduce risk of organ rejection after transplantation (Upton, 2001).

In our days it is always more difficult to find new pharmaceutical or agrochemical products by random synthesis. Screening for biological activities has made progress by exploring the (almost) unlimited richness of natural products (Anke, 1989). Among the sources of potentially bioactive metabolites, some organisms like Basidiomycetes have been strongly under-investigated. They give excellent promises for novel structures and biological activities (Anke, 1989). A pioneer work has been done by Anchel, Hervey, Wilkins et al. who have tested fruit bodies or mycelium of about 2000 species of the approximately 30'000 known, discovering several antibiotics. Since then an enormous amount of work has been done (see paragraph 2.5). Today it has become of great interest to the scientific world, not only to fight microbes but also against cancer, viruses and other causes of disease. The research in the phytosanitary field however is sparse. It would be very useful to dispose of new products to fight pathogenic fungi of plants or mushroom cultures.

Our laboratory aims to work only on cultivated mushrooms, thus restricting selected organisms to saprophytic mushrooms. Among them, we chose some species which were used traditionally in folk medicine on different continents. Knowledge to enhance health was slowly developed by communities over the centuries by the observation of the effects of the use of some mushrooms, thus increasing our possibility of finding an active molecule.

2.3.2 Therapeutic effects of Basidiomycetes

Higher Badisidiomycetes mushrooms have been used in Folk medicine throughout the world since ancient time (Wasser and Weis, 1999). Far from being an outdated curiosity, medicinal mushrooms earn ever growing popularity, stimulated by a high number of scientific studies. The discovery of antibiotics has convinced the public that fungi could produce particularly active substances and that the ancient traditions could have a part of truth. The paragraphs below only gives a rapid overview of the kind of activities found in mushrooms, as for each mushroom species studied in this work, a literature review is presented in paragraph 2.5.

2.3.2.1 Antitumoral

One of the key results of laboratory and human clinical studies on mushrooms concerns a number of mushroom compounds capable of modifying the biological response of the host by stimulation of the immune system provoking different therapeutic effects like inhibition of tumour growth and relief in immunodeficiant diseases such as aids. Japanese searchers in the 1970's, at a French convention, presented a new type of

antitumoral molecule found in a mushroom. This compound, a polysaccharide, does not act on the tumour by direct cytotoxicity like already known substances but acts by stimulation of the immune system (Takashi, 1995). Since then several other substances have been discovered. Several immunotherapeutic drugs have been commercialised in Japan, among them: PSK (Krestin) extracted from *Trametes versicolor* mycelium marketed in 1977 to treat digestive organ, lung and breast cancer. About 10 years later was commercialised a similar product, PSP. Lentinan from the *Lentinus edodes* fruit body commercialised in 1985 for stomach cancer, SPG (Schizophyllan) from *Schizophyllum commune* medium put on the market in 1986 against cervical cancer. In 1987 they represented 25,2 % of total anticancer agent sales in Japan (Mizuno, 1995). In Russia Befungin produced out of *Inonotus obliquus* fruit bodies and in the USA *Agaricus blazei* fruit bodies extracts were also commercialised (Wasser and Weis, 1999). This type of substance belongs to the biological therapies, fourth classic type of cancer treatment after surgery, chemotherapy and radiotherapy. They are not miracle drugs, and are more often used in combination with other classical treatments (Mizuno, 1995). Hundreds of Basidiomycetes extracts also showed antitumoral activity on mice or rats.

In addition, a number of high-molecular weight antitumor components were isolated from medicinal mushrooms, including heteroglycans, chitinous substances, peptidoglycan, proteoglycans, lectins, RNA components, dietary fibbers, and/or indegestible polysaccharide (Wasser and Weis, 1999). Low-molecular-weight substances such as terpenoids, steroids, novel gamma-pyrones, and novel phenols were also isolated (Wasser and Weis, 1999). *Ganodema lucidum, Inonotus obliquus, Trametes versicolor* are examples of species presenting low-molecular-weight substances presenting cytostatic activity (Lindequist et al., 1990).

2.3.2.2 Hypocholesterolemic

The major cause of mortality in western countries is cardiovascular disease. Relations between a high blood cholesterol level and certain diseases are known. One of the preventive measures against these diseases is to reduce the cholesterol level in the blood. This can be done by diet or by a therapeutical agent, like lovastatin (mevinoline) approved in 1987 and its derivates. Among Basidiomycetes, *Pleurotus ostreatus* has shown the highest lovastatine content. Other species are active by lowering blood cholesterol i.e. *Pleurotus cornuciopiae, P. eryngii, P. sapidus*, *Lentinus edodes, Polyporus confluens, Auricularia polytricha, A. auricula-judae, Flammulina velutipes* and *Ganoderma lucidum* (Wasser and Weis, 1999).

2.3.2.3 Anti-inflammatory effects

More than half of the people over 65 years old suffer from arthritis. Drugs on the market (aspirin, steroids) are of limited efficiency, because of potential secondary effects. In reason of the long traditional use of *Ganoderma lucidum* in Asia for rheumatism and stiff articulations, several studies have been done on this mushroom seeking a non steroidic drug. Particularly, oral administration had an activity similar to hydrocortisone after 6 hours, even better after 24 hours, but without the side effects. The possibility seems to be very promising on developing an anti-inflammatory drug from *Ganoderma* extracts. Several antitumoral β-D-glucans have also shown a non-specific activity for immune system stimulation. We also find heterogalactan-protein complexes with anti-

inflammatory and anti-allergic functions in *Ganoderma lucidum*. According to Wasser and Weis (1999) anti-inflammatory effects can be found in other mushroom products from *Tremella fuciformis, Schizophillum commune, Inonotus obliquus, Lentinus edodes, Flammulina velutipes, Marasmius androsaceus*.

2.3.2.4 Antibacterial

Fungi are well known for their important antibiotic production of penicillin; however antibiotic presence in the fruit bodies themselves has been far less studied and documented. Many, possibly all fungi are producing antibacterial substances to protect themselves in an overpopulated substrate where they grow. The pharmaceutical industry has been slow to explore mushrooms for antibiotic activity—until recently. We know now that more than 75% of Polypore mushroom species thus far surveyed show strong antimicrobial activity, and that 45% of 204 mushroom species analyzed—polypores and gilled mushrooms alike—showed activity (Suay et al., 2000). This higher concentration of effective antibiotics from Polypore mushrooms underscores that this barely explored group, in particular those with a long history of folkloric use by indigenous peoples, should be carefully surveyed (Atsumi, S., et al., 1990; Hirasawa et al., 1999). In Basidiomycetes the active compounds are mostly polyacetylenes, but also phenolic compounds, purines and pyrimidines, quinones and terpenoids (Delmas, 1989). Examples of known antibiotic producers among Basidiomycetes are: *Schizophyllum commune* against *Pseudomonas aeruginosa*, *Staphyllococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*, *Armillaria mellea* against *S. aureus*, *Bacillus cereus* and *B. subtilis*, *Dendropollyporus umbellatus* against *S. aureus* and *E. coli* (Wasser and Weis, 1999). All these activities were tested in-vitro or in animals but are not in human clinical studies.

2.3.2.5 Antifungal

Ecologically the production of antifungal substances is almost surely due to the constant need of the organism to compete against other fungi. There are indeed a lot of observations of aggressive action of one species towards another. It was in 1923 that Falck first recognised such an activity in *Sparassis crispa* (Delmas, 1989). Oudemansin has been isolated from mycelial cultures of *Oudemansiella mucida.* It exhibits strong antifungal properties and inhibits respiration in fungi, cells of the ascitic form of EHRLICH carcinoma, and rat liver mitochondria (Anke et al., 1979). Sterner et al. (1985) showed that sesquiterpenes isolated from *Lactarius vellereus* were active against *Candida utilis*.

2.3.2.6 Antiviral

Diverse basidiomycetous substances are effective against viral infections including AIDS. These are mostly substances which mobilise the body's humoral defences (Wasser and Weis, 1999). For instance, Lentinane extracted from *Lentinus edodes* suppressed the surface expression of human immunodeficiency virus on T cells when dosed in combination with aydothymidine (Tochikura et al., 1987). It was also active against vesicular stomatitis virus in mice, encephalitis virus, Abelson virus and adenovirus type 12. Other mushrooms like G*rifola frondosa, Trametes versicolor,*

Ganoderma lucidum have antiviral effects too (Wasser and Weis, 1999). Certain activities are effective *in vitro*, thus not implicating immunity mechanisms. For example, *Lentinus edodes* mycelium extract is able to inhibit the herpes virus *in vitro*.

2.3.2.7 Hypoglycemic

Diabetes is a disease due to the lack of regulation of the blood sugar level due to the malfunction or non function of specialised pancreatic cells. Certain mushrooms could have a positive effect on early stages of type 2 diabetes. For instance, ganoderans isolated from *Ganoderma lucidum* showed hypoglycemic activity in mice (Hikino et al., 1989). Two polysaccharides isolated from *Agrocybe cylindracea* were also active in normal and streptomicin-induced diabetic mice by i.p. administration (Kiho et al., 1994). According to Lo et al. (2005) *Tremella mesenterica* might be developed as a potential oral hypoglycaemic agent or functional food for diabetic patients and for persons with high risk for diabetes mellitus. *Lentinus edodes* and *Calocybe georgii* are two other examples of mushrooms showing hypoglycaemic activity.

2.3.2.8 Hypotensive

Certain compounds can maintain blood pressure at a normal level. Kabir et al. (1988) showed the effect on blood pressure of a powder prepared from *Ganoderma lucidum* fruit bodies on hypertensive rats. *Grifola frondosa, Lentinus edodes, Auricula auricula-judae* and *Armillaria mellea* are other mushrooms lowering blood pressure (Wasser and Weis, 1999).

2.3.2.9 Miscellanous activities

Other kinds of activities can be found in Basidiomycetes, i.e. cardiovascular (i.e. *Lentinus edodes, Auricularia auricula-judae, Armillaria mellea, Grifola frondosa, Volvariella volvacea*), antiallergic (i.e. *Agaricus campestris*), stimulation of NGF (i.e. *Hericium erinaceum*), hepatoprotective (i.e. *Schizophyllum commune, Dendropolyporus umbellatus, Trametes versicolor, Ganoderma lucidum, Lentinus edodes*).

2.3.3 Perspective for the future

A large view clearly shows that the higher fungi produce quantity of interesting pharmacological substances. If we compare the number of mushrooms studied with those of unknown chemistry and pharmacological effects, we realise that we have recognised only a tiny fraction of potentially active compounds elaborated by mushrooms. Look at *G. lucidum* (see below paragraph 2.5.11), it shows that one species can contain many different active compounds. Moreover, certain secondary active compounds can be linked to strain or culture conditions.

Scientists working on these organisms can hope to discover in future screenings a great number of new active compounds. Of all the active compounds studied *in vitro* or *in vivo* on animals it is clear that only a small part will correspond to needs of allopathic medicine; defined chemical composition for precise dosage, toxicology, pharmacodynamic and clinical studies. Moreover the production of the compound is important: the molecule should be synthesizable or there must be sufficient raw material to extract the active molecules. These molecules, if we know the chemical group

responsible for the biological activity, could be lead compounds for new molecule synthesis.

There is also a high potential for neutraceuticals, for which there is no need to know the active principle. An endless list of mushroom products is sold on the market with a multitude of claimed properties which are far from proved for some. The fashion is actually to return to nature including in food and remedies, and many people lack a critical sense toward so called 'natural' products. Thus, it could be important to develop these natural products but with scientifically proven effects.

Our laboratory is not equipped with the material or skilled for a large scale screening program. If the results of this work are encouraging, further collaborations would be necessary. However, we plan to develop a bioassay against the mycoparasite *Trichoderma viride* as there is an important need for a new antifungal compound.

2.3.4 Techniques for substances searches

The Basidiomycetes and fungi in general represent a wide pool of new molecules. In each species there can be hundreds of metabolites. Thus, as described by Hostettmann et al. (1997), the test system should be ideally simple, rapid, reproducible and inexpensive. Moreover the bioassays must be sensitive enough to detect principles present at low concentration in the crude extracts. Bioautographic assays can be considered as the most efficient way to detect antimicrobial compounds, because it allows localisation of the activity and thus a target-directed isolation of the active compound (Rahalison et al., 1991). Bioautobiography can be direct when microorganisms grow directly on the TLC plate, of contact when the compound is transferred from TLC plate to inoculated agar plates or agar overlay or immersion bioautography when the inoculated agar medium is applied on the TLC plate (Rahalison and al., 1991). Animal target organisms can not be replaced by such a technique.

2.3.5 Target organisms and compounds

2.3.5.1 Bacillus subtilis

B. subtilis is a Gram + bacteria, non pathogenic to humans, which presents no risk for the manipulator. A bacterium of the same genus, *B. anthracis* is responsible for anthrax disease, characterised by oedema, haemorrhage and tissular necroses. It is frequent in some animals and is sometimes transmitted to those manipulating it. It is also used as a biological agent in wars or in bioterrorism. Hopefully, if an extract is active against *B. subtilis* it could be active against *B. anthracis* as well, and eventually against other pathogenic Gram + bacteria like Staphylococci or Streptococci.

2.3.5.2 Escherichia coli

E.coli a Gram – bacteria, lives in our gastro-intestinal tracts without provoking harm. It can however, induce severe infections - sometimes deadly - to the patient with a depressed immune system (manuel Merck, 1988). Gram – bacteria cause much more problems than Gram + due to their different cell walls, the antibiotics of the penicillin and cephalosporin groups act on the synthesis of it. It is why the search for new antibiotic types is important for that group of organisms.

2.3.5.3 Candida albicans

C. albicans is a Deuteromycotina or imperfect fungi, a yeast that can also form mycelium. It is part of a normal flora of humans, particularly in the mouth and the vagina. In a depressed immune system (AIDS, chemotherapy, malnutrition, bad hygiene) or after long antibiotic cure, it can induce infections called candidoses. Different species of *Candida* provoke them, but *C. albicans* is the most ubiquitous one. It can affect almost all tissues, causing diseases going from a simple thrush in new-born babies to a fatal multi-organs infection. The main form of candidosis are mucous (muceo-cutaneous), cutaneous or systemic. *C. albicans* has been chosen because it is non pathogenic to the manipulator in normal conditions and it is an interesting target for research into new antifungal substances.

2.3.5.4 Cladosporium cucumerinum

C. cucumerinum is a filementous fungus of the Deuteromycotina subdivision. It is a pathogen of the Cucurbitaceae family. It is responsible for the cucumber cladosporiosis, but other species of the same genus are pathogenic to humans, causing for instance, chromoblastomycosis. The discovery of an active compound against this pathogen would be interesting, not only in agriculture but also in medicine. Possibly, such a compound could also be active against other filamentous fungi genus.

2.3.5.5 Trichoderma viride

Like largely discussed previously, *Trichoderma* spp represents a great problem in certain mushroom cultures provoking high losses. Moreover, except for the time consuming strain and culture condition selection, there are almost no available products to fight this pest. Thus we were particularly interested in developing an autobiographic assay to detect active compounds in Basidiomycetes that could be applied to fruit body extracts and to resistant mycelium selected by the method we plan to develop.

2.3.5.6 Aedes aegyptii

Yellow fever is, like malaria, leishmaniosis and tripanosomiasis, an infectious disease of tropical countries inoculated by mosquitoes. They are responsible for high annual mortality. *A. aegyptii* is the carrier of the pathogenic agent of yellow fever which touches South America and Africa in the regions of Equator (from 15° north latitude to 15° south latitude). Symptoms are strong fever with headaches and articular pain, haemorrhages and yellowing of the skin. It can bring to death by liver, kidneys and circulatory system failure. The only efficient measure against this disease is vaccination which offers protection for 10 years. Eradication of the disease passes by the elimination of the transmission agent in this case, mosquitoes larvae. Their elimination is, therefore essential to a permanent cure. The female mosquito transmits the pathogenic agent to a part of its eggs. Its' descendants are directly infectious without having to bite a sick person. Larvae live in stagnant water thus limiting zones to treat.

2.3.5.7 Biomphalaria glabrata

Schistosomiasis or bilharziose is a visceral parasitosis provoked by trematodes of the genus *Schistosomia*. This disease touches millions of people in intertropical regions of

the world. Only in sub-Saharan Africa it afflicts 200 millions people. Infection leads to the weakening of the body's defence system through blood-loss, malnutrition, and tissue and organ damage. Systemic parasitic colonisation predisposes individuals to other diseases and eventually to death from kidney or liver failure (WHO, 2000). Infection is from contact with water contaminated by cercaires which pass actively through the skin. The cercaires transform into adult worms which migrate to the bladder or the intestines in function of the parasitic species. Adults then lay eggs, which in turn become miracidies in need of an intermediate host to transform into cercaires capable of again infecting man. Intermediate hosts are different species of fresh water molluscs, *B. glabrata* being one of them. The only long term way to wipe out this disease is to eliminate the intermediate host and at the same time the parasite in the local population.

2.3.5.8 DPPH

Oxidation is one of the major causes of food and material degradation. It has been demonstrated that some molecules, in particular free radicals, react to oxygen, and are implicated in different diseases such as cancer and atherosclerosis, major public health problems in industrialised countries. Ageing could also be the result of reactions with free radicals occurring in our cells and tissues. Natural antioxidants could be an alternative to the use of synthetic substances in food and pharmaceutical technology or serve as lead compounds to develop new drugs to ameliorate the treatment of different diseases. That is why they are the object of growing interest (Cuendet, et al., 1997). The 1,1-diphenyl-2-picrylhydrazyle (DPPH) is a purple stable radical. If a compound captures this radical, it becomes yellow. This substance is antiradicalar or a free radical captor. It is hoped that it could also have the same properties against toxic free radicals in our cells. In this test it is a mode of action and not an activity which is tested, thus an antiradicalar in not necessarily an antioxidant, which is tested with β-caroten.

2.4 Plasticity of Grifola frondosa

2.4.1 Plasticity in mushrooms

Mushrooms seem to have certain plasticity. It might be interesting to use this potential not only to improve the smell, colour or conservation time of edible fungus, but also to induce or enhance production of potentially interesting substances such as active molecules. For instance, parapharmaceutical industries could be interested for producing mushroom extracts with higher active compounds content (i.e. anti-radicalar) or oligoelement rich extracts (this last subject is actually being studied by Gilles Farron in the course of his PhD thesis in our laboratory). Several factors are known to influence mushroom physiology and particularly metabolites profiles: substrate composition, culture conditions, strains genomes and applied treatment.

2.4.1.1 Influence of substrate composition

The type and quality of nutrient supplement has shown to modify *G. frondosa* morphology and yield (Shen and Royse, 2001). Substrate composition influences antagonism between Shiitake and *Trichoderma* spp. by allowing or not the production of a brown pigment at the colonies' contact zone (Tokimoto and Komatsu, 1995). Medium composition also influences fungi fatty acids (Müller et al., 1994), fungal

odorous profile (Jang and Birmingham, 1993), and flavour compounds (Gallois et al., 1990). In all the species of the genus *Pezicula* (Ascomycetes) melleine was or was not synthesized according to medium composition (Schulz et al., 1995).

2.4.1.2 Influence of culture conditions

Growth conditions can affect content and proportions of fatty acids (Szymczak, 1978a, 1978b, 1983), the production of volatiles (Jang and Birmingham, 1993). PH level and oxygen level modification could also provoke different substances produced in isolates in the Ascomycetes genus *Pezicula* (Schulz et al., 1995).

2.4.1.3 Influence of the strain

Genetic variation of the strains influenced production of volatiles (Jang and Birmingham, 1993) and triterpenes production (Nishitoba et al., 1986).

2.4.1.4 Influence of treatment applied to the collected fruit bodies

Little is known about the influence of different factors on metabolites degradation after the fruit bodies' harvest: boiling and storage has been shown to have an influence in *G. frondosa* (Takama et al., 1981), while other Basidiomycetes species metabolite profiles were shown to be influenced by damage to the fruit body (Pang et al., 1992; Pang and Sterner, 1991; Holzapfel and al., 1989, Sterner et al., 1985, Hilbig et al., 1985). Technique could also be the cause of disappearance of molecules or appearance by degradation or transformation of present molecules. For instance sesquiterpenes obtained were different both qualitatively and quantitatively when different extraction procedures were used (Sterner et al., 1985). Thus isolated or active compounds could well be artefacts.

2.4.2 The model organism: *Grifola frondosa*

Grifola frondosa was chosen as model organism to compare the metabolites profiles and some pharmacological activities in regards to strain, culture conditions, substrate composition and technical treatment. It was chosen because it is a prized edible Basidiomycete mushroom and the subject of numerous scientific studies on its' medicinal potential. It is also widely used as a health food in the form of tea, ground mushrooms, hot water extract powders, granules, drinks and tablets (Mizuno, 1999; Royse, 1997). Moreover this species is cultivated (Job and Schiff Giovannini, 2004; Stamets, 2000), but the cultivation conditions may change the chemical composition of this mushroom (Scherba et al., 1999). This could have an influence on the biological activities research and on commercialised medicinal products made with this species. Very few scientific studies, however, have been published concerning the physiology of this species (Shen and Royse, 2001; Mayuzumi and Mizuno, 1997; Kirchhoff, 1996; Stamets, 1993; Kunimoto, 1992).

2.5 Previous work on the studied species

All the species used during this work, for culture development, selection test, biological test and /or modulation of compounds are presented below. A brief description and the

review of the literature on common names, origin, general ecology, experimental or industrial culture in the world, uses in popular medicines, pharmacological studies and clinical studies are given. The review of pharmacological literature was mainly focused on the activities found in the fruit bodies; however some work on the mycelium was also reported especially in the case of their particular interest.

2.5.1 *Agaricus brasiliensis* S. Wasser et al.

Anciant name
The taxonomy of this mushroom has been confused until recently. It is now not known as *Agaricus blazei* anymore, but as a novel species.

Common names
Japanese: Himematsutake, kawariharatake. Locally called "Cogumelo de Deus" or "Mushroom of God" in Brazil.

Origin
Brazil

General ecology
It is a secondary saprophyte which in natural conditions is said to grow in specific soil conditions, with nocturnal temperatures of 20-25°, diurnal of 35° and regular rain in the evenings. Its main habitat is in the mountainous district of Piedade in Sao Paulo (Brazil) but it was also found a few times in Florida and South Carolina.

Culture in the world
First studies on the culture of this species were done at the Iwade Mushroom Institute in Japan and were attempted for commercialisation in 1978 (Mizuno, 1995). Iwade and Mizuno (1997) describe this culture on compost with added chemical and organic fertilisers at a pH of 6.5-6.8. When the hyphae have sufficiently invaded the substrate a soil covering at close to pH 7.0 is added. According to the authors it takes 40 to 50 days to obtain fruit bodies after inoculation of spawn whose development is achieved in about 10 days. Another Japanese publication mentions *A. blazei* culture development for harvesting fruit bodies with stable constituents for fundamental medical scientific researches (Higaki *et al.*, 1997, from English abstract). Dudukh et al. (2004) have published a book containing information about its cultivation.

Uses in traditional medicine
This mushroom was traditionally used against cancer, diabetes, hyperlipidemia, hypertension, atopic dermatitis, arteriosclerosis and chronic hepatitis (Takaku *et al.*, 2001; Higaki *et al.*, 1997 from English abstract). Some researchers noticed, a few decades ago, that the occurrence rate of certain adult diseases was extremely low in a mountain region (Ideate) near Sao Paulo, Brazil. They began studying *A. blazei* which was consumed regularly. Results showed its immune enhancing effect on the body in animal studies like described below.

Pharmacology
Antimutagenic activities have only been demonstrated in Salmonella and Chinese hamster V79 cells. Linoleic acid was isolated as a main substance having antimutagenic activity on Salmonella in *A. blazei* fruit body (Osaki *et al.*, 1994), while only mushroom

teas prepared from a 2,5% aqueous solution was used to indicate an antimutagenic activity when tested in V79 cells (Menoli *et al.*, 2001). A strong inhibition on VERO cells of cytopathic effects induced by western equine encephalitis virus was observer in the mycelial water extracts but not those of fruiting bodies (Sorimachi *et al.*, 2001). A fruiting body fraction comprised of protein and α (1-6)-β-D-glucan showed an antitumor activity by immune system enhancement in mice (Itoh *et al.*, 1994, Kawagishi *et al.*, 1989). Mizuno *et al.* (1998) have also proved a stimulation of lymphocytes by a hot water soluble fraction administered orally to mice. Fujimiya *et al.* (1998) have isolated a tumoricidal fraction consisting in more than 90% glucose, the main component being –1-4)α-D-glucan with (1-6)-β-branching in the ratio of approximately 4:1. This acid-treated fraction resulted in infiltration of the distant tumor (double-grafted tumor system) by natural killer cells with marked tumoricidal activity in Balb/c mice. Ergosterol was isolated as being the agent reducing significantly tumor growth in sarcom 180-bearing mice (Takaku *et al.*, 2001). The authors suggest that ergosterol or its metabolites may be involved in the inhibition of tumor-induced neovascularization. This is the first report of ergosterol as an antiangiogenic substance. A bactericidal substance was isolated and identified as 13-hydroxy cis-9, trans-11-octadecadienoic acid (13ZE-LOH) (Osaki *et al.*, 1994, from English abstract)

2.5.2 *Agrocybe aegerita* (Brig.) Sing.

Common names
French: Agrocybe du peuplier, pholiote du peuplier, pivoulade, albarelle, aloumère, aubadero, bolet de Salzé, champignon de saule. English: black poplar mushroom, the sword belt Agrocybe, south Poplar. Japanese: yangimatsutake. Chinese: Zhuzhuang-Tiantougou.

Origin
Mainly southern Europe and North America (Stamets, 1993), but also found in India (Kaul, 1981).

General ecology
Saprophyte growing on dead wood of diverse broad-leaved trees: poplar; willow, elder, maple, ash, elm. It needs a temperate and humid climate and fructifies from April to October in natural conditions.

Culture in the world
Agrocybe aegerita was probably the first mushroom cultivated in Europe. The method was empirical but it is still used today: poplar disks are rubbed with fresh carpophores lamellae and covered with some compost. They are buried in the ground in humid and warm atmosphere and watered to keep the wood humid. Cailleux has developed a culture method on organic wastes substrate. It is known to be cultivated on oak and alder sawdust/chips with a yield of about 16 to 20% (Stamets, 1993). It is one of the mushroom species of which the culture has been developed in our laboratory.

Uses in traditional medicine
In certain regions of China it is used as a diuretic and to stop diarrhoea (Hobbs, 1996).

Pharmacology
An antitumoral activity has been reported. It is due to a type of linear (1→3)-α-D-glucan, which show an activity against sarcoma 180 in mice (Yoshida *et al.*, 1996). Two hypoglycaemic polysaccharides have been isolated and characterised from a hot water extract of carpophores. It concerns AG-HN1 (a high molecular weight glucan) and AG-HN2 (a low molecular weight heteroglucan). These two substances have shown a hypoglycaemic activity both in normal and streptozotocin induced diabetic mice by intraperitoneal injection (Kiho *et al.*, 1994). Two substances extracted from a MeOH extract have shown an in vitro inhibition of lipid peroxidation in rat liver microsomes. This antiradicalar activity was dependant of the dose (Kim *et al.*, 1997).

2.5.3 *Dendropolyporus umbellatus* (Pers.:Fr.) Jül.

Common names
French: Polypore en ombelle. Chinese: Zhu ling or Chuling.

Origin
Europe, North America, Asia.

General ecology
On the ground in broad-leaved trees forests near *Quercus* and *Fagus*, but also *Carpinus*, *Populus* and *Acer*, often near tree stumps (Breitenbach and Kränzlin, 1986). It forms near black sclerotia (Stamets, 1993).

Culture in the world
In natural medium stump roots are inoculated by digging trenches into the already parasited root zones. In China the modified natural model is the main method of cultivation with collection of the sclerotia 3 years after inoculation (Stamets, 1993). Sclerotia production is stimulated by mycoflora in soils and by the absence of light, rendering it difficult to obtain in laboratory conditions according to Stamets (1993). He has obtained only short, lateral, hardened plateau with pored surfaces only a few centimetres high on remains of spent shiitake or reishi blocks (sterilized) and after 45-60 days incubation. Nothing was obtained on fresh starting material (i.e sawdust/chips/bran).

Uses in traditional medicine
It is one of the commonly used Chinese medicinal “herbs” (You *et al.*, 1994). This mushroom is used in TCM mainly in health imbalance where there is excess dampness: diuretic for painful urination, urinary tract infections, oedema, diarrhoea, jaundice and leukorrhea (Chang and But, 1987). It has also figured prominently in Chinese Pharmacopeia especially in treatment of lung cancer (Stamets, 1993).

Pharmacology
This species was already largely studied; however several studies on this mushroom have been published only in Chinese or Japanese, mostly prior to 1992. The information is therefore taken from English abstracts.

Ito *et al.* (1976; abstract) have studied the influence of sex of mice on antitumor effects and on the immunity against tumor of D. *umbellata* polysaccharides. The growth velocities were higher in male than female mice of Sarcoma 180, Ehrlich solid

carcinoma, pulmonary tumor 7423 and MF-sarcoma, with or without treatment, while the regression rate was higher in female mice. The growth velocity of Shionogi carcinoma 42 was not influenced by sex. On the other hand, both males and females which had experienced a regression of ascites tumor after the administration of polysaccharides rejected the re-implanted Ehrlich ascites carcinoma, Sarcoma 180, NF-sarcomma and Shionogi carcinoma 42. These results suggest that a strong enhancement of immune response occurs in tumors implanted in the host by administration of polysaccharides. Ohsawa *et al.* (1992, abstract) have isolated 7 new components, polyporusterone A, B, C, D, E, F and G, which showed cytotoxic action on leukemia 1210 cell proliferation. You *et al.* (1994) have shown that oral administration of "Chuling" extract, increased the life span of tumor-bearing mice (intrahepatic implantation of sarcoma 180 tumor cells) from 71,6%, compared to intraperitoneal injection of mitomycin C and the combination of both which respectively induced a 70,1% and a 119% life span increase. Zhang *et al.* (1993) mention that brucellosis in mice results in a distinct immunosuppression which may be abrogated by immunomodulators such as levamisole, bestatin, interleukin-2 and *Polyporus umbellatus*.

Ishida *et al.* (1999) have isolated 3 compounds from a 50% ethanol extract, acetosyringone, polyporusterone A and polyporusterone B, active in a hair-regrowth assay. Another compound 3,4-dihydroxybenzaldehyde, had been isolated previously by the same team (Inaoka *et al.*, 1994) out of a methanol extract. The assay was done with normal C3H/He mice from which telogen hair on the back had been removed.

Human clinical studies
A recent review of Chinese medicinal herbs for chronic hepatitis B using all the known randomised and quasi-randomised trials with at least three months follow-up mentions that *D. umbellatus* polysaccharides showed an effect on serum HbeAG and HBV DNA, however they conclude that the evidence is too weak to recommend it (Liu *et al.* 2001). Some clinical studies published mainly in Chinese were realised against urinary bladder cancer and chronic viral hepatitis.

2.5.4 *Fistulina hepatica* Schäff.:Fr.

Common names
French : fistuline hepatique, foie-de-bœuf, langue-de-bœuf, glu de chêne

General ecology
Grows at the bases of broad-leaved trees trunks and stumps, almost always on oak. Fructifies between July and October.

Culture in the world
To our knowledge, *F. hepatica* has not been cultivated elsewhere than in our laboratory.

Pharmacology
A hot water extract inhibits the growth of sarcoma 180 at 95% and of Ehrlich carcinoma at 90% in the white mouse (Ying *et al.*, 1987). If a strong antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus substilis* was found in mycelium extract and culture liquids (Bianco Coletto., 1981), Tsuge *et al.* (1999) have

demonstrated antibacterial activity of cinnatriacetins A and B, triacetylene derivates, from the fruiting body.

2.5.5 *Flammulina velutipes* (Curt.: Fr.) Sing.

Common names
French : flammuline à pied velouté, collybie à pied velu, collybie à pied velouté, patte de velours. English: snow puff, Velvet stem, Golden. Japanese: Enokitake.

Origin
Cosmopolite

General ecology
F. velutipes is saprophyte or parasite of weakness, especially on broad-leaves trees (willow, elm, ash and poplar). It fructifies from the end of autumn to the spring.

Culture in the world
Its culture is well developed in Japan where it is appreciated as well as in Extreme Orient; however this is not the case in Europe. Miles and Chang (1997) mention the culture of *F. velutipes* around 800AD already. The first attempt of culture in Europe was done in 1925 by rubbing some mature carpophores on tree trunks. This method had no success and its real culture started in 1956 in Japan. Light is required for carpogenesis for this species (Croan and Kim, 1997). The culture of this species was also developed in our laboratory.

Uses in traditional medicine
According to Traditional Chinese medicine, this mushroom would be used for preventing and curing diseases of the liver and of gastroenterical ulcers.

Pharmacology
Several substances showed antitumoral activities in animals: B-(1-3)-D-glucans (Leung *et al.*, 1997; Ikekawa *et al.*, 1982; Ohkuma *et al.*, 1982; Yoshioka *et al.*, 1973), protein-bound polysaccharides (Otagiri *et al.*, 1983; Ohkuma *et al.*, 1982), protein (Ko *et al.*, 1995). B-(1-3)-D-glucans have presented slight activity by injection but protein-polysaccharid complexes of low molecular weight have presented very strong activities given orally (and not by injection) against sarcoma 180 of the mouse. According to the promising results, it could be a very important substance as a BRM or immunopotentiator by oral administration (Ikekawa, 1995). Finally a report from the Nagano Prefectural Research Institute of Rural Industry, at Nagano in Japan (cited in Ikekawa, 1995) mentions a study to determine if the consumption of the mushroom is effective for reducing cancer. The epidemiological results indicate that apparently it does play a protective role. In the mycelium, proflamin has been isolated. This glycoprotein is similar but different from those isolated from the fruitbodies. It is active against allogenic and syngenic tumors by oral administration, and is particularly effective against the solid form of sarcoma 180, B-16 melanoma, 755 carcinoma and Gardner lymphoma (Ikekawa *et al.*, 1985). A comparative test has given better results than with krestin (PS-K) which is commercialised.

Fukushima *et al.* (2001) showed that the consumption of the fructification by rats lowered the serum total cholesterol level by enhancement of fecal cholesterol excretion,

and in particular, by enhancement of hepatic LDL receptor mRNA. *F. velutipes* also contains reducing substances with chemical properties similar to ascorbic acid (Okamura, 1994).

Toxicity
This mushroom contain a cytolitic and cardiotoxic toxin, called flammutoxin -first studied by Lin *et al.* (1975)- which resembles to phallolysin contained in *Amanita phalloides* by certain aspects (Bernheimer and Oppenheim, 1987). This toxin is not active when ingested and is destroyed by heat (100°C during 20 minutes).

2.5.6 *Fomes fomentarius* (L.:Fr.) Fr.

Common names
French: Amadouvier. Japanese: Tsuriganetake.

Origin
Cosmopolite

General ecology
It is a parasite (oak, poplar; beech) or saprohyte on dead broad-leaves tree trunks.

Uses in traditional medicine
Except its use to keep fire ember glowing, its' medicinal utilisation goes to the 5th century BC where it was used for cauterising wounds. This utilisation survived up to the 19th century and perhaps even later in Lapland and Nepal (Delmas, 1989). Its name "Agaric of surgeons" is due to its use for stoping light haemorrhages. Its flesh was beaten with a mallet to make it supple and applied as non caustic haemostatic. It was also traditionally used by barbers to stop the bleeding of razor cuts. In Khanty (West Siberia) folk medicine it was used in the same manner: its' flesh was pounded in a mortar until soft. This mass was applied on wounds to stop bleeding. They were also using it to make warm compresses for extremities and joints. The aching area was covered with cotton, the mushroom mass and then tied with cloth. It was left until the pain was gone. A magical use in this tribe consisted in burning the fruit bodies alone or with fir bark when a person died. This smoking was continued until the deceased was removed from the house. The people returning from the funeral also had to pass through the smoke so that the dead spirit would not have an influence on the living (Saar, 1991). In China it is used by indigestion and to reduce stasis of digestive vitality, for oesophageal cancer and gastric and uterine carcinomas (Ying *et al.*, 1987)

Pharmacology
Sparse references were found on this mushroom. One concerns an antitumoral polysaccharide fraction from its culture filtrate (Ito *et al.*, 1976). Hobbs (1996) mentions the patent of Sakagami and Kawazoe (1991) which contains a lignin from *F. fomentarius* and showed to completely inhibit the growth of *herpes simplex* virus in cultures. Ying *et al.* (1987) have showed that a liquid extract of sporophores reached a 80% effectivity rate against sarcoma 180 in mice. Moreover it would have antiviral substances with systematic effects (Aoki et al., 1993)

2.5.7 *Fomitopsis pinicola* (Fr.) Karst.

Common names
French: Unguline marginée

Origin
Europe, North America, Asia, Africa

General ecology
Saprophyte on broad-leaf and conifer dead wood, more rarely a parasite on standing or lying trunks or on stumps.

Uses in traditional medicine
This mushroom would have been used as haemostatic (stryptic) (Tyler, 1978, cited in Thoen, 1982) in the same manner as *Fomes fomentarius* and *Phellinus igniarius*. This use is also described in Hobbs (1996) who reports Beardsley's (1944) words that the Cree in Eastern Canada were using the "Mech quah" (red touchwood) as a styptic. The dried fruit body was reduced to powder and mixed to water to obtain a paste which was applied on free bleeding wounds. It was used in Europe against dysentery, chronic diarrhoea, nervous headache, neuralgia, increased flow of urine, ague cake, jaundice, bilious remittent fever as mentioned in King's *American Dispensatory* (1985, cited in Hobbs, 1996). According to Bauchet (1961, cited in Chapuis, 1984) it could have anti-secretive properties. It is also used daily to reduce inflammation of the digestive tract and increase general resistance or as a cancer-preventive.

Pharmacology
A moderate antitumoral activity was found against sarcoma 180 prior to 1973 but was not further studied; however a DCM extract of a wild fruit body has shown a strong bactericide activity in an autobiographic test on *Bacillus subtilis* (Keller, 1997). Five isolated steroids showed such an activity (Keller *et al.*, 1996).

2.5.8 *Ganoderma applanatum* (Pers.: Wallr.) Pat.

Common names
French: Ganoderme plan. English: Red mother fungus, the ancient ling zhi, the artist's conk.

General ecology
It often grows on broadleaf trees trunks and stumps.

Uses in traditional medicine
In China it is used for rheumatic tuberculosis and oesophageal cancer (Ying *et al.*, 1987).

Pharmacology
G. applanatum polysaccharides increase spleen cell proliferation in vitro and stimulate antitumor activity against sarcoma 180 in mice. They also increase spleen cell primary antibody responses to sheep red blood cells (Gao and Yang, 1991). Nucleic acids isolated from *G. applanatum* reduced the number of vaccinia virus plaques in chick embryo fibroblast tissue culture and i.v. doses conferred protection against tick-borne

encephalitis virus in mice. It induced a substance showing properties of interferon both in the tissue culture and in mice spleen (Kandefer-Szerszen *et al.*, 1979).

2.5.9 *Ganoderma lucidum* (Curt:Fr.) Karst.

Common names
French : ganoderme luisant, polypore luisant, polypore laqué. Chinese: ling zhi (="spirit plant"). Japanese: reishi. English: Ling-Zhi, Reishi.

Origin
Cosmopolite.

General ecology
In forests, parks, gardens, under bushes, near paths, on compost, and so on. It fructifies from the end of summer to autumn, more rarely in the spring. Frequent.

Culture in the world
The first culture in Europe was done by Constantin and Matruchot in 1898. The method is completed by Matruchot in 1908 who proposes a culture on leaf-stacks of different tree species in humid caves. In 1956, at the III^{ème} Congrès de Paris, Hullen and Witt present good results on humus. Other authors mention the obtention of fruit bodies on natural substrate or compost in boxes. It is the cultures done by Vaandrager and Visher in Holland and Brian *et al.* in Bordeaux (I.N.R.A.) which have allowed the adaptation to mushroom cave conditions (Delmas, 1989). In Asia artificial culture and cultivation of Mannentake were attempted initially by T. Henmi *et al.*, in 1937. Its mass production was first achieved by Y. Naoi in 1971 by cultivating the spawn using pots containing sawdust. Since then use of bed logs or sawdust has become established practice. For some purposes bagasse spawn bed method and bottle or bag cultivation may be used. Usually, for mass cultivation of high-quality Reishi, either outdoors or indoors, bed logs are used. There are several dozens of trees appropriate for growing *G. lucidum*. Used as a kind of medicinal fungus, *G. lucidum* must be cultured on broad-leaved tree logs just like in its wild life. People of China, Japan and Korea which are main producing areas of *G. lucidum*, hold that the fruiting body of *G. lucidum* cultured on substitute medium (such as sawdust for edible fungus) is useless for medicinal materials and the question of whether they can be used as a health-care food resource needs to be further discussed. The cultivation technique of this species was also developed in our laboratory on substitute medium.

Uses in traditional medicine
This largely studied mushroom during the last decades in reason of the numerous medicinal properties attributed to it has been used in traditional medicine in China and Japan for more than 2000 years. In popular Chinese tradition it was considered as a panacea, curing all ills. Among other properties, it was thought to cure hepatopathy, chronic hepatitis, nephritis, hypertension, arthritis, neurasthenia, insomnia, bronchitis, asthma and gastric ulcers (Kabir *et al.*, 1988) also mentioned as good for the heart (Yeung, 1985) for losing weight, prolonging life, giving eternal life or for love potions! There are many popular Chinese and Japanese stories about people with cancer or other degenerative diseases traveling long distances for it.

Pharmacology and Human clinical studies
Hundreds of studies have been published on this species, thus only a resume is given here on pharmacology and Human clinical studies. These studies confer on it the following properties: antitumoral by direct cytotoxic activity (Wu *et al*., 2001, Min et al., 2000) or by non specific stumulation of the immune system (Zhang et al., 2000; Wang *et al.*, 1997; Chen *et al.*, 1995), anti-allergic (Kohda *et al.* 1985), anti-inflammatory (Koyama *et al.*, 1996, Lin *et al.*, 1993; Stavinoha *et al.*, 1995), hypoglycemic (zhang and Lin), antiviral against HIV-1 (El-Mekkawy *et al.*, 1998; Min *et al.*, 1998), Epstein Barr virus, against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) (Kim et al., 2000) sedative on the central nervous system, healing of skin wounds (Su *et al.*, 1996), hypotensive (Morigiwa *et al.*, 1986), antifibrotic for the liver (Park *et al.*, 1997), anticholesterolemic (Komoda *et al.*, 1989), analgesic (Chang and But, 1986), bronchitis prevention by induction of the regeneration of bronchic epithelium, antibacterial against *Staphylococces*, *Sterptocoques* and *Bacillus pneumoniae* (Hsu, 1990), anti-oxydant in getting rid of free hydroxyl radicals (Lin *et al*.; Wang *et al.*, 1985), chemopreventive potential (Lee *et al.*, 2001), presence of neuroactive compounds (Cheung et al., 2000) stimulation of the proliferation of nucleated cells in the spinal cord, cardiotonic etc.

2.5.10 *Ganoderma resinaceum* Boud. in Pat.

Ecology
It is a rare fungus that persists throughout the year, found on *Quercus* and *Fagus*, on scattered woodland trees or in high altitude European mixed woods. It is on the provisional European Red List.

2.5.11 *Ganoderma tsugae* Murr.

Common names
Chinese: Song Shan ling zhi (=pine tree fungus or pine wound).

General ecology
It grows mainly on conifers especially fir and hemlock.

Culture in the world
It is widely cultivated on sawdust-polypropylene bag system.

Pharmacology
A significant hepato-protective activity from carbontetrachloride-induced liver toxicity was observed for ganoderic acid B –also found in *Ganoderma lucidum* when administered to mice (Su *et al.*, 1993). Seven polysaccharides isolated from the fruitbody showed strong antitumoral activity against sarcoma 180 in mice, the more potent ones being water soluble with a tumor inhibition of 95,1 to 100% and prolonged life span of 236,3 to 267,5 % (Wang *et al.*, 1993). The same group research made further studies on polysaccharides of fruitbodies and mycelium (Zhang *et al.*, 1994). A skin substitute made of *G. tsugae* fruit body residue was developed by Su *et al.* (1997) and named SACCHACHITINE because of its composition: polysaccharides and chitine. The wound healing effect was studied on rats and compared to Beschitin, a chitin sheet from crab shell which showed similar activity. They also studied its effect on wound healing and fibroblast cell proliferation and migration in female guinea pigs (Su *et al.*,

1999): wound size was significantly reduced compared to those covered with gauze, but similar to Beschitin, while it enhanced proliferation and migration of fibroblast cells. They also studied healing processes (Hung *et al.*, 2001). For further information on the activities discovered until 1995, see Mizuno et al. (1995).

2.5.12 *Gloeophyllum trabeum* (Pers.:Fr.) Murr.

General ecology
This quite rare brown rot appears all year long on dead trunks and broad-leaved and conifers beams. It seems to be thermophile, growing mainly in Southen Europe.

2.5.13 *Grifola frondosa* (Dickson: Fr.) S.F. Gray

Common names
French : polypore en touffes, coquille en muguet, poule-de-bois, pied-de-griffon.
English: Hen of the wood. Japanese: Maitake (=dancing mushroom).

Origin
Cosmopolite

General ecology
Primary saprophyte growing on the base of old broad-leaved trees, especially oak, hornbeam and chestnut. Fructification in summer and autumn.

Culture in the world
It is only in 1978 that the first culture technique is developed by Gramms, but the first commercial production began in Japan –major producer and consumer of *G. frondosa*- in 1981 (Delmas, 1989). The industrial production of this edible basidiomycete has increased every year as a response to the public demand because of their nutritional qualities. From an original 325 tonnes, production increased to 33'100 tons in 1997 (Yamanaka; 1997). Japan is the main consumer of *G. frondosa* and produces 98% of the world-wide production (Chang, 1999).

Most of the world cultures (mainly in China, Japan and North America) are realised on synthetic-logs. As a basal ingredient to provide good air exchange, a mixture of fine and coarse hardwood sawdust or a mixture of hardwood sawdust and wood chips (Stamets, 1993) are commonly used, sometimes partially replaced by cotton-seed hulls or spent substrate. This basal ingredient is usually supplemented with wheat bran (source of the essential B1 vitamin), lime or gypsum (for calcium), sucrose and sometimes soil.

In China and Japan sprawn run and primordia initiation and fruiting is done in two different rooms, the second being cooler. Primordia are usually formed in an enclosed bag while the fruitbody develops out of openings in the bag. Soil casing has been reported to produce higher yield when done with soil from humus-rich hardwood forests, with garden soil or with rice paddy soil (Wu, 1997, in Chinese reported by Chen, 1998, internet). Fruiting temperatures given in literature are variable: 10°C-16°C (Stamets, 1993), 16°C-18°C (Huang, 1997, op. citates), 15°C-20°C (Wu *et al.*, 1997). Although mycelia can grow in the dark, a low level of light facilitates primordia initiation (Wu *et al.*, 1997). The Chinese and Japanese methods usually prescribe a 50 lux light for sprawn run while it is done in total obscurity in the American method. Our

laboratory has developed a culture technique on ligno-cellulositic substrates (Job and Schiff Giovannini, 2004).

Uses in traditional medicine
G. frondosa is used in Chinese medicine under the name "Keisho". Shen Nong's scripture of herbal medicine states that it has been frequently used for improving spleen and stomach ailments, calming nerves and mind and treating hemorrhoids (Mizuno and Zhuang, 1995). Powdered basidiomes or extracts are also used in the production of many health foods such as granules, drinks and tablets (Mizuno, 1999).

Pharmacology and clinical studies
Only a short resume of this well studied species is reported. Most of the studies have been realised on antitumoral activities in mice or rats. Gf-1 fraction (Suzuki *et al.*, 1985; 1984), D-fraction (Noriko Kodama and al., 2002), Grifolan obtained from the mycelium (Ohno *et al.*, 1995; 1986; Adachi *et al.* 1994; Mori *et al.*, 1987; Suzuki *et al.*, 1987), the MD-fraction which seem to be more potent than the D-fraction (Kodama *et al.*, 2002; Mayell 2001) showed immunostimulant activity (Atsuyuki *et al.*, 2002; Nanba *et al.*, 2000;Mao *et al.*, 1999).

Other activity reported include: anticholesterolemic activity without HDL level modification (Kubo *et al.*, 1997) by enhancement of cholesterol excretion (Fukushima *et al.*, 2001), antidiabetic activity in rats (non insulin dependent mellitus diabetes model) (Hiroyuki and Masaru, 2001; Kurushima *et al.*, 2000; Kubo *et al.*, 1994) probably by enhancement of glucose metabolism (Kubo *et al.*, 1996) or by improvement of glucose tolerance in rats (Horio *et al.*, 2001), hepatoprotective activity in rats (Lee *et al.*, 2000; 1992), effect against hypertension in rats (Adachi *et al.*,1988) and in vitro inhibition of adipocytes conversion (Nakai *et al.*, 1999).

A HIV activity study was abandoned because of the toxicity of the product, a sulfated proteo-glucane. As a negative impact on health, the D-fraction has been shown to induce arthritis in mice (Shigesue *et al.*, 2000).

Preliminary clinical studies in the USA have demonstrated its efficiency by ingestion for breast and colorectal cancers. In China, this extract has shown its efficiency for patients with lung, stomach or liver cancers and leukemia. Recently a positive impact on HIV patients was observed in a long-term trial (Nanba et al., 2000). A drug was commercialised under the name Tohchyukaso and Mushikusa in China and Japan (Mizuno *et al.*, 1995)

2.5.14 *Hericium erinaceus* (Bull.:Fr.) Quél.

Common names
French: hydne hérisson. English: Monkeyhead, Bear's head. Japanese: yamabushitake, houtou.

General ecology
It mainly grows on oak and beech trees, more rarely on others. It fructifies from August to December, its distribution is very irregular and it is rare to very rare.

Culture in the world
The first cultivation of this species dates from 1960 (Miles and Chang, 1997). It is cultivated on a variety of substrates including sugar cane bagasse, sawdust, cottonseed hulls, chopped up paddy straw, corn cobs, to which may be added; rice, wheat bran, sucrose and gypsum. These mixtures are contained in plastic bags or plastic or glass bottles. In each case only one mushroom is produced at a time. Recently in Japan, cultivation of yamabusitake on fungus beds, featuring use of bags and bottles, has became established to supplement the original method with wood material so that this species now ranks among the cultivated mushrooms most freely available on the market. The culture of this species was also developed in our laboratory.

Uses in traditional medicine
This edible mushroom is used by ingestion in Chinese traditional medicine to heal indigestion, neurasthenia and general weakness. The mycelium is used to make pills to treat gastric duodenal ulcers, chronic gastritis and chronic atrophic gastritis, as well as gastric and oesophageal carcinoma (Ying *et al.*, 1987). In China health drinks called « Houtou » are made by infusing the dried mushroom in hot water (Mizuno, 1995). In the same country a drug prepared out of this mushroom by the Norman Bethune University of Medical Science is commercialised for chronic stomach problems.

Pharmacology
Seven substances obtained from an ethyl acetate extract accelerate NGF (Nerve growth factor) synthesis which could be implicated in Alzeimer disease (Kawagishi *et al.*, 1991). Lee *et al.* (2000) have isolated a new diterpenoid –Erinacine H- showing such an activity. A Lectin (HEL) isolated from an ethanol extract showed an inhibition activity of erythrocytes aggregation (Kawagishi et. al., 1994)

2.5.15 *Hypsizygus marmoreus* (Peck) Bigelow

Culture in the world
It recently became one of the most popular edible mushrooms in Japan. It is intensively cultivated in windowless rooms under high temperatures and humidity in polypropylene bottles containing sterilised wood substrate. Its culture has been developed in our laboratory.

Pharmacology
Ikekawa *et al.* (1992) have isolated an antitumoral polysaccharide from the fruit body in 1992 which showed remarkable inhibitory effect against tumor-growth of sarcoma 180. The action of this β-(1→3)-D-glucan is due to the enhancement of immunological response. Assays against carcinoma 180 were realised in mice with powdered fruit body and with the same powder heated and treated with α-amylase. Both samples, given orally, inhibited tumours by 48% and 60% respectively (Ikekawa, 1995). It has also been demonstrated in experiments with mice that the consumation of this mushroom is effective in preventing cancer. It is therefore probable that fruit body consumption inhibits carcinogenesis. However no clinical studies have been realised to our current knowledge. A protein –HM 23- inhibited the adhesion of Lewis lung carcinoma cells on type IV collagen substratum when treated (Tsuchida *et al.*, 1995).

A ribosome-inactivating protein was isolated and tested by Lam and Ng (2001) it showed and inhibitory action against mycelial growth of various fungus

(*Mycosphaerella arachidicola, Physalospora piricola, Fusarium oxysporum Botrytis cinerea*, …) as well as on the translation of the rabbit reticulocyte lysate system and the HIV-1 reverse transcriptase activity. Antiproliferative activity against mouse leukemia cells and human leukemia and hepatoma cells was also observed. English abstracts of Japanese publications (Matsuzawa *et al.*, 1998; 1997) mention the results of powdered fruit bodies or aqueous extract fed to mice on their antioxidant effect in mice plasma, suggesting that the increase of antioxidant activities in the plasma of tumor-bearing mice is one of the mechanisms of cancer prevention and that *H. marmoreus* might play a role in decreasing thiobarbituric acid reactive substances by controlling antioxidant activities induction.

2.5.16 *Laetiporus sulphureus* (Fr.) Murr.

General ecology
It grows on live or dead wood of broad-leaved trees (*Prunus, Pyrus, Robinia, Populus*) and sometimes on coniferes (*Larix*). The fruitbodies are formed from spring to summer. There are also imperfect fructifications.

Uses in traditional medicine
Dakota Indians slice young specimens and boil them during half an hour for consumption. In central Europe and notably in woody region of Bohemia the dried mushroom powder was used as a partial substitute for bread flour (citation of Pilat in Thoen 1982). Ainu from Hokkaido (Japan) eat the miniatus variety under the name "God-mushroom" or "mushroom of gods"(Thoen, 1982).

Pharmacology
A lectine with haemolitic and hemagluttinant activity was isolated by Konska *et al.* (1994), while Kang *et al.* (1982) have prepared a protein-polysaccharide fraction which inhibits sarcoma growth in mice by i.v. injection. According to Chapuis (1980) the agaricic acid or agaricin which is a B-cetylcitric acid obtained by alcoholic extraction is used as antihydrotic for tuberculous patients. It is also contained in the "Thaumasman" and the "Iminol" . The same author cites the use of this species in homeopathy as a tincture, obtained by the "dulcamara" type.

2.5.17 *Laricifomes officinalis* (Vill.:Fr.) Kotl. & Pouz.

Common names
French: polypore du mélèze, polypore officinal.

Origin
Common in the West of the USA, also found in Europe and Asia.

General ecology
It grows principally on dead or live larch (*Larix decidua*) trunks, standing or lying, at ground level or at several meters high. It is also known on *Pinus*, *Abies* and *Cedrus*. Encountered in alpine forests, mountains of South Europe and Near-Orient, Siberian forests and probably also in Japan and in North America. Rare.

Uses in traditional medicine

L. officinalis has a long history of use in the West. Pliny (first century A.C) is probably the first to have written about Agaricum (=*L. officinalis)*) in his "Natural History" (37 volumes). He mentions it for the following uses: spider and scorpion bites, protection against noxious drugs, after vomiting, as a component of the Mithridate formula, to strengthens the stomach, to ease breathing difficulties, to benefit the kidneys**,** to relieve sciatica and pains in the spine, to act as an aperient (mild laxative), for the disorder of the spleen, for troubles of the hypochondria and groin, diseases of the bladder and to eliminate stones, to cure stranguary, to cure injuries of the achilles tendon and shoulder pain, to help cure tuberculosis, to help indigestion, to cure epilepsy, to relieves the chills of fever, to cure dropsy, to cure jaundice, to help heal bruises and the bad effects of falls, to relieve hysterical suffocations accompanied by delayed menstruation, as an emmenagogue.

In his "De Materia Medica" Dioscoride (about 55 A.D) the only mushroom which seemed to have a therapeutic use was the "Agaric" or Agarikon or Agaricon. It is actually thought that it was *Laricifomes officinalis*. It was used as a stryptic to stop bleeding, against injuries, bruises, falls and fractured limbs, for kidney diseases with difficulty in passing urine, for menstrual insufficiency, for liver complaints and jaundice, for hysteria, dysentery, epilepsy, sallow complexion, internal weakness of the organs, asthma, colic, phtisis, pain in the hips, loins and joints, poisoning, snake and animal bites (Hobbs, 1996).

Following the description of Dioscoride *L. officinalis* was used as a panacea for the next 1600 years: it is mentioned in diverse Antidotarium of the 9th and 10th century as a remedy against different cancers.

Gerard, in his Herbal (1633) a known compilation of several herbals before this time, mentions the famous Agaric for cleaning the intestines, against jaundice, for menstrual difficulties, oedema, asthma, chronic fever and cough, to restore proper colour and treating tuberculosis.

Since the 17th century the fame of the Agaric lessened. it was considered by physicians as cholagogue and phlegmagogue until the 18th century where its antisudoral properties were recognised to fight night sweat of the phtysics.

Towards the end of the 19th century it enter in the composition of the famous Warburg Tincture, also known under the name Antiperiodica Tincture, probably the best known western herbal formula containing a mushroom as a major ingredient.The *British Pharmaceutical Codex* of 1934 mentions its use. It is also still used in the 20th century for a certain number of problems; for night sweats due to tuberculosis, for reducing excess secretion and as a digestive stimulant during tuberculosis and malaria (Hobbs, 1996).

Recently it entered in the composition of a natural deodorant. It has figured in a certain number of pharmaceutical Codexes until around 1950 and we still find it in the Swiss Codex in 1967, before being (almost) forgotten by herborists. It also entered diverse old formula like the composed tincture of aloes or long life Elixir which is an effective purgative. Marchand (1975, cited by Thoen, 1982) says that half a century ago the carpophores sold so well in the pharmacy that the highlanders kept the places secret and

that they kept an eye on the carrying trees! Now, uses of this mushroom are almost forgotten.

Dharmananda (1994) a famous Chinese herbalist has noticed the similarity with the description of tonics like ginseng in TCM. He says that *Laricifomes officinalis* could be a first class tonic (= enhance the immune system resistance and support kidneys functions), but no scientific studies have been realised. This mushroom is used in a number of other cultures from Asia to North America in the same type of applications.

Pharmacology
The fruitbody extract shows an inhibition rate of mice sarcoma 180 of 80%. The laxative effect of the fruit body could be due to agaricin, to water soluble salts and to mannitol.

2.5.18 *Lentinus conchatus* (Bull.:Fr.) Schroet.

Common names
French: Lentin coquillage, Panus en conche ou en éventail. English: Lilac Oysterling

General ecology
This mushroom is either quite common, rare or absent according to regions. It appears from June to autumn on broad leaf trees wood (logs and stumps), rarely on conifers.

2.5.19 *Lentinus edodes* (M.J. Berk.) Sing.

Common names
Japanese: Shiitake. Chinese: Shiang-gu, Shing ku, Hua Gu, Xiangu and Hoang-mo. English: black forest mushroom, black oak mushroom, golden oak mushroom, snake butter, pasania mushroom, oakwood mushroom, Japanese forest mushroom, Japanese mushroom.

Origin
Japan, China, Korea, Taiwan, north part of Thailand and Burma, Nepal, North Borneo, Philippines, New-Guinea. Also found in Europe and USA where it was introduced.

General ecology
Primary saprophyte which grows naturally on a wide range of deciduous trees.

Culture in the world
Shiitake mushroom is an economically important species, particularly in Eastern Asia. It is widely grown commercially for food and medicinal properties (Wasser and Weis, 1997).

Shiitake was probably one of the first mushrooms to be cultivated in the world after *Agrocybe aegerita*. It is indeed a very important mushroom in China and Japan, where it has been known for over 2000 years for food, medicine and religion. It might be in China that primitive forms of its'culture could have started. It was probably Chinese farmers who introduced these methods in Japan about 300 years ago (Singer, 1961). It is

only since 1930 that the study of genetic and sexual characters of Shiitake has allowed a big scale scientific industrial culture.

Many culture studies have been realised on logs. The world demand for shiitake has greatly increased in a few years, however the amount of available trees (mainly various species of *Quercus* and *Fagus*) to use as substrate is limited. Culture was then oriented toward substrates composed mainly of sawdust and liquid culture as spawn. Culture on synthetic sawdust has the advantage of a higher yield and a shorter period of time. The most popular basal ingredients of such substrates are sawdust (mainly from brod-leaf trees), straw and corn cobs (Wasser and Weis, 1997).

European growers have adopted the use of low temperature treated (pasteurized) wheat straw proposed by Delpech and Olivier (1991) to shorten the process and thus eliminating the formerly required expensive sterilisation (Savoie *et al.*, 2000). Now it is the second most cultivated mushroom in the world after the button mushroom. Our laboratory has developed its industrial culture technique on lignocellulositic wastes.

Uses in traditional medicine
Shiitake has a long history in oriental folklore for treatment of tumors, flu, heart diseases, highblood pressure, obesity, problems related to sexual dysfunction and ageing, diabetes, liver ailments, respiratory diseases, exhaustion and weakness.

Pharmacology
It is the most well known and better characterised mushroom used in medicine, source of diverse preparations with proved pharmacological properties (Wasser and Weis, 1997).

In the last quarter of century shiitake has been evaluated in several clinical studies with humans. It seems to be useful for treating diverse types of cancer, cardiac diseases, hyperlipidemia (included hypercholesterolemia), hypertension, infectious diseases and hepatitis, however most of the studies were realised on extracts or compounds from mycelium, like LEM, LAP or KS-2. We will therefore only focuse on fruitbody extracts or compounds.

Lantinan, either taken orally or by injection, presents a strong antitumoral activity in animals and humans. The important number of studies realised show its effectiveness; as an antitumoral agent (Nanba *et al.*, 1987; Chihara, 1970) against various kinds of bacterial, viral, and parasitic infections including AIDS (Mizuno, 1995; Aoki, 1984, Chang, 1981; Kanai and Kondo, 1981).

Eritadenine (= lentinacine = lentysine), isolated from shiitake lowers cholesterol and lipid levels in the blood of rats tested (Chibata *et al.*, 1969) by modification of hepatic phospholipid metabolism (Sugiyama *et al.*, 1995). Shiitake fiber also lower cholesterol level (Fukushima *et al.*, 2001). In a study on the efficiency of diverse natural substances in preventing tooth decay and periodontitis, shiitake was very promising (Hirasawa *et al.*, 1999). The in vitro tests results show that shiitake extract inhibit the main bacteria species responsible of the precited problems. Shiitake seems beneficial in lowering bronchial inflammation and in regulating urinary incontinence (Liu et Bau, 1980). Data shows that a solution of Shiitake inhibits mutagenicity induced by alkylating agents in mice (de Lima *et al.*, 2001).

Human clinical studies
Lentinan is a polysaccharide which was commercialised in December 1985 as an immunotherapeutic drug in Japan where it is used to sustain immunological functions of cancer patients in chemotherapy. It often increases the patient's survival time. It has been used particularly to prevent tumour recurrence and metastasis after surgical intervention. The Japanese government has listed it officially as a cancer treatment (Mizuno, 1995). However it is not active against all tumour types but it has been demonstrated that lentinan enhances survival time of patients with inoperable gastric cancer (Mashiko *et al.*, 1992 from english abstract) or advanced or recurrent gastric cancer (Taguchi *et al.*, 1987, 1982) also for recurrent breast cancer after surgical intervention (Kosaka *et al.*, 1985). It also showed favourable results in treating chronic persistant hepatitis (Lin *et al.*; 1987). The use of the powdered mushroom by patients with aids is being studied in England, Spain and Italy. It demonstrated effectiveness is some studies (Gordon *et al.*, 1998).

It is interesting to note that Lentinan also offers a potential treatment for other viruses like herpes, influenza, hepatitis and even against parasites. Lantinan mobilizes humoral defenses to help the body eliminate antibiotic resistant bacterial infections. The studies have been done with injections and not by ingestion. It is probable that it enhances our resistance against infection by its immunomodulator potential, shown by activity against low natural killer syndrome (LNKS) (Aoki *et al.*, 1987).

2.5.20 *Lepista nuda* (Bull.:Fr.) Cke.

Common names
French: pied bleu, pied violet, tricholome nu. English: Wood blewit.

Origine
Europe, North America, Australia

General ecology
In woods, parks, gardens, under bushes, near paths, among herbaceous plants also on compost. It fructifies from the end of the summer to the autumn, more rarely in the spring. Frequent.

Culture
The first culture dates from 1898 by Constantin and Matruchot, with a method completed by Matruchot which proposed a culture technique in humid caves on tree leave heaps in 1908.

In 1956, at the IIIrd Congrès de Paris, Hullen and Witt present good results obtained on humus. Studies on substrate requirements of European *Lepista* indicate that fruit body production is enhanced by the addition of 10% uncomposted straw to commercial *Agaricus* compost (Vaandrager and Visscher 1981). Guinberteau et al. (1989) reported that a cold shock was essential for the formation of fruit bodies of *L. nuda* and temperatures of 8° to 15°C have been found to be effective. It was cultivated semi-commercialy on *Agaricus bisporus* mushroom compost (Frische and vanLoone, 1989). Stott and Broderick (1996) showed that the optimum and minimum growth temperature

for Australian isolates was higher than for French isolates and that growth rate of Australian isolates was generally more than double the rate of French isolates. The same authors showed that the addition of 10% uncomposted cereal straw encouraged hyphal growth, but discouraged the initiation of fruiting bodies and was not beneficial to Australian isolates. A cold shock of 12°C encouraged more hyphal aggregations than 15°C, however, authors did not obtain fruit bodies.

Pharmacology
It is resistant to Gram+ and Gram- bacteria (Ying *et al.*, 1987).

2.5.21 *Lyophillum ulmarium* (Bull.:Fr.) Kühn.

Common names
French : pleurote des ormes

Origin
Europe

Culture
Our laboratory has developed a culture technique for this species, however it is already cultivated.

Uses in traditional medicine
It is used in China to stimulate blood circulation (Yang et Jong, 1989).

Pharmacology
Hot water extract would inhibit sarcoma 180 at 60% and Ehrlich carcinoma in mice by injection (Ying *et al.*, 1987).

2.5.22 *Meripilus giganteus* (Pers.:Pers.) Karst.

Common names
French: polypore géant.

Origin
Europe, North America.

General ecology
This mushroom grows on tree trunks, more rarely on living trees as weakness parasite. It is mainly found on *Fagus*, but also on other broad-leaved trees and more rarely on *Abies*. This species always appears on wood even when buried or on roots, giving the impression of a terrestrial species. The fruitbody appears in the summer and in the autumn.

Pharmacology
A mixture of saturated and unsaturated fatty acids and ergosterol peroxide were identified by Narbe *et al.* (1991) as immunosuppressive components. Among the fatty acids, palmitic, oleic and linoleic acid were determined.

2.5.23 *Perenniporia fraxinea* (Fr.) Ryv.

General ecology
This rare mushroom provokes a white rot on broad-leaf trees (*Fraxinus, Quercus, Juglans, Robinia*).

2.5.24 *Phellinus igniarius* (L.:Fr.) Quél.

General ecology
It is found on many deciduous tree species. It provokes a heart wood white trunk rot.

Pharmacology
Shon and Nam (2001) have shown that this species (polysaccharides extracts form culture broth and mycelia and water extract of fruitbodies) has antimutagenic activities and may play a role in cancer prevention by inducing NAD(P)H:quinone oxidoreductase and gluthatione-S-transferase activities and by increasing gluthatione level.

2.5.25 *Pleurotus citrinopileatus* Sing.

Common names
French: Pleurote jaune

Culture
It is cultivated in the same manner as other *Pleurotus* species (see *P. ostreatus*). Its culture has also been developed in our laboratory.

Pharmacology
A water soluble (FI) and two insoluble (FII, FIII) polysaccharides fractions extracted from the fruitbody have shown activity against implanted sarcoma 180 in mice by injection. They were better than PS-K used as a positive control (Zhang *et al.*, 1994).

2.5.26 *Pleurotus eous* (Berk.) Sacc.

Common names
French: Pleurote rose

Origin
India.

General ecology
On dead trees trunks in very hot valleys of India.

Culture in the world
It is cultivated about in the same manner as other *Pleurotus spp* (see *Pleurotus ostreatus*). Our laboratory has succeeded in cultivating this species.

2.5.27 *Pleurotus eryngii* (DC: Fr.) Quél.

Common names
French : Pleurote du Panicot, argouagne, oreille-de-chardon. English : King oyster, King Trumpet, Cardarello.

Origin
The entire Mediterranean basin.

General ecology
Contrary to other *Pleurotus spp.*, it does not grow on wood but on root stocks or roots of diverse Apiaceae cut the year before. It is found mostly in the spring and autumn, more rarely in the summer.

Culture in the world
It is cultivated in polypropylene bottles on various agricultural wastes; however it is more difficult and slower to grow than other oyster species. Thus high production costs due mainly to long incubation time, limits the diffusion of this edible species. Our laboratory has also developed its culture.

Pharmacology
Pleureryn, a protease isolated from fresh fruit bodies inhibited translation in a rabbit reticulocyte lysate system as well as acting against HIV-1 reverse transcriptase (Wang et Ng, 2001).

2.5.28 *Pleurotus ostreatus* (Jacq.:Fr.) Kumm.

Common names
French : pleurote en forme d'huître, pleurote coquille d'huître, pleurote en coquille, nouret, couvrose, poule-de-bois, oreille-de-nouret, oreille-de-noyer. English: white oyster, Gray oyster, oyster. Japanese: Hiratake.

Origin
Europe and temperate regions.

General ecology
Primary saprophytic mushroom that appears in autumn and winter on dead broad-leaved trees trunks, more rarely on conifer wood (*Abies*). It can also attack sick or wounded trees and induce severe damages.

Culture in the world
It is one of the easiest of wild mushroom to domesticate as it is able to digest a various agricultural lignocellulosic wastes or hardwood. It can even grow on conifer wood chips after treatment with another fungus. It is cultivated in many subtropical and temperate countries. This species cultivation technique was also developed in our laboratory

Uses in traditional medicine
Chinese people used to take powdered fruitbodies to cure lumbago, sore and heavy legs, blood vessels and strained tendons.

Pharmacology

Bobek and his team have published a lot on hypocholesterolemic activity of this mushroom. Their experiments were done mainly on just weaned Wistar rats. The results show that addition of the powdered mushrooms to their diet reduced cholesterol accumulation in serum and liver when fed a cholesterol diet. Several experiment aiming to explain the biochemical mechanisms have been realised. Thus the addition of 5% dried mushroom to the diet had the following effects: reduction of cholesterol in serum and liver (Bobek *et al.*, 1993) redistribution of cholesterol in favour of high-density lipoproteins by increasing the fractional turnover rate of VLDL (Bobek and Ozdin, 1994) reduced production of VLDL and LDL (Bobek and Ozdin, 1996) reduced cholesterol absorption (Bobek *et al.*, 1995) and reduced HMG-CoA (3-hydroxy-3-methylglutaryl CoA) activity in liver (Bobek *et al.*, 1995b). According to Gunde-Cimerman and Cimerman (1995) the HMG-CoA reductase is lovastatin, which was determined in the mycelium, primordia and different basidiocarpes parts by the authors. Dose and time-dependent effects were also studied with the same model showed that the reduction of cholesterol in serum and body organs was dependant on the amount of dietary mushroom administered. The dose of 1% had no affect while 5% did for the whole testing time (Bobek *et al.*, 1998). In Czech Republic and Slovakia the fruitbody is indeed an ingredient in dietetic preparations (e.g. HLIVETA Eritaden and PLEUROS 600) recommended for preventing high cholesterol levels (Opletal, 1993, taken from an English abstract, article in Czech).

Since 1999, Bobek and collaborators have been publishing on hypocholesterolemic and antiatherogenic effect experimenting on male rabbits (Chinchilla), then in 2001 about the effect of Pleuran, a β-1,3 glucan, in the treatment of ulcerative colitis in rats. Colitis was induced by intraluminal instillation of 4% acetic acid. Their results show that pleuran might have enhanced antioxidant defence of the colonic wall against the inflammatory attack (Bobek *et al.*, 2001) and a possible role in the immunomodulation (Nosal'ova *et al.*, 2001).

The product MycoVastatin (also known as Plovastin) is a biotechnological product produced in fermentor culture from the Oyster mushroom. The main active component is statins which inhibits cholesterol biosynthesis and prevent atherosclerosis. The product and the production process in fermentor culture have been patented in the U.S.A by the firm Med Myco's (Patent WO 2001/273005 A1).

In mice fed with this mushroom it is active against a mammar tumor system (inhibition rate 89,7 %), against sarcoma 180 (79,4 inhibition rate) (Mori *et al.*, 1986). An aqueous extract has shown an inhibition rate of 75% of sarcoma 180 and of 60% of the Ehrlich carcinoma in white mice. A more resent study on ICR mice treated with a carcinogen N-butyl-N'-butanolnitrosamine (= BBN) every day for weeks showed that bladder carcinoma was found in 100% of mice (10 in total) treated with BBN, while only in 65% (13 out of 20) of mice fed with *Pl. ostreatus*. Moreover chemotactic activity of macrophage and cytotoxic activity of lymphocytes against Yac-1 cells was suppressed in the first case but maintained normal in the second. Lymphocytes collected from mice fed with mushrooms also showed almost normal blastogenic response against concanavalin A (Kurashige *et al.*, 1997).

Wang *et al.* (2000) have isolated a dimeric lectin from fresh fruitbodies which prolonged survival in mice bearing sarcoma S-180 and hepatoma H-22, thus exerting

potent antitumor activity. It is interesting though that in another study, rats having free access to food and water would not eat the diet containing *Pl. ostreatus* even if emaciated. A lectin was isolated as the food intake-suppression principle by the authors Kawagishi *et al.* (2000). A diet containing only 0.1% lectins caused a 50% decrease in food uptake compared to control group.

A glycoprotein inhibited tranlation in a rabbit reticulocyte lysate system, exhibited low ribonucleolytic activity toward yeast tRNA and expressed an inhibitory activity toward human immunodefidiency virus-1 reverse transcriptase (Wang and Ng, 2000).

Human clinical studies
A clinical study shows that 25-20g of dried fruitbodies given daily during a month reduces hypercholesterolemia in some of the patients.

2.5.29 *Pleurotus tuber-regium* (Fr.) Sing.

Common names
French: Pleurote à sclérote. English: Tiger milk mushroom or sclerotia producing *Pleurotus*, Esan language in Nigeria: uhuenru.

Origin
Africa and Australasian-Pacific region

General ecology
Occurs both in tropical and subtropical regions of the world (Zoberi, 1972), distributed in Africa and the Australasian-Pacific region (Chen, 2001) this white rot growing in the wood of broad-leaf trees is the only know species of *Pleurotus* to produce a fruit body out of a real sclerotium (Isikhuemhen *et al.*, 1999). The sclerotia can be up to 25cm or even 30cm in diameter, are very dense, quite resistant to long periods of dry weather and can produce successive flushes during several seasons. It is dark brown on the outside and white on the inside (Oso, 1977).

Culture in the world
Primitive culture of this “royal truffle” by Nigerians and some African nations consist of picking up sclerotia, planting them in a favourable place and maintaining the needed humidity for carpophore formation.

Several authors have published their results on the cultivation this mushroom on various lignocellulositic wastes. It is grown on a wide range of substrates: cassava (*Manihot sp*.) peelings, corn (*Zea sp*.) straw, oil palm fruit fibers, rice (*Orizae sp*.) straw and wild grass (*Pennisetum sp*.) (Okhuoya and Okogbo, 1991), banana leaves, corn cobs, cotton waste and rice straw (Fasidi *et al.*,1993) and so on. Sawdust from broad-leaf trees can be used directly, while sawdust from tropical hardwoods or coniferous trees should first be fermented (Oei, 1996). However not all substrates allow sclerotia production (Okhuoya and Okogbo, 1990). Temperature is generally high, ranging from 15 to 40°C for sprawn run, from 24°C to 35°C for sclerotia formation and from 24°C to 35°C for fruiting (Braun *et al.*, 2000; Stamets, 2000; Oei, 1996). Only African isolates form sclerotia uniformely before fruiting in laboratory conditions. The australian strains sometimes fructify directly on the agar medium or on sterile wood substrate

(Isikhuemen *et al.*, 2000). The cultivation of *P. tuber-regium* has not yet reached a commercial scale in the world (Chen, 2001).

Uses in traditional medicine
Both the sclerotia and the fruitbodies are used for food and medicine in Tropical Africa (Oso, 1977). Sclerotia is usually dryed before eating, being bitter when fresh. The fruitbody is eaten very young as it becomes fibrous and tough when older (Chen, 2001). However Ogundana and Fagade (1981) have detected hydrocyanic acid in their tested mushroom samples.

It is mainly the fresh or dry sclerotia which are used by Nigerian native doctors in different herbal and other ingredient mixture. Topically, the most common use is for preventing or curing skin diseases (Oso, 1977). It has also been used by various African tribesmen to cure headaches, stomach pains, colds, fever (Oso, 1977), asthma, high blood pressure and even smallpox (Fasidi *et al.*, 1994; Oso, 1977). According to Chen (2001) it has been used topically for mammalian gland inflammation of breast-feeding mothers in Southwest China's Yunnan province.

Pharmacology
Very few studies have been published on the biological activity of the sclerotia, while none where found on the fruitbody except a preliminary investigation for the use of the powdered fruitbody as a tablet disintegrant instead of corn starch in paracetamol tablets (Iwuagwu and Onyekweli, 2002).

A potent homodimeric ribonuclease –pleuturegin- which showed strong ribonucleolytic activity toward Poly G, and slighter toward Poly U, Poly A and Poly C was isolated from fresh sclerotia. This protein also inhibited cell-free translation in a rabbit reticulocyte lysate. (Wang and Ng, 2001a, 2001b). Hot alkali extracts of nonstarch polysaccharides belonging to β-glucan type were effective given i.v. in inhibiting tumor proliferation in BALB/c mice implanted with solid tumor Sarcoma 180. It also had an antiproliferation activity against human tumor cell lines HL-60 and HepG2. The authors (Zhang *et al.*, 2001) therefore postulate that the antitumoral effect of nonstarch polysaccharides from the sclerotia is probably host-mediated and cytocidal.

2.5.30 *Polyporus brumalis* Fr.

Common names
French: Polypore d'hiver.

General ecology
Typically in the winter (Courtecuisse, 2000) on buried wood or fallen broad-leaf trees.

2.5.31 *Polyporus ciliatus* Fr.

General ecology
This frequent species in spring and autumn has a wide distribution (Courtecuisse, 2000).

2.5.32 *Polyporus squamosus* Fr.

Common names
French: Polypore écailleux, Polypore du noyer, chouette. English: Scaly Polyporus.

General ecology
This species of white rot is common in the spring and summer, rarely in autumn on trunks and stumps, parasite or saprophyte on various broad leaf trees, very rarely on fir (Romagnesi, 1995).

Pharmacology
The main activity found in this mushroom was from a mycelium extract, PS-64. Multiple injections of extract, administered before the primary or secondary immunisation with ovalbumin (OA) resulted in a dose-dependent inhibition of IgE and IgG antibody response to OA in mice. The 3 week experiment showed IgE inhibition to the end while IgG response was back to control level by the third week of the second immunisation. Histamine release was not influenced but the PS-64 extract inhibited in vitro proliferation of human mononuclear cells upon phytohemagglutinin stimulation and also a delayed type hypersensitivity reaction and skin graft rejection similar to the effect noted with Cyclosporin A (Babakhin *et al.*, 1997).

2.5.33 *Pleurotus tuberaster* (Pers.:Fr.) Fr.

Common names
French : polypore à sclérote; champignon pierre; (pierre à champignon)

Origin
Italie

Culture in the world
This mushroom known in Italy since ancient times has only been cultivated primitively about in the same manner than *Pleurotus tuber-regium*: slices of the sclerotia are buried in rich soil kept humid. Incubation temperature over 20oC allows fruiting in a few weeks (Delmas, 1989).

Uses in traditional medicine
The sclerotia seem to be the Tuckahoe eaten by eastern USA Indian tribes. The “white men” called these sclerotia –Indian bread- suggesting this use (Thoen, 1982). American Indians and notably the Crees used the sclerotia as a poultice against rheumatism (Johnston, 1970 and Brodie, 1978, cited by Thoen, 1982). Césalpino in his “De Plantis” from 1583 describes the use of this species which grows on the “Pietra fungaia” against renal stones. The popular belief was that this “Pietra” was the concretion of Lynx (*Lapis lyncurium*) urine and by analogy was thought effective against renal lithiasis (cited in Chapuis, 1984).

2.5.34 *Pycnoporus cinnabarinus* (Jacq.:Fr.) Karst.

Common names
French: Tramète rouge cinabre, polypore rouge cinabre. English: cinnabar-red polypore

General ecology
A white rot which is found on tree trunks, stumps, fence wood of many broad leaf trees, rarely on conifers, fructifies all year long, but mainly from August to December. Can be regionally quite rare or common.

Culture
To our knowledge only the mycelium is cultivated for laccase production or other products (i.e. vanilline).

2.5.35 *Schizophyllum commune* Fr.:Fr.

Common names
French: schizophylle commun

Origin
As indicated by its name this species grows on all continents, probably the most well known of mushrooms.

General ecology
This ubiquitous species fructifies all year on all kinds of ligneous substrates.

Culture in the world
This species is cultivated in a number of ways in the world due to its affinity with so many different substrates.

Uses in traditional medicine
In traditional Chinese medicine this mushroom is recommended against general weakness (Ying *et al.*, 1987) and to cure gynaecologic diseases (Yang et Yong, 1989). It is also cooked with eggs as a cure for leucorrhea.

Pharmacology and clinical studies
This species is one of the mushrooms which has been extensively studied. Hundreds of publications exist so the following is a resume of results.

The main activity is the antitumoral which led in 1986 to the commercialisation of a polysaccharide extracted from the medium product and called schizophyllan or Sizofiran or SPG. It is prescribed in Japan by injection as an adjuvent to classical therapy against cervical cancer. Publications up to 1969 were often in Japanese. An example of in vitro studies is the publication of Sakagami *et al.* (1988) concerning interferon-γ and interleukine 2 production increases in human peripheral blood mononuclear cells by schizophyllan. Examples of animal studies shows the positive implication of T-lymphocytes and macrophages on the anti-tumor activity in mice (Suzuki *et al.*, 1982) and a combination of radiation and Sizofiran on tumor growth and metastasis in squamous-cell carcinoma in mice (Arika *et al.*, 1992). Clinical trials were realised on the evaluation of sizofilan as assistant immunotherapy in the treatment of cervical carcinoma (Miyazaki *et al.*, 1995). A rapid recovery of the immunologic parameters impaired by radiotherapy in head and neck cancer (Kimura *et al.*, 1994). Patients had a better prognosis on resectable gastric cancer when sizofiran was prescribed with antitumor drugs (Fujimoto *et al.*, 1991, 1984).

Sizofiran also induced modulation of cellular and humoral immune responses in patient with chronic hepatitis B (Kakumu *et al.*, 1991). Growth inhibition of *Bacillus subtilis* and *Sarcenia sp* was described (Parag and Parag, 1974). An activity against *B. subtilis* observed from culture liquid and mycelium (Coletto 1981). Grant *et al.* (1990) found a muramidase enzyme effective against several Gram-positive bacteria produced when *S. commune* was grown on heat-killed *B. subtilis*. Shrimp tested on their survival, hemocyte phagocytosis and superoxide anion production showed enhancement of all three when schizophyllum is administred in their diet, showing a later return to pre-test levels (Chang *et al.*, 2000).

2.5.36 *Sparassis crispa* Wulf.:Fr.

Common names
French: sparassis crépu; clavaire crépue

Origin
Europe

General ecology
Grows on conifers roots, mostly pine. Distribution is wide but quite rare. It fructifies from August to November.

Culture in the world
According to Ohno *et al.* (2000) it has recently been cultivable in Japan. However our laboratory has already succeeded in its culture since several years.

Uses in traditional medicine
According to Fourré (1982) *Sparassis crispa* could be cultivated in China for its therapeutic effects to treat coughs, fatigue and childhood diseases.

Pharmacology
Very few scientific studies have been published. Three polysaccharides fractions of 6-branched 1,3-β-glucans showed antitumor activity in mice against solid Sarcoma 180. Beside, strong vascular dilatation and hemorrhagic reaction were observed. These fractions, given i.p. of p.o. to leukopenic mice induced by cyclophosphamide, showed enhanced hematopoietic response (Ohno *et al.*, 2000). Mycelium cultivated in liquid medium has furnished 3 antifungal compounds active against *Cladosporium cucumerinum*, a pathogen of cucurbitaceae. These compounds are all derivates of orsellinic acid, found in wood invaded by *Sparassis crispa,* active against bacteria and fungi (Woodward *et al*, 1993).

2.5.37 *Sparassis laminosa* Fr.

Origin
Europe

General ecology
This species, more frequent than *S. crispa* is bound to broad-leaved trees.

Culture in the world
Our laboratory has established the industrial culture method for this species.

2.5.38 *Stropharia rugosa-annulata* (Farlow ap Murr.) K. & R.

Common names
English: king stropharia, the Garden Giant.

General ecology
Saprophyte able to attack all kind of wood or compost substrates (i.e. sawdust, branches).

Culture
This edible species –when young- is commercially grown on a wide range of substrates like straw, compost, sawdust, mulch or wood chips. A variety of kits are sold on the market for amateur gardeners. Outdoors, it fructifies in the summer and autumn.

2.5.39 *Trametes gibbosa* (Pers.: Fr.) Fr.

Origin
Europe, Asia.

General ecology
On dead wood of broad-leaved trees in particular on *Fagus*, exceptionally a parasite of weakness, fructifies all year (Breitenbach and Kräntzlin, 1986).

2.5.40 *Trametes hirsuta* (Wulf. ex Fr.) Pil.

General ecology
Very common, almost exclusively saprophytic on all kinds of dead broad-leaved trees woods: stumps, trunks, branches, fructifies all year.

2.5.41 *Trametes suaveolens* (Fr.) Pil.

Origin
Europe, North America, Asia

General ecology
On old trunks of alive or dead *Salix* and *Populus*.

Uses in traditional medicine
According to Cetto (1965, cited in Chapuis, 1984) it was used powdered by Sartorius in 1785 in the treatment of pulmonary tuberculosis.

2.5.42 *Trametes versicolor* (Fr.) Pil.

Common names
French: polypore versicole. The English work "Turkey Tail" is due to its resemblance with the given birds. In Chinese it is yun-zhi, and in Japanese, kawaratake.

Origin
Cosmopolite

General ecology
On dead wood of broad-leaved trees, more rarely on conifers. On branches, trunks, tree stumps. Sometimes a parasite of weakness.

Uses in traditional medicine
Alfaro *et al.* (1983, cited in Hobbs, 1996) mentions that in Mexican folk medicine the fungus is used to cure ringworm or impetigo of the skin. In TCM it is used to clear dampness, reduce phlegm, heal pulmonary disorders (Ying *et al.*, 1986 in Hobbs 1996), and strengthen the physique, increase energy, and benefit people with chronic diseases (Yang et Yong, 1989 cited in Hobb, 1996).

Pharmacology and clinical studies
Hundreds of publications exist on the pharmacology and clinical studies of this species, mostly on PSK also called Krestin which is a protein-bound polysaccharide extracted from the mycelium. In Japan, it is commercialised since 1977 as an immunotherapeutical drug against cancer of digestive organs, lung and breast (Mizuno *et al.*, 1995). In 1987 in Japan PSK represented 25,2% of total national expenses for anticancerous agents which is the mirror of its efficiency. Many publications are in Japanese as the principle research was done there. In Japanese trials since 1970, PSK significantly extended survival to five years or more in cancers of stomach, colon-rectum, oesophagus, nasopharynx and lung cancer, as well as in a HLA B40-positive breast cancer subset (Kidd, 2000). This substance was also shown to prolong the average survival time of mice infected with a variety of gram-negative bacteria pathogens or with *Candida albicans*. This activity was correlated with activation of mouse macrophages (Mayer and Drews, 1980). It presented antiradicalar activity by mimicking superoxide dismutase action. Tumours bearing rats and human patients with digestive tract cancer suffering from oxidative stress were relieved by a single intraperitoneal administration of PSK or a 1day per oral prescription (Kariya *et al.*, 1992).

Other polysaccharides like PSP extracted from mycelium and SPCV were also studied. PSP was classified as a biological response modifier because of its' actions: on animals it increased γ-interferon and interleukin-2 production and T-cell proliferation. Its' antiproliferative activity against tumour cell lines and in vivo antitumor activity has been demonstrated. Administered to patients with oesophageal, gastric and lung cancer undergoing radiotherapy or chemotherapy it helps alleviate symptoms and prevents immune status decline (Ng, 1998). Subjected to Phase II and III trials in China, it significantly extended five-year survival in oesophagial cancer (Kidd, 2000) while SPCV (small polypeptide) showed potential cytotoxic effect on human tumour cell lines and immunopotentiating effects (Yang *et al.*, 1992).

2.5.43 Summary

Table 2.1 shows if the studied mushrooms have been cultivated, if they were/are used in traditional folk medicine, if they have been submitted to pharmacological study and if they are producers of commercialised drugs.

Species	Cultivated	Used in traditional medicine	Pharmacological study	Commercialised drugs
Agaricus brasiliensis	X	X	X	
Agrocybe aegerita	X	(X)	X	
Dendropolyporus umbellatus	X	X	X	
Fistulina hepatica	X		X	
Flammulina velutipes	X	(X)	X	
Fomes fomentarius		X	(X)	
Fomitopsis pinicola.		X	(X)	
Ganoderma applanatum		X	X	
Ganoderma lucidum	X	X	X	X
Ganoderma resinaceum				
Ganoderma tsugae	X		X	
Gloeophyllum trabeum				
Grifola frondosa	X	X	X	X
Hericium erinaceum	X	X	X	
Hypsizigus marmoreus	X		X	
Laetiporus sulphureus		X	X	
Lariciformes officinalis		X	(X)	
Lentinus conchatus	X	X	X	X
Lentinus edodes	X			
Lepista nuda	X		(X)	
Lyophillum ulmarium	X	(X)	(X)	
Meripilus giganteus			(X)	
Perenniporia fraxinea				
Phelinus igniarius			(X)	
Pleurotus citrinopileatus	X		(X)	
Pleurotus eous	X		(X)	
Pleurotus eryngii	X			
Pleurotus ostreatus	X	X	X	
Pleurotus tuber-regium	X	X	X	
Polyporus brumalis				
Polyporus ciliatus				

Species	Cultivated	Used in traditional medicine	Pharmacological study	Commercialised drugs
Polyporus squamosus			(X)	
Polyporus tuberaster	X	X		
Pycnoporus cinnabarinus				
Schizophyllum commune	X	X	X	X
Sparassis crispa	X	(X)	(X)	
Sparassis laminosa	X			
Stropharia rugosa-annulata	X			
Trametes gibbosa			(X)	
Trametes hirsuta				
Trametes suaveolens		(X)		
Trametes versicolor	X	X	X	X

Table 2-1: overview of the studied mushrooms. X: correspondence to the top heading. (X): only sparse indications.

3 EXPERIMENTAL PART

3.1 Species and strains

All the Basidiomycetes species and strains used during the present work are presented in table 3.1 with their strain identification code and their sources. All the species studied belong to the sub-division Basidiomycotina and to the Class Hymenomycetes. Classification into this Class and synonyms of all species are presented in tables 3.2 and 3.3

Isolation of wild strains and generation (F1, F2…) of strains was realised by inoculating a piece of the center of the carpophore into a 0.02% steptomycin 2% AM medium. Cultures were observed by optic microscope, to ensure the absence of contaminants.

All the strains are maintained at 4°C on 2% AM medium in slanted tubes and transferred every year to a new tube. Two tubes per strains are usually kept in the mycotheca of the University.

When there was several strains of one species (see table 3.1), dual confrontation have been done by inoculating 2 dicaryotic mycelium plugs of each of the two tested strains facing each other (fig. 3.1) in a 2% AM plate. Plates were incubated at 25°C in the dark, until contact of the colonies, according to Worrall (1994). Somatic incompatibility was demonstrated by the presence of a thick line, sometimes discoloured by orange, brown or black pigmentation, at the meeting point of the two colonies.

Species	Strain	Source
Agaricus brasiliensis	MJFGL2 (=AbST)	Kindly provided by Mr Stamets (USA)
Agrocybe aegerita	MJFAA2	University of Neuchâtel
Dendropolyporus umbellatus	Chine	Kindly provided by Mr Moos. Chinese origin.
Fistulina hepatica	FH1.2.2	University of Neuchâtel
Flammulina velutipes	Flam.velMY	University of Neuchâtel
Fomes fomentarius	MUCL 35117	MUCL culture collection, Louvain-La-Neuve, Belgium)
Fomitopsis pinicola	MUCL 30544 MUCL 31677 K1 K2 K3	MUCL culture collection, Louvain-La-Neuve, Belgium) University of Neuchâtel, collected and identified by Dr Keller
Ganoderma applanatum	No87 (=K99) No78 (=NeuF269)	University of Neuchâtel
Ganoderma lucidum	MJFGL2	University of Neuchâtel
Ganoderma resinaceum	K1	University of Neuchâtel, collected and identified by Dr. Keller
Ganoderma tsugae	Chine	Kindly provided by Mr. Moos
Gloeophyllum trabeum	56	University of Neuchâtel

Species	Strain	Source
Grifola frondosa	MJFGF3 (=GfNE)	University of Neuchâtel (industrial strain)
	GfSt	Stamets (USA)
	GfJ	Japan
	MUCL 31544 (=B)	MUCL culture collection, Louvain-La-Neuve, Belgium)
	GfJ1397	Recombinant strains between the Japanese strain and the GFNE strain
	GfK00	University of Neuchâtel, collected and identified by Dr Keller
Hericium erinaceum	HerieriJP	Foreign industrial culture
Hypsizigus marmoreus		University of Neuchâtel
Laetiporus sulphureus	No41 I1	University of Neuchâtel
Lariciformes officinalis	No70	University of Neuchâtel
Lentinus conchatus	LcK	University of Neuchâtel
Lentinus edodes	MJFShii2F	University of Neuchâtel
Lepista nuda	4010	Somycel (France)
Lyophillum ulmarium	LyulmJmwGf2	University of Neuchâtel
Meripilus giganteus	K1	University of Neuchâtel, collected and identified by Dr Keller
Perenniporia fraxinea	K1	University of Neuchâtel, collected and identified by Dr Keller
Phellinus igniarius	MUCL 39333 MUCL 30499	MUCL culture collection, Louvain-La-Neuve, Belgium)
Pleurotus citrinopileatus	P.citrinow	Foreign industrial culture
Pleurotus eous	MJFPr1	University of Neuchâtel
Pleurotus ostreatus	HK35	Somycel (France)
Pleurotus tuber-regium	M	Kindly provided by Mr. Moos. Strain of African origin
Polyporus brumalis	No22	University of Neuchâtel
Polyporus ciliatus	No42 (=Neu 270)	University of Neuchâtel
Polyporus squamosus	Neu F271	University of Neuchâtel
Polyporus tuberaster	No53	University of Neuchâtel
Pycnoporus cinnabarinus	No71	University of Neuchâtel
Schizophyllum commune	No79 (=Neu F8) No80 (=Neu F14)	University of Neuchâtel
Sparassis crispa	Sc1F2	University of Neuchâtel
Sparassis laminosa	S.lam5	University of Neuchâtel
Stropharia rugosa-annulata	Kit	Commercialized kit for home cultivation
Trametes hirsuta	No11	University of Neuchâtel
Trametes gibbosa	No28	University of Neuchâtel
Trametes suaveolens	No73	University of Neuchâtel

Species	Strain	Source
Trametes versicolor	No2 Chine	University of Neuchâtel China

Table 3-1: Species, strains and sources of the studied mushrooms.

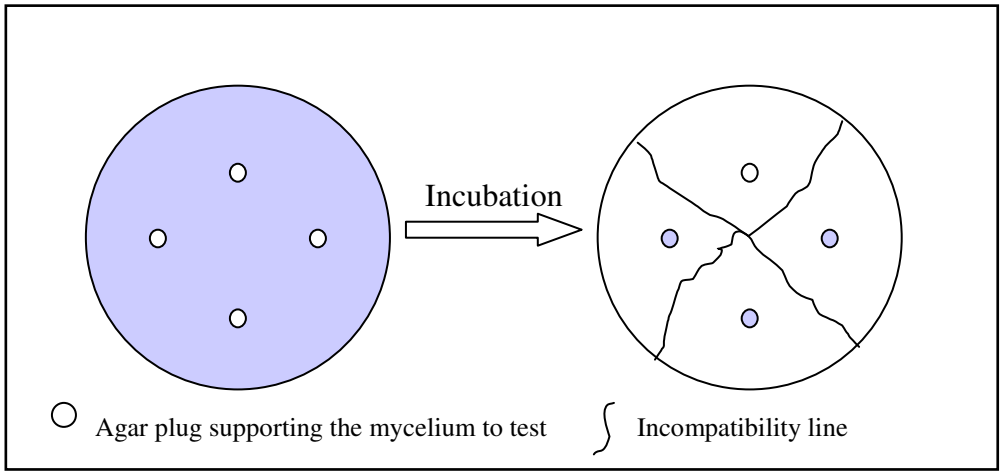

Figure 3-1: somatic incompatibility test.

Family	Species	Synonyms
Agaricaceae	***Agaricus brasiliensis*** S. Wasser et al.	
Bolbitiaceae	***Agrocybe aegerita*** (Brig.) Sing	*Agrocybe cylindracea* (Dc. ex Fr.) Maire *Pholiota aegerita* (Grig.) *Pholiota cylindracea* (Dc. ex Fr.) Gill.
Fistulinaceae	***Fistulina hepatica*** Schäff.: Fr.	
Ganodermataceae	***Ganoderma applanatum*** (Pers. ex Wallr.) Pat.	*Boletus applanatus* Pers. *Effvingia megaloma* (Lév) *Elfringia applanata* (Blum. et Nees:Fr.) Kuntze *Fomes applanatum* (Pers.;Wallr.) Gill. *Ganoderma lipsiense* (Batsch) Atk. *Ganoderma tornicatum* (Fr.) Pat. *Polyporus applanatus* Pers.:Wallr. *Polyporus megaloma* Lév.
	Ganoderma lucidum (Curt.: Fr.) Karst	*Boletus lucidus* W. Curt *Ganoderma polychromum*(Gilbertson und Ryvarden) *Ganoderma sessile* Murr. *Polyporus lucidus* (Curt.) Fr. *Polyporus polychromus*
	Ganoderma resinaceum Boud. in Pat.	
	Ganoderma tsugae Murr.	

Family	Species	Synonyms
Hericiaceae	***Hericium erinaceum*** (Bull.: Fr.) Quél.	*Dryodon erinaceus* (Bull. : Fr.) Karst.
Hymenochaetaceae	***Phellinus igniarius*** (L.:Fr.) Quél.	*Fomes igniarius* (L. :Fr.) J. Kicks. Fil.
Polyporaceae (s. stricto)	***Lentinus conchatus*** (Bull.: Fr.) Schroet.	*Lentinus carneotomentosus* (Batsh) Schröt. *L. torulosus* (Pers.) Lloyd *Panus conchatus* (Bull.: Fr.) Fr.) *P. flabelliformis* Schaef *P. torulosus* Pers.
	Lentinus edodes (Berk.) Sing.	*Agaricus edodes* Berk. *A. russatipes* Berk. Apud Cooke, Grevillea *Armillaria edodes* (Berk.) Sacc. *Collybia shiitake* Schroet., Gartenfl. *Cortinellus berkeleyanus* Ito et Imai *C. edodes* (Berk.) S. Ito et Imai *C. shiitake* (Tanaka) P. Henn *Lentinula edodes* (Berk.) Pegler. *Lentinus shiitake* (Schroet.) Sing. *L. tonkinensis* Pat. *Lepiota shiitake* (Schroet.) Tanaka *Mastaleucomyces edodes* (Berk.) O. Kuntze. *Pleurotus russaticeps* (Berk.) Sacc. *Tricholoma shiitake* (Schroet.) Lloyd. *Tricholomopsis edodes* Sing.
	Pleurotus citrinopileatus Sing.	Certain authors consider it as a variety of *Pleurotus cornocopia*
	Pleurotus eous (Berk) Sacc.	*Agaricus eous* M.J. Berk *Pleurotus salmoneo-stamineus* Vasiljeva
	Pleurotus ostreatus (Jacq.: Fr.) Kumm.	*Agaricus ostreatus* Jacq.: Fr. *Pleurotus colombinus* Quél. (sometimes considered as an other species)
	Pleurotus tuber-regium (Fr.) Sing.	*Agaricus tuber-regium* Fr. *Lentinus cyathus* Berk & Broome *L. tuber-regium* (Fr.) Fr.
Polyporaceae (s. lat.)	***Dendropolyporus umbellatus*** (Pers.:Fr.) Jül.	*Grifola umbellata* (Pers. ex Fr.) Donk *Polyporus umbellatus* Fr.
	Fomes fomentarius (L.:Fr.) Fr.	*Ungulina fomentaria* (L.: Fr.) Fr.
	Fomitopsis pinicola (Fr.) Karst.	*Fomes marginatus* (Fr.) Gill. *F. pinicola* (Fr.) *Polyporus marginatus* (Pers. ex Fr. *P. pinicola* (Fr.) *Ungulina marginata* (Pers. ex Fr.) Pat.

Family	Species	Synonyms
Polyporaceae (s. lat.)	***Gloeophyllum trabeum*** (Pers.: Fr.) Murr.	*Lenzites trabea* (Pers. ex Fr.) Fr.
	Grifola frondosa (Dickson: Fr.) S.F. Gray	*Polypilus frondosus* (Dickson: Fr.) Karst *Polyporus frondosus* Fl. Don.
	Laetiporus sulphureus (Fr.) Murr.	*Polyporus sulphureus* (Bull. ex Fr.) Fr.
	Lariciformes officinalis (Vill. : Fr.) Kotl. & Pouz.	*Agaricum officinale* (Vill.: Fr.) Donk. *Boletus purgans* Pers. *Fomes laricis* (Jack.) Murr. *F. officinalis* (Vill.:Fr.) Faull. *Fomitopsis officinalis* (Fr.) Bond. & Sing. *Polyporus officinalis* Vill. (Fr.)
	Meripilus giganteus (Pers.:Pers.) Karst.	*Polyporus giganteus* Pers.: Fr.
	Pereniporia fraxinea (Fr.) Ryv.	*Fomitopsis cytisina* (Berk.) Bond. & Sing. *Haploporus cytisinus* (Berk.)
	Polyporus brumalis Fr.	
	Polyporus ciliatus Fr.	*Polyporus lepideus* Fr.
	Polyporus squamosus Fr.	*Melanoporus squamosus* (Huds.) Pat.
	Polyporus tuberaster (Pers. : Fr.) Fr.	*Polyporus forquignoni* (Quél.) Sacc. *P. lentus* Berk.
	Pycnoporus cinnabarinus (Jacq. : Fr.) Karst.	*Trametes cinnabarina* (Jacq. ex Fr.) Fr.
	Trametes gibbosa (Pers.: Fr.) Fr.	
	Trametes hirsuta (Wulf. ex Fr.) Pil.	
	Trametes suaveolens (Fr.) Pil.	
	Trametes versicolor (Fr.) Pil.	*Boletus versicolor* Rostk. *Coriolus versicolor* (L.: Fr.) *Polyporus versicolor* (L. :Fr.) *Polystictus versicolor*
Schizophyllaceae	***Schizophyllum commune*** Fr.: Fr.	
Sparassidaceae	***Sparassis crispa*** Wulf.: Fr.	
	Sparassis laminosa Fr.	*Sparassis brevipes* Krombh.
Strophariaceae	***Stropharia rugosa-annulata*** (Farlow ap. Murr.) K. & R.	*Geophila rugosa-annulata* *Nematoloma ferrii* (Bres.) Sing. *Stropharia elegans* Murr. *Str. ferrii* Bres.

Family	Species	Synonyms
Tricholomataceae	***Flammulina velutipes*** (Curt. ex Fr.) Sing.	*Agaricus nigripes* Bull. *Agaricus velutipes* Curt. ex Fr. *Collybia velutipes* Quél. *Pleurotus velutipes* Quél.
	Hypsizigus marmoreus (Peck) Bigelow	*Lyophillum shimeji*
	Lepista nuda (Bull.: Fr.) Cke.	*Rhodopaxillus nudus* (Bull.: Fr.) Mre. *Tricholoma nudum* (Bull.: Fr.) Kumm.
	Lyophillum ulmarium (Bull.: Fr.) Kühn.	*Eulyophyllum ulmarium* *Hypsizygus tesselatus* (Bull.: Fr.) Sing. *Hypsizygus ulmarium* *Pleurotus ulmarius* (Bull.: Fr.) Quél.

Table 3-2: classification and synonyms of all the species tested for culture, selection test, biological test or modulation.

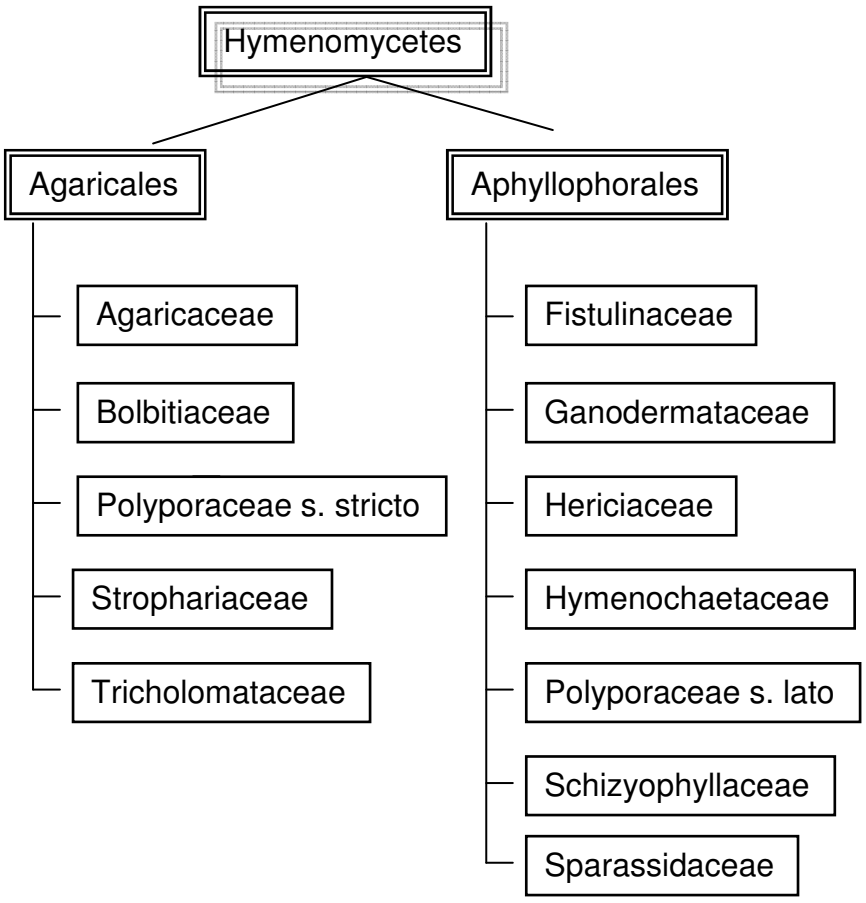

Figure 3-2: SYSTEMATICS: repartition of the Hymenomycetes mushrooms studied.

3.2 Culture

3.2.1 Inoculum

The used inoculum was generally birdseeds (seeds of the grass *Phalaris canariensis*) colonised by the mycelium to inoculate. Other cases are mentioned punctually. Organic birdseeds were soaked for12 hours in water or water with 2% malt. After draining the seeds were poured into a glass or plastic jar and sterilised in the autoclave 90 minutes at 121°C. The mycelium was then inoculated on the cold seeds with agar plugs or the seeds of an already colonised inoculum and allowed to grow at 25°C in the dark.

3.2.2 Substrates preparation and inoculation

3.2.2.1 Main substrates

Most of the preliminary tests were realised on 4 basic substrates varying in their C/N coefficient from 75 to 265 (table 3.3): substrates A, B, C and D, in the order of increasing values (or decreasing nitrogen content). Substrate A was designed initially for *Agrocybe aegerita*, substrate B designed for *Grifola frondosa*, substrate C for *Lentinus edodes* and D for *Sparassis crispa*. These substrates were generally prepared at Fermenta SA, Payerne, Switzerland according to Job and Schiff Giovannini (2004): the base constituents of the substrates were mainly sawdust and straw with various complements (table 3.3) the substrates were mixed and soaked during 24 hours in water with pH adjusted to about 6.5-6.8. The final C/N coefficient of substrates A, B and C were obtained by the adjunction of NO_3NH_4. After having drained off the substrate, it was packed into polypropylene bags with incorporated filter (bottom area 12 x 20 cm, height 35 cm). Bags of substrates A, B and C weighted about 1.8 kg while bags of substrate D weighted about 2.2 kg. The substrates were then pasteurised 10 hours at 90°C.

Substrate type	Wheat straw [% dry weight]	Sawdust [% dry weight]	Birdseeds [% dry weight]	Wheat flour [% dry weight]	Compost [% dry weight]	Moisture [% fresh weight]	C/N coefficient
A	40	40	15.7	3	1.3	73-76	75
B	80	20	0	0	0	73-76	90
C	40	40	10	0	10	68-71	125
D	10	60	10	0	20	68-71	265

Table 3-3: composition of the substrates with their final water content and C/N coefficient.

L. conchatus was also tested on Dcaf which is a D substrate with Chinese reed instead of wheat straw.

Some cultural tests were performed in polypropylene 2 litres cylindrical pots, 15 cm height, 15 cm diameter in which various quantities of substrates (500g to 800g) substrate was incorporated before being sterilised at 121°C during an hour. It is the case of *G. applanatum* on substrates A and B, of *P. tuberaster* and *P. ciliatus* which were tested on the four basic substrates A, B, C and D in pots only.

When followed by the letter "m" it means that these substrates were realised at the University of Neuchâtel instead of at Fermenta. In theses cases pH was not adjusted and substrates were always filled in 2 litres cylindrical pots before sterilisation.

3.2.2.2 Substrates composition for A. brasiliensis culture development

All the experiments with *A. brasiliensis* were performed in polypropylene pots. Casing was performed on all substrates (table 3.4) except abl.2 after total invasion of the substrate with an industrial compost at pH about 6.5-7 (COOP, Switzerland) on AB1 and AB2 and with sterilised silt-chalky soil at pH 7-8 on Abl.1, Abl.3, Abl.4 and Abl.5.PH was measured in the following manner: the substrate to be tested was placed in a recipient with 2.5 times its volume of desionised H_2O and the whole was mixed. After an hour, during which the mixture was shaken several times, measures were realised with the pH meter and the pH paper.

Substrate type	**B substrate colonised by *G. frondosa* mycelium [g]**	**B substrate colonised by *F. fomentarius* mycelium [g]**	**Compost (Coop, Switzerland) [g]**	**Industrial mixture* for *G. frondosa* [ml]**	**Industrial mixture* for *A. ae*gerita [ml]**	**Tap water**
AB1	100		100			600
AB2	70		180			600
Abl.1		90	90	1		375
Abl.2		90	90		1	375
Abl.3		90	90			375
Abl.4		50	200	0.25		375
Abl.5			400			

*Table 3-4: substrate composition for A. brasiliensis culture development. * Composition is intentionally not given.*

3.2.2.3 Substrates compositions for *L. officinalis* culture development

Table 3.5 and 3.6 show substrate composition elaborated especially for *L. officinalis* and contained in polypropylene pots.

Substrate type	Sawdust [g]	Straw [g]	Reed straw [g]	Compost [g]	Rice powder [g]	Rice [g]	A substrate colonised by *L. officinalis* mycelium [g]	B substrate colonised by *L. officinalis* mycelium [g]	D substrate	Pure lignin [g]	Malt [g]	Tap water [ml]
Lo 1	100									0.56	1	650
Lo 2	50	50									1.4	500
Lo 3	80		20									500
Lo 4	80		10		10							500
Lo 5	35		60		5					1	1.2	600
Lo 6							280			0.5	0.83	520
Lo 7								280				520
Lo 8							280				1.8	520
Lo 9				45		15			250			520

Table 3-5: substrates composition for L. officinalis culture development

Substrate type	Sawdust [g]	Straw [g]	Compost [g]	Rice [g]	Malt [g]	Asparagin D/L [g]	Mixture for *G. frondosa* [g]	$ClNH_4$ [g]	Yeast extract [g]	Tap water [ml]
LO	56	140	56	28	2.5	0.4	0.25			520
LO2	40	100	60	20	3	0.45		0.45	1.2	600

Table 3-6: substrates composition for L. officinalis culture development

Two pots of LO were also inoculated with the 78 and 87 strains of *G. applanatum* and two with *S. rugosa-annulata* FTA wild strain.

3.2.2.4 Substrates composition for *S. rugosa-annulata* culture development

The two substrates prepared and placed in polypropylene pots were RC composed of 100% reed straw dampened with tap water, and RCM composed of 100% reed straw dampened with tap water mixed with 7g/l malt.

3.2.3 Incubation and fruiting conditions

Incubation was usually performed at 25°C +/- 1°C in the dark or at room conditions (natural light, room temperature varying between about 20°C and 23°C). Transfer to the fructification room was realised at different times according to the strains, but generally after total invasion of the substrates. Fruiting was allowed in a ventilated room (Cenviron, Canada) at 18-19°C with high humidity level (91-100%) with 6-hour artificial light cycles per 24 hours.

T. suaveolens was also placed at 9°C and at 16°C for fruiting stage.

Ganodermataceae species were also cultured at room temperature and natural light.

For *P. tuber-regium*, incubation of the bags inoculated with Neu-M strain was performed at laboratory room conditions (cycle natural day/night; temperature varying from 20 to 23°C). Incubation of the bags inoculated with NEU-MF1 strain was done in a dark room at 25°C +/- 1. For fructification induction, sclerotia were generally buried in humid compost (COOP, Switzerland) in a polypropylene 2 litres cylindrical pots (15 cm height, 15 cm diameter) and placed either in room conditions or at 29°C in a desiccator's vessel with water on the bottom and constant incoming air, or in the standard fructification room. CO_2 shocks were provoked by covering the propylene pots with parafilm.

3.2.4 Harvesting, yield and biological efficiency (BE)

Harvesting was usually performed when caps where fully mature; however fungal contamination sometimes forced us to an early harvest. Yield was expressed as a percentage and determined as the ration of fresh sclerotia or fruit body weight per kg of fresh substrate. The estimated biological efficiency (BE) is expressed as a percentage and is determined as the ratio of the fresh sclerotia or fruit body weight per kg of dry substrate.

3.3 Selection test

3.3.1 Strains

Grifola frondosa.- GfNE, GfSt, GfJ, GfB (=MUCL 31544), GfNE-J1397 (table 3.1).
Fomitopsis pinicola.- FpK1, FpK2, FpK3, MUCL 30677 and MUCL 30544 (table 3.1)
Trichoderma strain was isolated from *G. frondosa* GfNE contaminated substrate B bags and determined as *Trichoderma viride* Pers. by optic microscope characteristics.

3.3.2 Growth measurements on 2% AM medium

For measuring mycelium front advance, a few days precultivated agar slant supporting the strain was inoculated in the center of the Petri dish containing 2% AM medium. Mycelium was allowed to grow at 18°C, 25°C and 30°C in the dark. Mycelial elongation was measured (mm) between day 2 and day 5 and between day 5 and day 8 after inoculation, on 4 radial lines drawn before inoculation. Two replicates were realized.

3.3.3 Tubes preparation

For testing the strains' invasion speed and resistance in different substrates, both Pyrex glass tubes and translucide, autoclavable Teflon® FEP tubes (Fisher Scientific, Wohlen, Switzerland) open on both sides were used. The Teflon tubes, internal diameter 14,4 mm, were cut at a length of approximately 15 cm. Both cotton and metallic caps for 16mm test tubes were used to close the two open sides after filling with the different pasteurised substrates. A, B, C and D substrates (table 3.3) were used. The tubes were sterilised in an autoclave at 121°C for ½ hour. The strain to be tested was inoculated sterilely with a 12 mm diameter disk of young mycelium on 2%AM medium at one end

of the tube, placed in a box with water on the bottom to maintain humidity and the mycelium was allowed to grow in the dark at 25°C.

3.3.4 Vegetative resistance tests in Petri dish

For testing resistance of the strains, both tests on 2%AM medium in Petri dish and on the four different substrates in tubes were realised. Resistance of the strains on 2% AM medium was evaluated by inoculating a young conidiating mycelium plug from *T. viride* at the other end of the Petri dish than the tested species colony with different diameters: 2, 4 and 6 cm and on the mycelium itself. Incubation was realised in the dark at 25°C and *T. viride* progression observed daily until no more changes happened. Resistance was determined by the time, in days, the strains could resist *T. viride* when in contact.

3.3.5 Experiments

3.3.5.1 *Experiment 1*- evaluation of the antagonism between 5 strains of *G. frondosa* and *Trichoderma* spp on substrates A, B, C and D

The substrates were filled into the Teflon® tubes arbitrarily to a length of 7cm after maximum compression and then natural extension, however not with the same density as in the industrial bags. Cellulose caps were used. Sterilisation induced the substrates to swell slightly up to 7.4 cm. Measures of invasion rate were realised twice after *G. frondosa* inoculation and mean value represented the average between faster and slower growth. 1ml of *Trichoderma viride* conidial suspension was inoculated on the other side of the tube 54 days after the start of the experiment after total invasion of the substrates. *Trichoderma* progression in the colonised substrate was observed and noted five times between day 60 and day 87. Resistance was determined by *T. viride* progression in the substrate fully colonised by *G. frondosa* in percent. 3 replicates.

3.3.5.2 *Experiment 2*- influence of substrate quantity on *F. pinicola* FpK2 strain on substrate A

1g, 3g, 5g, 7g and 9g substrate A were filled in Pyrex tubes with cellulose caps covered with parafilm. *F. pinicola* was inoculated at one side of the tube with an agar plug and colonisation rate was measured twice (6 and 9 days after inoculation) and was the average between faster and slower growth measured. *T. viride* was inoculated on the other side of the tube after total invasion of the substrate by *F. pinicola* once with an agar plug 20 days after the start of the experiment and once with a conidial suspension 21 days later. *Trichoderma* penetration in the substrate colonised by *F. pinicola* was measure 47 days after the start of the experiment. Two replicates were done.

3.3.5.3 *Experiment 3* - influence of B substrate quantities on 5 *G. frondosa* strains resistance.

Teflon® tubes were filled with 3g, 5g, 7g or 9g Bm substrate compressed at the maximum and then allowed to extend naturally. After autoclaving the substrate lengths were about 35mm, 55mm; 75mm and 90mm for respectively 3g, 5g, 7g and 9g

substrate, with variations of a few mm between tubes. Three lines were drawn along the tubes to determine place of measures for colonisation rate and *Trichoderma* progression. *G. frondosa* strains were inoculated in the same manner as previously. Measures of colonisation rate were realised 6 times along these lines. 41 days after the start of the experiment 500µl of a suspension of *Trichoderma viride* conidia was inoculated in each tube. The number of conidia –counted by a dilution test technique- was between 2.8 and 3.6 x 10^7 spores per 100µl. *Trichoderma* penetration in the substrate colonised by *G. frondosa* was measured along the lines by observation of the green conidiation on day 57, and 69. Three ml of a conidia suspension was added on day 70. *Trichoderma* progression was measured on day 78 and on day 103 Two replicates were realised.

3.3.5.4 *Experiment 4*- influence of B substrate quantities on 5 *F. pinicola* strains resistance

The tubes were prepared in the same manner as in experiment 2. Substrate colonisation rate measures and *Trichoderma* inoculation were realised the same days as in experiment 3, except that it was only inoculated once. *Trichoderma* penetration was measured in the same manner but 52 days after the start of the experiment. Two replicates per treatment were done.

3.3.5.5 *Experiment 5*- influence of colony size of *G. frondosa* strains GfSt and GfJ on the resistance

24 tubes were filled with 7g substrates with the same density as in the culture bags. Substrate length in the tubes was originally 67mm but autoclaving induced the elongation of the substrate up to 72mm in some tubes. Six lines were drawn along the tubes. Twelve tubes, noted by the letters "a" to "l", were inoculated with each of the strains. 0.3ml of *Trichoderma* spore solution was inoculated at the opposite ends of the substrate colonisation (Table 3.7). Tubes "k" and "l" were used as blanks.

Species and strains	**days**				
	Tubes a-b	**Tubes c-d**	**Tubes e-f**	**Tubes g-h**	**Tubes i-j**
***G. frondosa* GfSt**	6	20	35	58	58
***G. frondosa* GfJ**	6	20	35	65	65
***F. pinicola* FpK1**	5	7	10	20	25
***F. pinicola* FpK2**	5	7	10	16	21

Table 3-7 : Time of inoculation of T. viride *expressed in days after the start of the experiment.*

3.3.5.6 *Experiment 6*- influence of the colony size of *F. pinicola* FpK1 and FpK2 on the resistance

The experiment was realised like the previous one except than 3 longitudinal lines were drawn on each tube. Days of *Trichoderma* inoculation are shown in table 3.7.

3.3.5.7 *Experiment 7*-final evaluation of invasion rate and resistance of *G. frondosa* and *F. pinicola* strains on substrate A, C and D

In each tube 9g of each mentioned substrate was filled at the same density as in the culture bags. Six lines were drawn along the tubes. Six strains of *G. frondosa* –GfB, GfSt, GfJ, GfNE, GfNE-J1397 and GfK00- and the 5 usual *F. pinicola* strains were inoculated in the tubes with 2 replicates per treatment. Invasion rate was measure twice until day 13, and *Trichoderma* inoculated on day 30 after total invasion of the substrates. *Trichoderma* penetration was measured in the same manner as previously described.

3.3.6 Statistical test

The results having been interpreted according to the Student's-T test (Job and Rajchemberg, 1988) and the ANOVA test (Diorio et al., 2003)

3.4 Activity tests

3.4.1 Extractions procedures

To reduce chemical modifications, fruit bodies were generally frozen immediately after collection in order to be lyophilised. After lyophilisation, mushrooms were kept in a freezer at –20°C until extractions were done in a cold and dark room (5°C). However this was not applied to the 8 first tested species in the Institute of Pharmacognosy and Phytochemistry of the University of Lausanne (see below). In these cases mushrooms were mostly dried, the extraction realised at room temperature and extracts were kept at room temperature.

Air dried or lyophilised (Lyolab A, LSL Secfroid, with Pascal vacuum pump, Alcatel) fruit bodies were extracted three times for 24 hours with DCM and 3 times with MeOH. The solvents were evaporated under reduced pressure on a Büchi RE 111 rotavapor equiped with a Büchi 461 Water Bath and a Büchi B-171 Vacobox or with a Büchi rotavapor equipped with a Vacuubrand diaphragm vacuum pomp type MZ 2C and a Glaskeller vakuum-Regler at a maximum bath temperature of 40°C.

A hot water extract was realised with dried *Pl. ostreatus*, and lyophised *A. aegerita* and *S. crispa*. The mushrooms were powdered and mixed with distilled water then boiled for 1 hour. They were then filtered before evaporation of the water.

Extracts were then lyophilised and kept in a freezer at –20°C until use for biological tests or chromatographic analysis.

3.4.2 Species extracted

Yield between fresh, dried or lyophilised fruit bodies is presented in table 3.8. *A. aegerita, F. hepatica, F. velutipes, G. lucidum, G. frondosa, H. erinaceum, H. marmoreus, K. mutabilis, L. edodes, L. ulmarium, P. nameko, P. citrinopileatus, P. eous, P. eryngii, P. ostreatus, S. crispa* and *S. laminosa* were obtained by the cultivation method developed by Dr Daniel Job. *L. nuda* came from the St-Sulpice caves. The other

species' cultures were developed during this work (see chapter 4.1)**.**Yield between dried mushroom and extract is presented in table 3.9.

Species	Fresh weight [g]	Dry weight [g]	Yield [%]	Average yield [%]
Agrocybe aegerita	309.8	21	6.8	7.9
	606*	43	7.1	
	285.8	28.1	9.8	
Fistulina hepatica	378	25	6.6	8.4
	445	43.2	9.7	
	250	22.2	8.9	
Flammulina velutipes	104.5	19.2	18.4	11.5
	64	9.4	14.1	
	300	22.7	7.6	
	80.1	9.7	12.1	
	63.8	5.1	8	
	14.1	1.2	8.5	
	12.6	1	7.9	
	7	1.1	15.7	
Ganoderma applanatum	100.7	44.5	44.1	43.8
	9.2	4	43.5	
Ganoderma lucidum	106.8	33	30.9	32.2
	35.8	8.8	24.6	
	49.7	21	42.3	
	33.6	10.4	31	
Grifola frondosa NE (dried)	177	14.1	8	
Grifola frondosa 1	431	31,3	7,3	
Grifola frondosa 2	452	23,5	5,2	
Grifola frondosa 3	486	43.1	8.9	
Grifola frondosa 4	372	30	8.1	
Grifola frondosa 5	437	34.1	7.8	
Grifola frondosa 6		45		
Grifola frondosa 7	252	30.5	12.1	
Grifola frondosa 8	525	62.9	12	
Grifola frondosa 9	262	30.4	11.6	
Grifola frondosa 10	910.5	110	12.1	
Grifola frondosa 11	361	28.7	8	
Grifola frondosa 12	400.5	43	10.7	
Grifola frondosa 13	250	65.6	26.2	
Grifola frondosa 14	175	29.5	16.9	
Grifola frondosa 15	238	33.4	14	
Grifola frondosa				11.26

Hericium erinaceum	399	34.2	8.6	9.1
	311.6	30.5	9.8	
	300	26.3	8.8	
Hypsizigus marmoreus	141.8	12.3	8.7	10.8
	195	22.1	11.3	
	36.8	4.6	12.5	
Species	**Fresh weight [g]**	**Dry weight [g]**	**Yield [%]**	**Average yield [%]**
Lentinus conchatus	44.7	4.7	10.5	12.05
	153.2	19	12.4	
	36	4.6	12.8	
	68.7	10.4	15.1	
	203.8	21.3	10.5	
	50	5.5	11	
Lentinus edodes (dried)	166	15.5	9.3	9.5
	156	15.1	9.7	
Lyophillum ulmarium	165.5	13.2	8	8
Meripilus giganteus	184	18.4	10	10
Pleurotus citrinopileatus	368	33.8	9.2	9.35
	390	36.9	9.5	
Pleurotus eous	138.9	9.5	6.8	7.35
	175	13.8	7.9	
Pleurotus eryngii	253	20.7	8.2	8.2
Pleurotus ostreatus	159.5	15	9.4	9.15
	678.7	60.7	8.9	
Polyporus ciliatus No42	26.8	4	14.9	12.7
	30.3	3.3	10.9	
	9	0.7	7.8	
	28.3	2.5	8.8	
	19	2.9	15.3	
	18?	3.2	17.8?	
	15.4	1.7	11	
	19.6	2.9	14.8	
Polyporus squamosus	170.5	13.5	7.9	7.35
	150	13.5	9	
	252.3	17.7	7	
	353	23.5	6.7	
Polyporus tuberaster	38	3.8	10	9.7
	68.5	6.3	9.2	
	65	6.3	9.7	
	33.5	3.7	11	
	16.5	1.5	9.1	
	30	2.7	9	
	9.2	1	10.9	
	11.8	1.1	9.3	
	20	1.8	9	
Schizophyllum commune No80	24.8	4.5	18.1	21.5
	47.2	8.9	18.9	
	9.4	2.7	28.7	

	9.6	1.6	16.7	
	9.6	1.6	16.7	
	113.5	22.8	20.1	
	57	10	17.5	
	8	3.4	42.5	
	14	2.8	20	
	57	9	15.8	
Species	**Fresh weight [g]**	**Dry weight [g]**	**Yield [%]**	**Average yield [%]**
Sparassis crispa	160*	13.7	8.6	9.2
	246	24.8	10.1	
	246*	33.2	13.5	
	370	17	4.6	
Sparassis laminosa	71.6	11	15.4	9.9
	135.8	13	9.6	
	51.5	3.8	7.4	
	55.5	4	7.2	
Trametes gibbosa	15	5	33.3	23.4
	31.7	6.8	21.5	
	8.7	2.5	28.7	
	22.2	3.5	15.8	
	162	29	17.9	
Trametes hirsuta	16.5	3.1	18.8	21.7
	90.9	12.7	14	
	31.8	8.2	25.8	
	56.8	16	28.2	
Trametes suaveolens	63	38.4	61	50.3
	82	35.6	43.4	
	111	51.6	46.5	
Trametes versicolor	17	3.5	20.6	20.6

Table 3-8: yield between fresh and dried mushroom species

Species	**Quantity extracted [g]**	**Solvant**	**Extract [g]**	**Yield [%]**
Agrocybe aegerita	20	DCM:MeOH	1.8835	9.4
		H2O	9.9661	49.83
Agrocybe aegerita (air dried)		DCM	4.4	
		MeOH	20.88	
Flammulina velutipes	20	MeOH	3.7417	18.7
Ganoderma lucidum (air dried)		DCM	4.93	
		MeOH	4.9	
Grifola frondosa GfNE cap (air dried)	20	DCM	0.92	
		MeOH	15.57	
Grifola frondosa GfNE stipe (air dried)	20	DCM	0.81	
		MeOH	9.95	
Grifola frondosa GfNE stipe 2nd extract		DCM		
		MeOH	89.42	

G. frondosa mycelium in substrate		DCM MeOH	3.94 8.61	
Grifola frondosa 1	20	DCM MeOH	0,3803 1,5365	1,9 7,7
Grifola frondosa 2	20	DCM MeOH	0,3439 1.4417	1,7 7.2
Species	**Quantity extracted [g]**	**Solvant**	**Extract [g]**	**Yield [%]**
Grifola frondosa 3	20	DCM MeOH	0.3363 3.7931	1.7 19
Grifola frondosa 4	20	DCM MeOH	0.3603 4.1056	1.8 20.5
Grifola frondosa 5	20	DCM MeOH	0.3533 4.6475	1.8 23.2
Grifola frondosa 6	20	DCM MeOH	0.3128 2.9876	1.6 14.9
Grifola frondosa 7	20	DCM MeOH	0.3325 2.7585	1.7 13.8
Grifola frondosa 8	20	DCM MeOH	0.3776 3.1596	1.9 15.8
Grifola frondosa 9	20	DCM MeOH	0.5344 3.0398	2.7 15.2
Grifola frondosa 10	20	DCM MeOH	0.442 2.1061	2.2 10.5
Grifola frondosa 11	20	DCM MeOH	0.3276 2.5186	1.6 12.6
Grifola frondosa 12	20	DCM MeOH	0.4338 2.0016	2.2 10
Grifola frondosa 13	20	DCM MeOH	0.2995 3.0244	1.5 15.1
Grifola frondosa 14	20	DCM MeOH	0.3813 4.3204	1.9 21.6
Grifola frondosa 15	20	DCM MeOH	0.7060? 3.9007	3.5 ? 19.5
Lentinus conchatus	40g	DCM MeOH	0.2411 3.0962	0.24 7.7
Lentinus edodes (air dried)		DCM MeOH	2.06 19.57	
Lentinus edodes	20	DCM:MeOH	2.4616	12.3
Lepista nuda		DCM MeOH	3.28 27.83	
Pleurotus citrinopileatus	20	DCM :MeOH	1.6536	8.3
Pleurotus eous (air dried)		DCM MeOH	1.28 15.29	
Pleurotus eous	20	H2O DCM :MeOH	3.7226 3.7226	18.6 18.6
Pleurotus eryngii	20	DCM MeOH	0.5942 4.5132	3 22.3
P. eryingii (air dried)		DCM	1.45	

		MeOH	25.9	
Pleurotus ostreatus (air dried)		DCM MeOH	1.23 14.48	
Pleurotus ostreatus	20	DCM:MeOH	1.4154	7.1
Polyporus squamosus	37.6	MeOH	3.6093	9.6
Polyporus tuberaster	20	MeOH	2.7244	13.6
Species	**Quantity extracted [g]**	**Solvant**	**Extract [g]**	**Yield [%]**
Schizophyllum commune No80	20	DCM MeOH	0.1888 1.4344	0.9 6.5
Sparassis crispa	20	H2O DCM :MeOH	1.9158 4.4397	9.6 22.2
Sparassis laminosa	20	DCM :MeOH	1.9457	9.7

Table 3-9: species extracted with their yield in different solvent.

3.4.3 Biological tests

3.4.3.1 Antifungal tests against *Trichoderma viride*,

Preliminary assays: To determine which agar concentration was the best for a thin layer on silicium, 3 AM medium containing respectively 5g/l, 10g/L and 15g/l agar were poured on TLC. For incubation conditions testing, plates inoculated with the different target species were incubated either at 25°C in the etuve with light, or in the dark incubation room, in the etuve at 30°C or 37°C in the dark. *Trichoderma viride* strain F16 was the target organism.

Final technique: A spore solution (strain F16) was prepared a few days before the tests by putting a certain quantity of young culture conidia in sterile water. After shaking for separating the conidia, a dilution technique was used to count spore numbers by ml. The solution was diluted until obtention of a solution containing approximatively 10^{-6} conidia per ml. Extracts (200µg) were applied on the TLC with the DU-SIN surface detergent (Dudler, Switzerland) as positive control diluted at 1% applied at 20µl for *Trichoderma*. Two plates were realised in parallel. 10 ml of the diluted conidia suspension was added in sterile 2% AM medium at 60°C. The medium was spread directly in a thin layer over one of the TLC plates (maintained on a heating plate at 35°C) with a sterile Pasteur pipette, while the other was observed under UV light at 366nm and nm then revealed with Godin reagent. The incubation conditions of the plates for the biological tests were 25°C with light for *Trichoderma viride*. The fungi were then killed by spraying with H_2SO_4 or a 94% ethanolic solution.

3.4.3.2 *Bacillus subtilis* bioassay (Hamburger & Cordell, 1987)

20µg or 100µg of each extract was applied on glass-backed silica gel GF254 TLC plates. Chloremphenicol was used as positive control at 0,01µg. Plates were developed in an appropriate solvent system: ligroïne-AcOEt 1:1 for DCM extracts and $CHCl_3$-MeOH-H_2O 65:35:5 for MeOH extracts. After drying, an inoculum of *Bacillus subtilis* strain ATCC 6633 in Agar-LB medium at 40°C was applied in a thin layer. The inoculum was prepared in the following manner: the bacteria was inoculated in a liquid

LB medium and left at 25°C under constant shaking. Of this solution 1ml (approx. 10^8 cells/ml) was added to 50ml of Agar-LB medium at 40°C which was used for applying the TLC. The chromatograms were incubated overnight at 30oC in a moist atmosphere in a polyethylene box. Plates were revealed by spraying an aqueous methylthiazoyldiphenyl-tetrazolium bromide (Fluka) solution (2,5 mg/ml). After a few minutes, inhibition spots appeared clearly on a blue background.

3.4.3.3 *Candida albicans* bioassay (Rahalison et al., 1991)

Each extract was tested at a concentration of 100µg on glass-backed silica gel GF254 TLC plates. Plates were prepared in the same manner as for the *B. subtilis* test, with a few differences: the positive control was amphotericin B (1µg). The inoculum was prepared in the following manner: a colony of *Candida albicans* (given by the CHUV) was inoculated in 50ml liquid Sabouraud medium contained in a 250ml flask and agitated during 15 hours. Of this solution 1ml was added to 50ml of Sabouraud medium and agitated for 8 hours. 1 ml was taken and added to an 50ml of an Agar-Malt medium at a temperature of 40-48°C. 20 ml (approx. 10^5 cells/ml) of this inoculum was poured on the TLC with a sterile pipette. Plates were incubated one night at 30°C in humid atmosphere in polyethylene boxes. TLC were sprayed with the tetrazolium salt and incubated during 4 hours in humid atmosphere to develop colour.

3.4.3.4 *Cladosporium cucumerinum* bioassay (Homans and Fuchs, 1970)

100µg of each extract were applied on TLC aluminium-backed silica gel GF_{254} plates and developed as for the *B. subtilis* assay with some differences: propiconazole (0,01g) was used as positive control. 2ml of a lyophilised spore solution were inoculated in 20ml of Sabouraud medium. It was shaken several times during one half hour and sprayed on TLC which was then incubated in a humid atmosphere at room temperature during 72 hours. Fungi were killed by spraying 94% ethanol. Inhibition zones appeared white on dark grey background.

3.4.3.5 *Aedes aegyptii* bioassay (Cepleanu et al., 1994)

Eggs of *Aedes aegyptii* on filter paper were provided by the Swiss Tropical Institute in Basel. A small portion of the paper carrying the eggs was incubated 24 hours in 25°C river water. Twenty larvae at the 2nd larval stage were collected and placed in a test tube containing 5,5 mg of the crude extract dissolved in DMSO. The volume was adjusted with water to 10ml to give an extract solution of 500 ppm with a DMSO concentration of less than 1%. Tubes were incubated at 27°C and observed after 30 minutes and 24 hours. 100% larval mortality indicated an extract activity. The positive control was β-asarone at a concentration of 15 µg/ml.

3.4.3.6 *Biomphalaria glabrata* bioassay (Hostettmann et al., 1982)

Extract were suspended in distilled water. Two aquarium bred snails of the species *B. glabrata* approximately 1cm in diameter were placed in an aqueous solution containing 400µg/ml of the extract. Mortality after 24 hours was determined by observing

heartbeats with the binocular. Bayluscide was used as a positive control at a concentration of 0,3 µg/ml.

3.4.3.7 Antiradicalar bioassay (Cuendet, 1995)

TLC were prepared in the same manner as for the *Cl. cucumerinum* assay and were sprayed with a DPPH radical [1,1-diphenyl-2-picrylhydrazyle or 1,1-diphanyl-2-(2,4,6-trinitrophenyl)hydrazyl] at 0,2% methanol solution. Plates were observed after half an hour. Antiradicalar activities appeared yellow-white on purple background by reduction of the DPPH radical.

3.4.4 Chromatographic methods

3.4.4.1 Thin layer chromatography (TLC)

TLC has been used for biological bioassays, to determine the methods to separate constituents and to test fractions after a column. For separation on normal phase, pre-coated silica-gel $60F_{254}$ aluminium sheets (Merck) or glass were used. They were developed in conventional TLC chambers. The mobile phases were as EtOAc-Hex 7:3 for lipophilic constituant and DCM-MeOH 9:1 for hydrophilic constituant. To test fractions obtained after fraction 4 *G. frondosa* DCM extract separation RP-18 TLC plates were used. Detection of the compounds were realized by fluorescence extinction under UV light at 254 nm, fluorescence under UV light at 366 nm and by revelation with the following chemical reagents: Godin's reagent (Godin, 1954), Ninhydrin/ chlorhydric acid reagent, Dragendorff reagent (Auterhoff and Kovar, 1985) and Gibbs reagent, and with particular reagent like a silver nitrate reagent

Chemical reagents:

- **Godin's reagent** (Godin, 1954): an equal volume of an 1% ethanolic solution of vanillin (Merck 1.08510) and a 3% aqueous solution of $HClO_4$ are mixed. The plates are sprayed with reagent and afterwards with a 10% ethanolic solution of H_2SO_4. The compounds appear as differently colored spots after heating the TLC plate up to 100°C.
- **Dragendorff reagent**: it is composed of 2 solutions. To obtain solution A 0.85g bismuth nitrate in 10ml cristallisable acetic acid and 40ml water. To obtain solution B 8g potassium iodure are dissolved in 20ml water. Just before use 5 ml of solution A is mixed with the same volume of solution B and completed to 100ml with water. After spraying, reaction is immediate, showing reductive compounds (alkaloids for instance) as orange or green spots.
- **Gibbs reagent**: the TLC is sprayed with a 1% dichloro-2,6-quinonechlorimide ethanolic solution and exposed to the action of ammoniacal vapors. Phenols appear as blue spots.

3.4.4.2 Open column chromatography

Chromatography on silica gel: Columns have been prepared as follow: stationnary (silica gel 60) and mobile phases were mixed before being introduced in the column. After stabilisation, the sample was adsorbed on 2-3 times its weight of stationnary phase and deposited on the column covered by a thin layer of mobile phase. The separation of

the DCM *G. frondosa* extract was performed on a 450x30 mm column with 35-75µm diameter particles (Merk). The extract was fractionated by a solvent gradient of increasing polarity: ligroïne: EtOAc 5:1, EtOAc, and MeOH.

Gel filtration: the *G. frondosa* 4-5 fraction was fractionated by exclusion chromatography on a Sephadex LH-20 column (Pharmacia). Stationnary and mobile phases were mixed and introduced in the column. After stabilisation, sample was introduced in a liquid form. Separation was realised by $CHCl_2$: MeOH 1:1.

3.4.4.3 Low Pressure Liquid Chromatography (LPLC)

Fraction 4 of *G. frondosa* DCM extract was separated on a ready to use 2400x110mm RP-18 lobar column with 40-63µm diameter particles (LiChoprep®, Merck). LPLC was equiped with a CFG Duramat pump and with an ISCO Retriever II fraction collector. Flow rate was 0.5ml/mn for a pressure of 1-2 bars. The sample was introduced with a seringue in liquid form. The fraction was separated by 200ml of CHCl2: MeOH 1:9.

3.5 Plasticity of Grifola frondosa

3.5.1 Organisms and strains

Grifola frondosa: strains GfNE, GfSt, GfJ, GfB (MUCL 31544), GfNE-J1397, GfJ1397 (Table 3.1).

Target organisms: *Bacillus subtilis*: strain Neu1. *Escherichi coli*: strain 1096 =E. coli Hfrx11 blue. *Trichoderma viride*, strain F16.

3.5.2 Extracts

Various dried or lyophilised fruit bodies (table 3.10) were extracted with DCM and MeOH as described in paragraph 3.4.1.

Extract number	Strain	Substrate	Culture conditions/treatments
1	GfNE-J1397	B	Cut, frozen then dried at room temperature. Yellow to brown-black aspect.
2	GfNE	B	Cut, frozen then dried at room temperature. Blackened.
3	GfNE-J1397	B	Cut and dried at room temperature. Centre of stipe yellow.
4	GfNE	B	Cut and dried at room temperature. Border of stipe yellow
5	GfNE	B	Cut and dried in a drying oven at 60°C. Border of stipe yellow

6	GfNE	B	Frozen on foot and lyophilised.
7	GfNE	B	Entire fruit body malformed by lack of oxygen. Frozen and lyophilised.
Extract number	**Strain**	**Substrate**	**Culture conditions/treatments**
8	GfNE	B	"Daylight" neon lighting. White fruit body. Cut, frozen and lyophilised.
9	GfJ1397	wild strain	Picked up and determined by Dr. J. Keller in 97. Frozen and lyophilised.
10	GfNE-J1397	B	Cut, frozen and lyophilised.
11	GfNE	B	Fruit body cut with a razor blade on foot and frozen after 15 minutes, lyophilised.
12	GfNE	B	Normal brown fruit body grown in greenhouse for the last four days. Frozen and lyophilised.
13	GfNE	B	Stipe deformed by high CO_2 level. Frozen and lyophilised.
14	GfSt	D	Frozen and lyophilised.
15	GfSt	C	Frozen and lyophilised.

Table 3-10: Strains, substrates, special culture conditions and treatments of the Grifola frondosa *extracted fruit bodies*

3.5.3 Chemical composition profiling

TLC was performed on pre-coated silica-gel 60 F254 aluminium sheets (Merck). They were developed in conventional TLC chambers with dichloromethane/methanol (9/1, v/v) or ligroin/ethyl acetate (1/1, v/v). Detection of the compounds were realized by fluorescence extinction under ultraviolet (UV) light at 254 nm, fluorescence under UV light at 366 nm and by revelation with the following chemical reagents: Godin's reagent (Godin, 1954), Ninhydrin/ chlorhydric acid reagent, Dragendorff reagent (Auterhoff and Kovar, 1985) and Gibbs reagent.

HPLC analysis of chemical compounds were carried out on a Hewlett Packard Series 1050 instrument equipped with an HPLC DAD detection system HP 1040A. The interface was HP 35900. Separation was performed using an analytical RP-C18 column (nucleosil 100-7-C18, 7 µm, 250 x 4.6 mm, Bischoff, Leonberd, D) with a precolumn of the same material. Chromatographic analysis conditions were a gradient from 10% MeOH and 90% H2O to 90% MeOH and 10% H2O in 60 minutes with a flow rate of 1ml per min. Extracts were dissolved at a concentration of 5mg ml-1 in HPLC quality MeOH and a volume of 50 µl was injected. Detection was made at 254 nm and the UV spectra recorded on-line at 220, 254, 280, 320 and 450 nm.

Cluster analysis was performed to compare similarities between the 15 extracts. Simple matching coefficients of Sokal and Michener (symmetrical binary coefficient) was used as a measure of similarity between samples and a dendrogram was computed using complete linkage agglomerative clustering (Legendre and Legendre, 1998).

3.5.4 Biological tests

Bacillus subtilis and *Trichoderma viride* bioassays were realised as described above. *Escherichi coli* bioassay was performed in the same manner as *B. subtilis* test, except for the positive control used at a concentration of 0.1 µg and that incubation took place at 37°C.

4 RESULTS AND DISCUSSION

4.1 Culture

4.1.1 Strains isolation and interstrain incompatibility tests

Nine strains of 7 species were isolated during the present work, the rest of the strains analysed coming from other sources (Table 3.1). When there was more than one strain for a species, somatic incompatibility tests were realised showing that none of the strains used during this work were compatible with one another. The somatic incompatibility was demonstrated by the presence of a thick line sometimes discoloured by orange, brown or black pigmentation at the meeting points of the two colonies.

4.1.2 Species cultivated with already developed techniques

We have obtained 14 species fruit bodies with techniques previously developed in the laboratory (table 4.1). Moreover *F. hepatica* and *G. lucidum* were fructified on 2 and 3 different substrates respectively. The production of these mushrooms in controlled conditions was performed to obtain material for the pharmacological tests as well as to be able to compare the mechanism of fructifications with the newly developed cultivation techniques during this work.

Species	Substrate	Fresh weight [g]	Yield [%]	Average of the yield [%]
Agrocybe aegerita	A	309.8	17.2	25.45
		606	33.7	
Fistulina hepatica	D	378	17.2	18.6
	FJ2	445	24.7	
		250	13.9	
Flammulina velutipes	A	64	3.6	8.7
		300	16.7	
		104.5	5.8	
Ganoderma lucidum	A	106,8	5.9	3.5
	C	41.5	2.3	
	D	49.7	2.3	
Grifola frondosa GfNE	B	431	23.9	22.25
		372	20.7	
		437	24.3	
		361	20.1	
		400.5	22.25	
Hericium erinaceum	B	399	22.2	19.45
		300	16.7	
Hypsizigus marmoreus	C	141.8	7.9	9.4
	C	195.3	10.9	
Lentinus edodes	C	166	9.2	9.2
Lyophillum ulmarium	A	165.5	9.2	9.2

Species	Substrate	Fresh weight [g]	Yield [%]	Average of the yield [%]
Pleurotus citrinopileatus	A	368 390	20.4 21.67	21
Pleurotus eous	A	138.9 175	7.7 9.7	8.7
Pleurotus ostreatus	A	159.5 678.7	8.9 37.7	23.3
Sparassis crispa	D	160 246	7.3 11.2	9.25
Sparassis laminosa	D	71.6 135.8 51.5 55.5	3.2 6.2 2.3 2.5	3.55

Table 4-1 Species cultivated according to techniques developed by Dr. Job, with their substrate code and yield obtained.

4.1.3 Development of new cultivation techniques

4.1.3.1 Agaricus brasiliensis

Experiments development: All experiments were realised in polypropylene pots and casing was performed by adding 1 to 2 cm of sterilised silt-chalky soil at pH 7-8 except in Abl.2, to evaluate the effect of casing on fruiting process. In a first experiment, AbSt strain was tested on the two substrates AB1 and AB2. A small fruit body obtained on AB2 was used to isolate first generation AbSt F1. This strain was then tested on 5 substrates, Abl.1 to Abl.5. The second generation F2 was isolated from a fruit body arising from Abl.3 substrate. Figure 4.1a shows spawn run time, casing, presence of primordia and fruit bodies, of these experiments. Figure 4.2 shows fruit bodies arising from Abl.1 substrate. The ones formed on Abl.3 substrate looked similar, but with about 15 more primordia.

Culture conditions: figure 4.1b shows the culture conditions applied to the different substrates. After casing, incubation was performed at room temperature (approx. 22°C) and natural light for substrate AB1 and in the fructification room at 18°C for substrate AB2 to evaluate what conditions were best for fructifying. However as fungal contamination appeared in the AB2 casing material 4 days after casing, the polypropylene pots were transferred to the same conditions as AB1 to see if contamination was due to a weakening of the strain in colder temperature. It seems to be confirmed as contamination was totally recovered by *A. brasiliensis* mycelium in only a few days. At room conditions however *A. brasiliensis* was not able to induce fruiting cycle in our substrates. Moreover the substrate surface tended to dry. Therefore all pots were eventually transferred to the fructification room (see fig 4.1b).

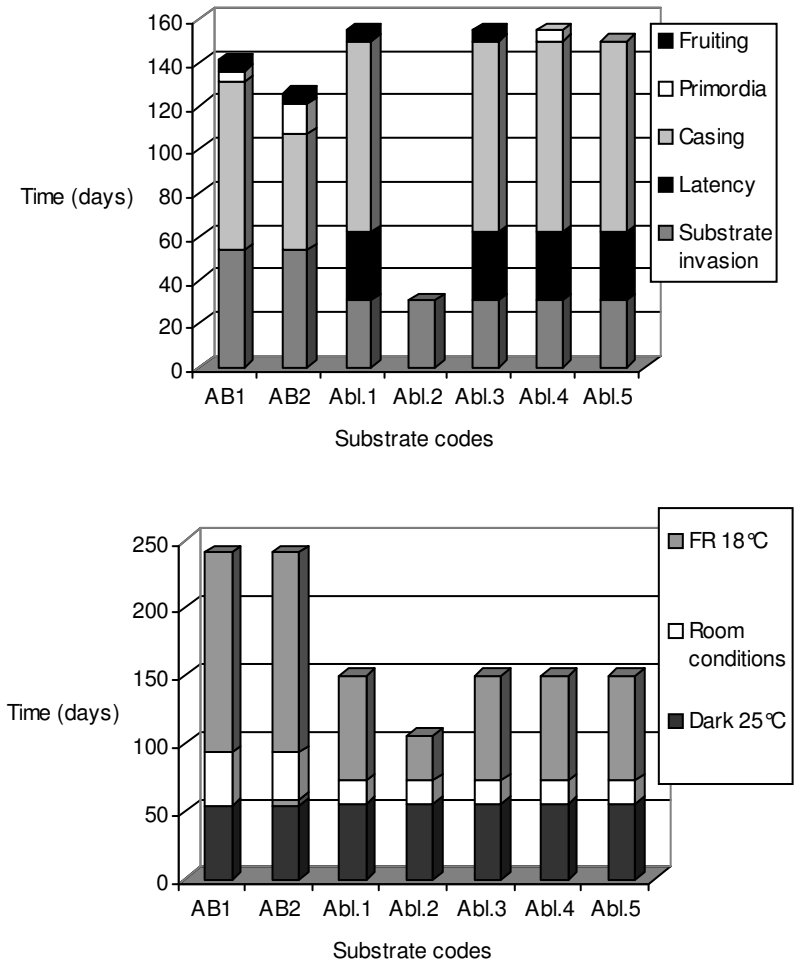

Figure 4-1:a. Time in days for substrate invasion, casing, primordia formation and fruiting of strain AbSt on AB1 and AB2 and of strain AbST F1 on the other substrates. B. Culture conditions applied to the different substrates.

Figure 4-2: A. brasiliensis growing on Abl.1 substrate

Contaminations: 4 days after casing, an undetermined fungal contamination appeared on AB2 casing material in the fructification room but in room conditions was rapidly overgrown by *A. brasiliensis*. Later both AB1 and AB2 substrate and their 1 to 2 cm high unopened fruit bodies were invaded by fly larvae. To limit the pest damage, substrates were soaked in water to kill the larva and the pots then covered with gauze. However, fruiting could no longer be induced. Abl.2 and Abl.5 were slightly contaminated with *Trichoderma viride* at the end of incubation time, however only the first one was totally contaminated after 106 days of experiment.

Discussion: *A. brasiliensis* was the only secondary saprophytic mushroom tested during the present work. Until now only primary saprophytic mushrooms had been tested in our laboratory. The aims were:

1. to obtain fruit bodies at a lower temperature than normal for an eventual introduction on the list of species cultivated in Europe,
2. to evaluate the necessity of casing,
3. to evaluate effects of different substrates partly realised out of spent substrates from cultivated primary saprophytic mushrooms.

Results show us that:

1. Incubation at 25°C in the dark was fine for mycelium invasion. It is thus adapted to our infrastructures for this cycle stage. In comparison to the average of natural conditions which go from about 20°C to 35°C; fructification could take place at 18°C.
2. No fruit bodies were obtained without casing. It corresponds to description of culture developed by Iwade and Mizuno (1997) and to other *Agaricus* spp. Moreover invasion of the casing layer and fruiting seems to be bound. Indeed invasion of the top soil by the mycelium was marked on substrates Abl.1 and Abl.3 which formed fruit bodies, was less important for substrate Abl.4 which only initiated primordia and was almost nonexistent for substrate Abl.5 which did not fructify.
3. The only cased substrates where *A. brasiliensis* did not initiate fruiting was the Abl.5 substrate containing 100% compost, while the one containing compost in the proportion of 80% bulk material only formed primordia. Compost proportion of 72% allowed fruiting initiation until the primordia was invaded by fly larvae. The best substrates seem to be half compost, half spent substrate without the influence of other additives. Thus too high of a content in compost does not seems ideal for fruiting.

Time taken before collection in our experiments was about 3 times that taken to obtain fruit bodies according to Iwade and Mizuno (1997).

These preliminary results were used by Delphine Delavy as the start of her diploma work entitled “Developpement d’un système de culture *in vitro* d’ *Agaricus brasiliensis* Murrill. Etude des conditions trophiques et environnementales de croissance et mesure de l’assimilation du zinc et du fer par le mycélium et les fructifications” (Development of an *in vitro* cultivation system for *Agaricus brasiliensis* Murrill. Study on trophic and environmental growth conditions and zinc and iron uptake by the mycelium and the fruit bodies).

4.1.3.2 Dendropolyporus umbellatus

Experiment development: One industrial bag of each of the substrate A, B, C and D were inoculated with the Chinese strain. Only small primordia were formed on A, B and D substrates.

Culture conditions: dark room at 25°C during 96 days then fructification room. Bags of substrate A and B were transferred to room conditions 153 days after the start of the experiment to try to induce primordia evolution toward fruit bodies.

Spawn run: density of the mycelium was different in function of the substrates: on A the younger mycelium border was sparse for a few centimetres and then had a very net limit of dense mycelium, while on the other substrates, the mycelium was sparse everywhere except near the birdseed inoculum. Substrate A was totally invaded in 32 days while in the other substrates the mycelium stopped growing before total invasion, visibly because of a too much humidity in the lower part of the bags. Mycelium was generally very sparse, except on the top of substrate A and B. The substrates B, C and D were very soft. On day 39 exudation started on the top of substrate A.

Primordia: they appeared in 32 and then 45 days on D substrate, in 96 and 153 days on A with thick flesh formed on the top of that substrate, and in 153 days on B with pores at some places beside the primordia. However all primordia quickly aborted when the size was maximum 1 cm. All formed pores on their surface.

Contamination: The bag of substrate C and D were totally contaminated by *T. viride* on day 130 and 153 respectively.

Discussion: This experiment was not continued because it would probably be too long to develop its culture. No fruit body evolved from the primordia suggesting that further study should be realised by modifying substrate D for 2 reasons. First, the behaviour of *D. umbellatus* seems to be much like the one of *Sparassis crispa* for which substrate D was specially developed. *D. umbellatus* formed primordia early in the cycle while *S. crispa* is not capable of fructifying in our laboratory conditions on totally invaded substrates. Second, the primordia appeared after only one month thus potentially allowing a shorter term experiment.

No sclerotia were produced during this experiment. This is in accordance with Stamets (1993) who mentions that sclerotia production is stimulated by mycoflora in soils and that absence of light rends it difficult to obtain in laboratory conditions. However we have obtained primordia on fresh starting material, contrary to this author who has obtained only short, lateral, hardened plateau with pored surface a few centimetres height on sterilised remains of spent shiitake (*L. edodes*) or reishi (*G. lucidum*) blocks, but not on fresh material.

4.1.3.3 Fomes fomentarius

Experiment development: strain MUCL 35117 was first inoculated on one bag of each of the four basic substrates A, B, C and D. Fruiting cycle was induced on A, B and C substrates, however was only completed on A and B. Time for fruit body formation was much shorter on B substrate. Thus the same strain was inoculated into 2 bags of substrate B (B2 and B3). Fruit bodies were obtained on both bags. In all cases fruit bodies were growing very slowly with growth phases, growing for a while, then growth stopping and starting again after a while. The limits between these growth phases were very visible.

Culture conditions: All bags were placed vertically as the fruit bodies of this mushroom naturally arise from vertical surfaces. They were first incubated in the dark room at 25°C, and then were submitted to different treatments.

- Bag of substrate A was transferred to the fructification room after 181 days of the experiment. The filter was removed to enhance gas exchange 11 days later and the plastic around the surface removed on day 206 when the primordia had evolved to fruit body.
- The first bag of substrate B was transferred to the fructification room after 116 days of the experiment with the plastic around the substrate removed to allow a good aeration of the forming fruit body. As the fruit body had stopped growing it was transferred to room conditions in a aerated and humidified desiccator's contender. Because of a start of contamination it was again transferred to the fructification room to see if it would stop the contaminants' progression.
- The second bag of substrate B (B2) had its filter removed after 96 days of experiment, because primordia appeared. On day 130 all the plastic around the top surface was removed and the bag was transferred to the fructification room because the primordia had evolved to a fruit body.
- The last bag (B3) also had its filter removed 96 days after the start of the experiment for the same reason as bag B2, however it was also transferred to the fructification room immediately. The plastic around the surface was removed on day 112.
- The bag of substrate C was transferred to the fructification room on day 12 of the experiment as primordia were formed. Filter was removed on day 38 as the primordia had aborted. When no other primordia were formed, the bag was transferred to room conditions on day 172 to see if fruiting cycle could be induced. However it was returned to the fructification room 9 days later as the substrate tended to dry.
- Bag of substrate D stayed in incubation conditions. As no primordia were formed, the filter was removed after 225 days of experiment to observe if aeration would induce fruiting cycle on that substrate.

Spawn run: Mycelial growth was very rapid as it colonised the whole substrate in about 15 days. In all bags, mycelium first took a fawn colour and exuded black liquid, then became dark pigmented and harder.

Primordia: A, B and C substrates allowed fruiting induction. First primordia formed early after the start of the experiment: after 12 days on C substrate, after 21 days on A and B (bag B1) substrate. However they all aborted in the next 11 days, perhaps because the filters were not removed. On C substrate a new primordia formed much later, 225 after the start of the experiment, however it also aborted in the next month. A and B substrate also allowed the formation of new primordia: on day 181 for A, 73 for B1, and 87 for B2 and B3. These evolved to fruit body forms.

Fruit body: substrates A and B (3/3 bags) allowed the evolution of the primordia to fruit bodies. Fruit body evolution was very slow: on substrate A it took 25 days since the first observation of primordia to the apparition of pores, while it took 43 days for the 3 bags of substrate B. All the fruit bodies then grew very slowly by phases as described in the paragraph "experiment development" above. The fruit body on substrate A had two growth phases in 84 days. It had 3 growth phases in 138 days on B1, one growth phase in 100 days in B2 and 2 growth phases in 100 days on B3.

Contamination: No contamination occurred on the mycelium, however all fruit bodies obtained during these experiments showed the same behaviour toward *T. viride* contamination. Indeed in all cases the pathogenic fungus colonised the older parts of the fruit bodies, but generally not the growing margin or the hymenium. The only exception is the fruit body on substrate B (bag B1) where the margin and the pores surface were also covered by the pathogen at the end of the experiment (day 254). *T. viride* however, formed very few conidia, and mostly mycelium which is quite unusual for this high rate disseminator fungus. It was normal on the older parts.

Discussion: Spawn run in the dark at 25°C suited this species very well as it grew very rapidly. Results show us that if the fruiting cycle could be initiated on A, B and C substrates, it could only be completed on A and B substrates (lower C/N ratio). Thus for this species nitrogen limitation seems to be an inhibitory factor to fruiting success.

It seems important to enhance gas exchanges by taking off the filter when the primordia are initiated. Indeed primordia aborted in the two cases when filters were not removed on substrates A and B. After filter or plastic removal, primordia evolved to fruit bodies (formation of pores).

Contamination by *T. viride* during these experiments was very interesting. *F. fomentarius* is probably synthesising an inhibiting substance in the growing margin which disappears afterwards. No antifungal activities have been reported from that species. However this "Agaric of surgeons" was used for stopping light haemorrhages for centuries. Thus some active compounds might well be found in this mushroom in the future. Unfortunately due to *T. viride* contamination, none of the fruit bodies could be used for extractions.

4.1.3.4 Fomitopsis pinicola

Experiments development: 3 strains were tested for this species. Strains, MUCL 30677, MUCL 30544 and K2 were inoculated once on each of the substrates A, B, C and D. The bags were incubated vertically as the fruit bodies of this species arise from vertical surfaces. Table 4.2 shows the stage achieved by the strains on the four substrates.

Strain	Substrate type			
	A (C:N=75)	B (C:N=90)	C (C:N=125)	D (C:N=265)
MUCL 30677	F	-	F	F
MUCL 30544	P	-	P	F
K2	F*	-	F*	P

*Table 4-2: Stages achieved by the three tested F. Pinicola strains on substrates A, B, C and D. P= primordia, F= fruit body. * Abnormal fruit bodies.*

Strain MUCL 30677

Culture conditions: spawn run was allowed in standard conditions. After 21 days all the bags were transferred to the fructification room. Filters of both A and C substrates were removed because there were primordia on C substrate and flesh with pores on A. The plastic was removed around the growing fruit bodies on substrate A, C and D on day 38 to enhance gas exchange, while the filter was removed from bag B to attempt primordia formation.

Spawn run: it took 21 days for the strain to fully colonise the four substrates, however the mycelium was denser on substrate A than on the other substrates, even forming a fleshy layer at some places. Pores had formed directly on the mycelium surface on substrates A and B. Abundant exudation liquid started to be visible in all the bags after total colonisation.

Primordia: first primordia appeared 21 days after inoculation on substrate C. However they were small (2 mm) and already bearing pores. Two new primordia were visible 6 days later (approx. 10 mm) on the same substrate also bearing pores.

Fruit bodies: 38 days after the start of the experiment 3 primordia had evolved to a normal fruit body shape on substrate C, 1 on substrate D and 1 on substrate A. When the experiment was ended after 81 days of experiment (because of contamination), there were 2 fruit bodies on C substrate weighting a total of 18.1g. There were also 2 fruit bodies on D substrate weighting 14.4g. The one growing on A remained small but was not weighted as it was totally recovered by *T. viride*.

Contamination: 81 days after the start of the experiment the mycelium of the four substrates were totally contaminated by *T. viride*. Most of the fruit bodies carried small contamination spots, thus they could not be used for extraction.

Strain MUCL 30544

Culture conditions: spawn run was allowed in standard conditions. Bags of substrate A, C and D were transferred to the fructification room with their filter removed after 21 days, and bag of substrate B after 38 days.

Spawn run: it took 21 days for the strain to totally colonise the four substrates, however the mycelium was sparser on substrate B than on the other ones. Pores had formed directly on the mycelium surface of bag D. There were brown exudation droplets on substrate A and B. Pores had also formed on the mycelium surface growing on B substrate, 32 days after the start of the experiment. In bag D there was abundant exudation liquid on day 38.

Primordia: first primordia appeared on substrate C after 21 days of the experiment. Like for strain MUCL 30677 they already bared pores, but without sporulating or having a fruit body shape. New ones were visible 17 days later while the first ones aborted. Primordia –some already with pores- were also formed on B substrate on day 32. On day 38, there were also primordia on substrate A and D (10 mm), however on D they had a brown-red discolouration, while all the other primordia were grey.

Fruit bodies: After 81 days of experiment a 9.7g fruit body on D substrate was collected.

Contamination: some flesh was formed in bag of substrate A along the plastic and became slightly contaminated after 38 days of experiment. After 81 days of experiment the same type of contamination as for strain MUCL 30677 occurred in all bags.

Strain K2

Culture conditions: spawn run was allowed in standard conditions. Bags of substrate A and B were transferred to the fructification room on day 32. The two other bags were transferred 6 days later with their filter removed. The same day the filters were also removed from bag A as

a primordia was growing and of bag B. The plastic of bag A was partly removed 77 days after the start of the experiment to see if enhanced aeration would help the primordia to evolve to a normal fruit body.

Spawn run: Flesh formed near the plastic of substrate C after only 13 days of incubation. The mycelium growing on substrate D was very sparse in some places and very dense in others. It took 32 days for the mycelium to totally colonise all the substrates. Abundant exudation liquid was accumulated at the bottom of the propylene bags, which was poured off on day 38 by the hole left by the removed filter. Substrate B was very soft. Liquid kept on accumulating and was poured away on day 45 again. The liquid kept on accumulating, especially in bags of substrates A and C, but this time with an unpleasant smell and a dark brown discolouration.

Primordia: after 32 days of experiment, a primordia had formed through an accidental hole in the polypropylene bag containing substrate A. It kept on growing until it was accidentally broken on day 45. However another one was visible at the same place four days later. Primordia also were also visible at that time on substrate D.

Fruit bodies: Primordia growing on A substrate had formed pores 77 days after the start of the experiment, which were able to sporulate later (day 112), however without ever forming the normal shape of a fruit body. The same was observed on C substrate on the same day.

Contamination: There was an accidental hole in the plastic of bag A. After 13 days of incubation we could see a contamination of the non invaded substrate by *Penicillium* sp., however, there was no contamination up to a distance of 3.5cm from *F. pinicola* mycelium front. The mycelium then overgrew the fungal contamination showing a good resistance potential of this strain on this substrate. On day 96 however *T. viride* contamination was visible on the mycelium growing on substrate A, while it had totally recovered the mycelium growing on B substrate. It also overgrew totally the mycelium and the primordia on D substrate 16 days later, and on A and C substrates 24 days later.

Discussion: Spawn run in the dark at 25°C suited this species very well as it grew quite rapidly, especially the two MUCL strains. Moreover first primordia appeared early after inoculation, always when the substrate was just fully colonised. The 3 strains induced the fruiting cycle on A, C and D substrate but never on B substrate. Interestingly it is the only substrate which does not contain birdseeds and compost. These components or at least one of them might be important for fruiting induction in vitro. This remains to be studied. Evolution of the fruiting cycle to well formed fruit bodies however, doesn't allow us to draw a general conclusions for the species, as the three tested strains behaved differently (table 4.2). However these results seem to indicate that strain MUCL 30677 adapted more easily to a wider variety of substrates for completing life cycle.

One of the problems encountered during these experiments is that there was abundant exudation liquid in many bags. This could indicate bad environmental conditions for this species, or humidity in the fructification room might be too high.

All the experiments were ended because of a total invasion by the mycoparasite *T. viride*. Thus none of the strains tested for this species seem to be able to resist that pest on any substrate. It is interesting to note that the fruit bodies (except the MUCL 30677 strain on A Substrate) were generally only slightly contaminated compared to the mycelium. This could either be due to a chemical compound present in the fruit body, either be due to the difference

in water content of the vegetative and reproductive parts of the mushroom. If the strains were not resistant to *Trichoderma*, strain K2 was able to stop *Penicillium* sp at distance and then to overgrow it. This surly indicates the production of an antifungal compound in the medium. No references were found in the literature about antifungal compounds in the fruit body, in the mycelium or in the culture medium of this species. For this species only antibacterial activities were described for the mycelium extract (Keller, 1997; Bianco Coletto, 1981). In the course of his diploma work in our laboratory, Abdelaaziz Hmamda further studied the cultivation techniques for this species as well as resistance mechanisms of the mycelium and fruit body toward different bacteria and fungi.

4.1.3.5 Ganoderma applanatum

Experiments development: two strains were tested for this species. Strain 78 was inoculated on B (1x), C (1x), Bm (5x + 2x), Jm (2x) and LO (1x) substrates in polypropylene pots and in 1 bag of substrate A and B and 2 bags of substrate C. Strain 87 was only tested on Bm (5x), Jm substrate (2x) and LO substrate (1x) in polypropylene pots. The pots and bags were usually placed on the side because fruit bodies arise from vertical surfaces. Table 4.3 shows whether fruiting was achieved in the different substrate for both strains.

Substrate container	**Polypropylene pots (approx. 400g)**					**Industrial bags (approx. 1800g)**		
Substrate type	**B**	**C**	**Bm**	**Jm**	**LO**	**A**	**B**	**C**
Strain 78	F	-	F	F	F	F	F	F
Strain 87	ND	ND	P	-	-	ND	ND	ND

Table 4-3: Fruiting results obtained on the different tested substrates for the two strains 78 and 87. F= fruit body, - =no fruiting induction, ND= no determined.

The first fruit bodies obtained were collected for extraction, while the others were left on the substrate for spore collection since the cultivation technique of this species was also developed to be able to collect pure spores for a clinical study on their allergic effect. Thus the yield is not always quantified.

Strain 78

Culture conditions: polypropylene pots of substrates B and C were incubated in room conditions. All the other substrates were incubated in standard conditions.

Transfer to the fructification room: B and C substrates in polypropylene pots (with their caps removed) after 22 days of incubation, both industrial C bags after 23 days (with the plastic cut around the surface). A pot of substrate Bm (second experiment) was transferred after 56 days of experiment. Bag of substrate B was transferred after 41 days of experiment. Filter was removed 8 days later to enhance gas exchanges to try to induce fruiting. Plastic was removed on day 61 because water was condensing inside.

Transfer to room condition: All the 5 Bm substrate pots first tested after 21 days. One of these pots was transferred to the fructification room 44 days after the start of the experiment just as it was forming primordia. The Jm substrate pot after 31 days. A pot of substrate Bm (second experiment) was transferred after 56 days of experiment. Bag of substrate A was transferred after 41 days of incubation and its filter removed 8 days later to enhance gas exchanges

because of a fruit body appearance. Plastic was removed on day 61 because of condensation inside.

On day 90 the covers of both Bm substrates of the second experiment were removed and the one in room conditions was transferred to the fructification room.

Spawn run: Interestingly, the strain formed pores on the surface of substrate A and B in industrial bags. Moreover there were melanised zones on B substrate (day 37). 12 days later the mycelium formed a thick layer on these two substrates. The strain also formed a thin layer of dark pigmented mycelium on the surface of LO substrate 26 days after the start of the experiment.

Spawn run time, primordia and observation of fruit bodies are indicated in table 4.4 for all substrates tested.

Primordia: most of the time, we did not observe primordia, but directly small fruit bodies. Thus differentiation seems to happen very quickly.

Fruit bodies: the fruit bodies growing on Bm in the second experiment stopped growing early after their formation. Most of the other fruit bodies grew steadily and sporulating continuously until contamination by fly larvae, fungal contaminants or mites.

Substrate container	**Polypropylene pots (approx. 400g)**					**Industrial bags (approx. 1800g)**		
Substrate type	**B**	**C**	**Bm**	**Jm**	**LO**	**A**	**B**	**C**
Spawn run (days)	22	22	21	Jm1:15 Jm2:31	26	37	37	23
Primordia						7	7	
Fruit body collection (g)	9.2g (day 177)	0	Spores collection: Bm1: days 69 to 84 Bm2 and Bm3: days 90 to 100	spore collection: Jm1 and Jm2 days 31 to 69	Day 62: numerous small malformed fruit bodies, removed on day 94 because of fly larvae Days 108-144: 2 fruit bodies	49-161	124	C1: 47.9g (day 125) + 21g (day 303) C2:52.8 (day 125) + 9.1 (day 303)

Table 4-4: Strain 78 spawn run time, primordia appearance and observation of fruit bodies on the 6 tested substrates and in different containers.

Contamination: There were two major contamination problems: *Trichoderma viride* and fly larvae. . Four Bm substrates and substrate C in polypropylene post were totally contaminated by *Trichoderma*. Fruit bodies of bag B were totally contaminated after 124 days and those on A after 161 days.

The last Bm substrate was invaded by fly larvae about 2 ½ months after the start of the experiment. Both Jm substrate pots were invaded by fly larvae after 69 days of experiment and spore collection had to be interrupted. First fruit bodies on LO substrate were invaded by

fly larvae 94 days after the start of the experiment, they were removed but new ones appeared. The whole substrate was again invaded by larvae 144 days after the start of the experiment.

In a second experiment with Bm substrate (2x), both pots were invaded by mites. Soaking the substrates allowed their elimination, however the fruit bodies, which had stopped growing were all colonised by an undetermined mould on day 133.

Strain 87

Culture conditions: standard conditions were applied for incubation. After 21 days all the Bm substrate pots were transferred to room conditions. The only substrate which had not been contaminated was transferred to the fructification room 44 days after the start of the experiment to try to induce fruiting cycle.

Spawn run: the mycelium formed a thick layer on the top of all substrates tested with pores. Table 4.5 shows spawn run time and primordia observation of strain 87 on the 3 tested substrates.

Primordia: they appeared 5 days after the transfer of Bm substrate from room condition to the fructification room, but aborted rapidly.

Substrate container	**Polypropylene pots (approx. 400g)**		
Substrate type	**Bm**	**Jm**	**LO**
Spawn run (days)	21	15	26
Primordia (days)	49	-	-
Fruit body collection (g)	-	-	-

Table 4-5: strain 87 spawn run time, primordia and fruit body appearance.

Contamination: the same problems as for strain 78 occurred. Four out of the 5 Bm substrates were totally covered by *T. viride* after 44 days of experiment. The last one was full of fly larvae after 2 months of experiment. However the strain had formed black melanised lines around the contaminated zones. Both Jm substrate pots were invaded by fly larvae after 69 days of experiment. Interestingly, one of them had a cucumber smell which was not present before the fly invasion. It might be a reaction to the larvae. After 62 days of experiment substrate LO was also invaded by fly larvae. It had formed a melanised layer, surely in reaction to the larvae invasion.

Discussion: Of the two tested strains, strain 78 was the better for fruiting success and its' better resistance to fly larvae. One reason might be that strain 78 produces melanised zones early in the cycle while strain 87 did so only later in fly invasion. Melanisation has previously shown to influence resistance toward the mycoparasite *Trichoderma* in *L. edodes* cultures (Tokimoto, 1980). This type of mycelium might have a slightly toxic effect on larvae. It remains to be proved.

Strain 78 was able to fruit on all tested substrates (A, B, C, Bm, Jm, LO). This species seem to grow on a wide variety of substrates. The fruit bodies however were formed very slowly as the results show. Moreover, yield was low, as the most obtained was 3.8% on C substrate in an industrial bag. Standard environmental factors normally applied in our culture experiment

seem not to be adequate for this species. Indeed it seems that the fruit bodies were healthier, when in room temperature and light, even though humidity was too low there.

Contamination mostly by insects, but also sometimes by pathogenic fungi was a big problem, certainly favoured by the long wait for the fruit bodies to form. Interestingly, there were some other mushroom species tested at the same time which were not attacked by flies (it will be mentioned for the concerned species). The apparition of a cucumber smell in one of the substrates after fly larvae invasion might be correlated to a reaction of the mushroom species. It has previously been demonstrated for *Lentinus edodes* culture that toxicity of the antifungal fraction after *Trichoderma* attack was higher than before the attack (Tokimoto 1985). At least six antifungal substances were detected in the antifungal fraction prepared after conttact between *T. polysporum* and *L. edodes*, but only three or four before (Tokimoto et al., 1987).

4.1.3.6 Ganoderma resinaceum

Experiments development: Strain K1 was first inoculated on each one of the basic substrates A, B, C and D. Later another C substrate was inoculated. The sides of bag D were covered by black plastic after 5 months of experiment to see if light had an influence on primordia initiation. This strain was able to induce and achieve fruiting cycle on A, C and D, Substrate B being contaminated early on in the experiment.

Culture conditions: incubation was performed in standard conditions.
Bag of substrate A was transferred to the fructification room with the filter removed after 15 days because of the presence of primordia. The plastic was removed from bag A which was then placed in room conditions because of a mould after 71 days of experiment. There, primordia were formed and the bag was again transferred to the fructification room.

The first bag of substrate C was transferred to room conditions after 15 days because of *T. viride* contamination.

The second bag of substrate C was placed at room conditions after 42 days of experiment to try to induce the evolution of a primordia to a fruit body. Finally the bag with a sporulating fruit body was transferred to the fructification room with the filter removed on day 146. It was transferred again to room conditions 14 days later, as the fruit body had stopped growing.

Bag D was placed at room condition at the appearance of primordia after 35 days of experiment. The sides of bag D were covered by black plastic on day 153 to see if light had an influence on primordia and fruit bodies' formation. On day 189 the filter was removed to enhance gas exchanges to try to induce evolution of the primordia to fruit bodies.

Spawn run: Mycelium growth on substrate D was unusual, growing slightly under the substrate surface in contact with the plastic. Moreover it formed zones of dense mycelium and zones of sparse mycelium delimited by thin dark pigmented mycelium lines. In the second bag of substrate C was there black pigmented hard mycelium line almost everywhere after 34 days of experiment (2 weeks after total colonisation of the substrate).

Primordia: They were first observed on substrate A, only 15 days after the start of the experiment when approx. 90% of the substrate was colonised by the mycelium. Their size was already up to 7cm in diameter and up to 4cm high. The primordium however aborted and was

contaminated by an undetermined mould. New ones were then formed on day 80 which evolved to fruit bodies.

A primordia had formed on the first bag of substrate C on day 49, then aborted at that stage. In the second experiment with substrate C, there were already one big primordium (9cm x 5-6cm) and 2 small ones after 20 days of experiment, when the substrate was totally colonised by the mycelium. They kept on growing horizontally while new ones had appeared 22 days later. The bag was placed vertically to try to induce opening of the caps on the side. However they kept on growing along the substrate until day 139 were finally the biggest primordia started to differentiate into a fruit body.

Two primordia were observed on the sides of substrate D, 35 days after the start of the experiment which evolved to fruit bodies, and new ones on day 173 on the top surface after 20 days after we covered the sides by black plastic.

Fruit bodies: It took 12 days for the primordia observed on A substrate on day 80 to evolve to fruit bodies. Only the primordia just under the plastic surface of the sides evolved to pored structures!

In the second bag of substrate C the fruit body started to sporulate 7 days after its visible differentiation from primordia on day 146. The fruit body stopped growing the next 2 weeks so the bag was transferred again to room conditions on day 159. Surprisingly, 3 new caps formed on the old one on day 184, but stopped growing again 19 days later. No more evolution was observed.

Some primordia on D substrate had evolved to small fruit bodies on day 71, 36 days after fruiting initiation. Like on substrate A, only primordia under the plastic surface evolved to fruit bodies. Other primordia formed on day 173 had evolved to fruit bodies on day 198. Only one kept on growing: it was 5cm wide and 4.5cm in projection on day 217 and sporulating generously before attacked by contamination.

Contamination: Substrate B was totally contaminated by *T. viride* 8 days after the start of the experiment and had to be rejected. The primordia on A substrate were contaminated by an undetermined mould which then colonised the top of the substrate, however it did not keep the strain from forming a new primordia. Finally it was mites which forced rejection of that substrate after 124 days of experiment. The first bag of substrate C presented only a slight contamination by the same pathogen. A week later the strain had formed a hard and dark pigmented mycelium at the margin of the contaminant colonies. It then kept the contamination steady. It was unfortunately invaded by mites after 71 days of experiment and had to be rejected. The same thing occurred for bag D about 8-1/2 months after the start of the experiment.

Discussion: This species appears to be able to fructify on various substrates. Indeed the tested strain could form fruit bodies on 3 out of the 4 tested, the last one (B) being contaminated early in the experiment. If primordia formed quite early in the experiment, however only few of them evolved to fruit bodies. Moreover the fruit bodies grew very slowly as observed for *G. applanatum*. Standard environmental factors generally applied during our culture experiment do not seem to suit this species. Insufficient aeration made the stipe elongate without the cap opening. This is also observed with *G. lucidum* (Job, pers. obs.). Moreover even when the bag was placed vertically the fruit body grew toward the filter, thus seemed not

to be influenced by gravity but by aeration. Light seem to be important also: on substrate D, primordia was produced on the side of the bag, but after covering the sides with black plastic, primordia only formed on the top and evolved to fruit bodies. In general fruit bodies grew better when in room conditions with natural light wavelength than in the fructification room with artificial light. The same thing was already observed with *G. applanatum*.

There were several interesting observations on resistance to contamination: the C substrate tested in the first experiment resisted to *T. viride invasion* first by forming a black line around the contamination and then by forming a whole black hard crust. At that moment the pest had invaded at least 70% of the substrate but could not progress further. *G. resinaceum* even produced a primordia which could not evolve to a fruit body because of mite invasion. Very thin black lines were also formed on D and the second C substrates but without visible contamination. This is similar to what was observed in *Lentinus edodes* cultures. Indeed the formation of black line at the contact of the mycelia of the two antagonists *L. edodes* and *Trichoderma* spp. is common (Tokimoto, 1980). This brown line has shown to stop the progression of *Trichoderma* spp significantly for a certain laps of time.

In general this species behaved quite like *G. applanatum* concerning environmental conditions for fruiting, time taken for fruit body formation and antagonism to fungal pathogens. We have not yet found the adequate conditions in which to cultivate these two species.

4.1.3.7 Ganoderma tsugae

Experiments development: The Chine strain was inoculated first on one bag each of the substrates A, B, C and D. B substrate was contaminated early. Primordia formed on the other three substrates; however they generally continued lengthening up to a tenth of centimetre without forming a cap. Later a second C bag was inoculated, as well as an A bag covered by black plastic on the sides and bottom to eliminate influence of light on fruiting induction. Again both substrates allowed fruiting initiation only without black plastic, however only one primordia evolved to a fruit body on A substrate.

Culture conditions: All the bags were incubated in room conditions. After 15 days bags of substrate B and C were placed at room conditions because of the presence or contamination, and bags A (primordia) and D in the fructification room. On day 35, bags A and C were placed in a desiccator vessel at room temperature with water on the bottom and incoming air to determine if natural light had an influence on the growing primordia as observed for *G. applanatum* and *G. resinaceum*. The top plastic of both bags was opened. 8 days later however this bag was transferred to the fructification room again as 11 out of 18 primordia had stopped growing. They all had stopped growing 4 days later. Temperature of the fructification room was elevated to 20°C to see if it could have any influence on growth.

On day 48 primordia had stopped growing on substrate D, so the bag was placed at laboratory conditions. After the appearance of new primordia on day 71 this bag was left in the same conditions until day 92 where it was transferred into the fructification room to evaluate the different environmental factors. The primordia stopped growing, so that on day 112, bag D was transferred again to room temperature and light and placed vertically without success.

On day 32 the sides of the second bag of substrate C were covered with black plastic to see the influence of light. Holes were made in the filter to enhance gas exchanges on day 59 and

the filter removed 7 days later. On day 130 the bag was transferred to the fructification room to see if it would induce the fruit body to restart growth.

The second bag of substrate A was wrapped in black plastic since the start of the experiment. It was transferred to the fructification room after 77 days of experiment after the fruit body stopped growing to see if environmental changes could induce the fruit body to grow again.

Spawn run: The shortest time taken by the mycelium to colonise the whole substrate was 13 days on C, while the longest was 22 days on D. However the mycelium looked different on substrate D compared to the others: it had some black lines separating white and yellow mycelium zones, while on the other substrates mycelium was white except around contaminated zones on substrate C.

Primordia: The first primordia were visible after only 15 days on the first A substrate tested. They elongated and 10 primordia were about 7 cm high on day 35. All stopped growing on day 48.

In the second A bag tested (surrounded by black plastic), observed after 26 days of experiment, 3-4cm long primordia were all formed on the top surface and turned toward the light (opposite the room's wall). They kept on growing, forming sometimes several branches. On day 33, bag was rotated 180° to observe the reaction to light from the growing primordia. It seemed conclusive as the fruit body nearest the light source formed a bend in it's stipe to grow towards the light. It is the only bag where one primordia formed a cap after 58 days of experiment.

Primordia were visible after 22 days of experiment on C substrate despite *T. viride* contamination. 6 grew up to 6 cm high in 13 days. Two grew until day 92 but then stopped. On day 122 they were removed and weighed (17.1g) but were rejected because of contamination.

Primordia were already visible after 13 days of experiment in the second C bag tested. They kept on lengthening, on day 32, new ones appeared on the sides. On day 59 the primordia were already about 15 cm long. On day 77, a primordia had grown downward toward the filter opening, showing an influence of aeration more important than gravity. However no primordia formed a cap until they had stopped growing on day 130.

On D substrate only one malformed twisted primordia was visible after 35 days of experiment. A crust was growing alongside the plastic, on day 48 the primordia stopped growing. On day 71 there were 3 new twisted primordia on D. On day 92 they had continued to grow against the plastic and had an unusual yellow-sulphur colour compared to the others which were had a shiny brown-red colour, but had stopped growing on day 112. On day 133 it had started growing again with a normal colour, however, directed downwards. On day 152 it was still elongating and starting to form a new branch from the middle part of the growing primordia. Growing continued until day 216. The stipe was extremely long and crooked but never formed a cap. The lyophilised bag was kept for demonstration.

Primordia looked exactly like those of *G. lucidum* cultivated in our laboratory on A and C.

Fruit bodies: The only real fruit bodies were observed in the second bag of substrate A tested. After 58 days of experiment and 32 days after observation of the first primordia, one of

them formed a cap through the hole left by the removed filter. It kept growing during 14 days before stopping (size of the cap: 6.5cm wide and 7cm in projection), without sporulated and never resumed growth again despite the change in environmental conditions.

Contamination: Substrate B and C were contaminated with *T. viride* after 15 days. On C however *G. tsugae* had formed a thick black layer where in contact with the pest, becoming yellow-brown in the side of *G. tsugae* colony. Interestingly a week later, substrate B was totally contaminated by the pathogenic fungus, while on C substrate, *G. tsugae* had stopped the progression and even overgrown it. Thus pigmentation of the mycelium clearly had an influence on the resistance of that strain on substrate C in comparison to substrate B. On day 122 *Trichoderma* had overgrown *G. tsugae* and even colonised the primordia. Thus the bag had to be rejected for the same reason as that of substrate A.

Discussion: *G. tsugae* seem to have a large choice of possible substrates to induce fructification, as it was induced on the 3 non-contaminated substrates. The difficulty was that primordia tended to keep on elongating without forming a cap, with one exception on A substrate. With no sporulation, however, the fruiting cycle was not completed.

Like for the two previous Ganoderma species tested, it seems that our trophic and/or environmental conditions are inadequate. Light is a first factor to consider: except the malformed primordia on substrate D, all primordia were formed in the presence of natural light. It seems to be confirmed by the last experiments where the sides of the bag where covered by black plastic, under which no primordia were formed. Moreover in this same experiment all the stipes were growing toward light as shown by the 180° turn of the bag which induced a bend in one stipe growing toward the light source of the room (windows). Moreover natural light seems essential. Indeed each time the bags were transferred into the fructification room with artificial light, the primordia stopped growing. When transferred again to room conditions, with natural light on substrate D, primordia started growing again. However primordia growth on substrate C of the second experiment stopped even in natural light more than $3^{1/2}$ months after the fruiting initiation.

Moreover it seems clear that substrate composition has an influence on the morphology of this species: while the primordia on A and C substrates looked like the ones of *G. lucidum* with a shiny brown-red colour, the ones growing on C were in general darker and thinner. On substrate D they were all crooked with a mat sulphur-yellow colour. It is also on that substrate that the mycelium had a different look. This substrate should be abandoned for further studies, because it seems unsuitable for this strain, the reverse of the expected, as it is the substrate with the highest quantity of sawdust, the main component in industrial cultures of this species.

Resistance behaviour of *G. tsugae* was similar to that of *G. resinaceum*, of the 4 basic substrates tested, B and C were contaminated, but both species resisted the *T. viride* invasion on substrate C only, forming brown zones where in contact. Both formed primordia. It would be interesting to test the antagonism between these two species and *T. viride* on different substrates to evaluate the influence of substrate composition on resistance.

4.1.3.8 Gloeophyllum trabeum

Experiment development: strain K00 was inoculated once on each of the substrates A, B, C and D. Malformed fruit bodies were observed on A and D substrates.

Culture conditions: the bags were incubated in standard conditions. All were transferred to the fructification room 34 days later, placed vertically because fruit bodies of this species generally arise from vertical surfaces. The filter was removed from all the bags on day 54 to try to induce fruiting cycle by enhanced gas exchanges.

Spawn run: substrates A and B were fully colonised by *G. trabeum* yellow mycelium after 18 days of incubation despite the fungal contamination, and substrates C and D after 34 days only as it more difficulty overgrew the contamination.
Fruit bodies: no real fruit bodies were observed, but very small (less than 1 cm) soft, round, black coloured structures baring pores on substrates A and D at the end of the experiment (day 117).

Contamination: All the bags had a few *T. viride* contamination spots 6 days after inoculation. Thus the inoculum might have been itself contaminated. However it did not seem to bother mycelial growth which overgrew the contamination. Since day 54 the same contamination was visible inside the plastic of bags A and B but not on the mycelium. The bags had finally to be rejected because of invasion of fly larvae in the substrates after 117 days of experiment.

Discussion: This preliminary test shows that this strain is very aggressive, overgrowing easily a fungal contamination on all the substrates. It was however slower overgrowing it on C and D substrates than on A and B. Thus higher nitrogen content seems to favours *G. trabeum* against *T. viride*. This is the reverse of the observation by Tokimoto and Komatsu (1979) in antagonism between *L. edodes* and *Trichoderma* spp. which was favoured by nitrogen rich substrates.

Fruiting was unsatisfying on A and D substrates in the fructification room; the fruit bodies were very small, did not have a normal shape and took more than 3 months to appear.

4.1.3.9 Grifola frondosa

Experiments development: this species merited special attention as it has already been largely studied in our laboratory and is the only species used in all parts of this work. Five different strains were tested, not necessarily at the same time. The reference GfNE strain is the industrial strain developed by Dr. Job for fructifying on the B substrate and allowing a good average yield of nearly 25% in industrial assays (Job and Schiff-Giovannini, 2004). The GfNE strain grown in different environmental conditions for chemical profiling will be mentionned in chapter 4.4. Here only new strains tested in the experiments are described.

Strains GfJ, GfSt, GfB, and GfK00 were all inoculated on the four substrates in industrial bags. Some cultivation assays were also realised in polypropylene pots, strain GfB on the four substrates and GfJ and GfSt on C substrate. Stages achieved in the fruiting process are presented in table 4.6.

	Substrate A	Substrate B	Substrate C	Substrate D
***G. frondosa* GfSt**	F	F	F	F
***G. frondosa* GfJ**	-	-	F	-
***G. frondosa* GfB**	P	-	F	-
***G. frondosa* GfK00**				
***G. frondosa* GfNE**	-	F	-	-

Table 4-6: Stages achieved by the 5 tested G. frondosa strains on substrates A, B, C and D. P= primordia, F= fruit body.

Strain GfJ

This strain was inoculated on the four substrates in industrial bags and on substrate C in polypropylene pot.

Culture conditions: All substrates were incubated in standard environmental conditions. A bag of substrate B was transferred to the fructification room 6 months after inoculation when it presented a primordia. Substrate C in polypropylene pot was placed after more than 5 months in a dark room with a higher temperature than usual (approx. 22°C).

Spawn run: After 3 months *G. frondosa* had formed pores on A substrate surface.

Primordia: substrate B allowed the formation of small primordia after 6 months of experiment that afterwards aborted.

Fruit bodies: substrate C in a polypropylene pot is the only one which allowed the formation of a fruit body. Interestingly, conditions were totally different than those normally applied for fruiting cycle of GfNE strain. Indeed it fructified in the dark at high temperature with the pot still covered, that is to say with a quite low gas exchange. It weighted about 27g for an initial substrate weight of 400g (yield = 6.75%). The fruit body could not be used for extraction as it was slightly contaminated.

Contamination: Industrial bags of substrate C and D were rejected after 22 days of experiment because of *T. viride* contamination. The bag of substrate A was totally contaminated after 3 months of experiment, and the bag of substrate B after about 7 months.

Strain GfSt

For this strain two bags of substrates A (A1 and A2), B (B1 and B2) and C (C1 and C2) and 3 bags of substrate D (D1, D2 and D3) were inoculated, as well as a substrate C (400g) in polypropylene pot.

Culture conditions: Bags of substrate A1 and A2, B2, and C2 were incubated at room conditions. These others were incubated in standard conditions. Both B and C substrate bags were transferred to the fructification room after 68 days of experiment.

Spawn run: After 36 days of incubation all substrates were totally colonised by the mycelium. Mycelium on B and C substrates was yellowish on the surface. On day 56 there was an orange colour around the birdseeds inoculum of these two same substrates.

Primordia: There were aborted primordia on bag B1 68 days after inoculation. There were also two aborted primordia on A1 after 73 days. On day 74 small primordia (less than 1cm in diameter) were also visible on all C and D substrates. They aborted on C1, D2 and D3.

Fruit bodies: One fruit body grew in the non opened bag, A2 still at room conditions after 73 days of experiment. It was collected and weighted 238g. It was darker than normal fruit bodies obtained with GfNE strain and had a much stronger "forest smell". Moreover there was almost no stipe.

On substrate C in a propylene pot a 15.7g fruit body was collected 210 days after inoculation, while it was still in room conditions but very dark (fig. 4.3).

A 238g fruit body was collected from bag C2 after 86 days of experiment, only 13 days after observation of the small primordia.

A 175g fruit body was collected from bag D1 after 86 days of experiment, like the one on C2. The morphology of the fruit body was no conform to the others: it had a big stipe with "leaves" totally opened and turning upwards (fig. 4.4)

Figure 4-3:G. frondosa strain GfSt fruit body growing on C substrate in polypropylene pot

Figure 4-4: G. frondosa strain GfSt fruit body growing on D substrate in industrial bag

Contamination: Bag of substrate B1 was totally contaminated by *T. viride* after 94 days of experiment. It is interesting to note that in bag A2 contamination only occurred on the remains of the cut fruit body a few days after collection, even though it neighboured bags of *Fomitopsis pinicola* totally contaminated with *T. viride.* Substrate D3 was totally contaminated by the same pathogen after 108 days of experiment, while all the remaining bags were invaded by mites after 183 days, bringing an end to the experiment.

Strain GfB

This strain was inoculated once on each of the substrates (approx. 400 g) in polypropylene pots and on one industrial bag of substrate A, B and C and 3 of D–D1, D2 and D3-.

Culture conditions: All the substrates in polypropylene pots and substrate A in industrial bag were incubated at room conditions and transferred to the fructification room after total colonization of the substrate by the mycelium. All the other bags were incubated in standard conditions. The A, B and C substrates in industrial bags, as well as D1 bag were transferred to the fructification room after total colonization of the substrate (56 days). The filter of bag C was removed on day 82 and the plastic was cut all around to allow better aeration when primordia were differentiating into fruit body (day 94).Filter of bag D1 was removed to try to induce a change on day 94, without success.

Spawn run: 56 days after inoculation, all the substrates in industrial bags were totally colonised by the mycelium. On substrates A, B and D the mycelium was dense, forming a thick flesh on the entire top surface. There was exudation on all the substrates

Primordia: Primordia only formed on substrates in industrial bags. A primordia formed after 66 days of experiment on substrate A (10 days after transfer to the fructification room) but aborted when it was 2.5 cm in diameter.

On substrate B grey spots had given small blisters-like protuberances after 76 days of experiment, but did not evolve, except for the formation of pores.

On substrate C a small grey primordia, darker than on other substrates was visible after 66 days of experiment. On day 82 it was 3 cm in diameter with 2 other small ones starting to differentiate into a fruit body. The removed filter allowed detecting the strong hazelnut smell characteristic of *G. frondosa*. Another primordia was visible on day 94.

Only one of the 3 D substrates presented tissue differentiation. Without being real primordia, grey spots with pores were visible on the top of D1 substrate after 94 days of experiment.

Fruit bodies: Fruit bodies were only obtained on C substrate in an industrial bag. On day 94, there were 3 stipes so close ones to the others, that it looked like one fruit body coming straight from the substrate. Another fruit body with a strong hazelnut smell was forming on a big primordia. It was very dark grey with a rusty grey colour in some places and pores were formed below the "leaves" with a small stipe (less than 1 cm high). It was collected on day 111 (192g).

Contamination: all the substrate in polypropylene pots were contaminated by *T. viride* 3 to 4 months after the start of the experiment. Substrate C in an industrial bag was contaminated by *T. viride* on day 130, 19 days after the fruit body collection. Substrates A and B in industrial bags were completely contaminated by the same pathogen after 147 days of experiment. Bag D3 was contaminated after 2 months of experiment, and D2 after 6 months without any differentiated tissue formation.

Strain GFK00

This strain was inoculated once on each of the A, B and C and D substrates in industrial bag.

Culture conditions: all the bags were incubated in standard conditions and transferred into the fructification room after total colonisation of the substrate by the mycelium (after 42 days). The filter of substrate A was removed on day 69 to enhance aeration for fruit body formation.

Spawn run: on all substrates, growth was good with flesh production near the birdseed inoculum. On day 42 all the substrates were totally invaded and the strain had produced dense fleshy tissue layer on the top of all the substrates, however on substrate A it was darker yellow and harder. There were also some salmon colour spots on the flesh with starting primordia on substrate C. On day 55 there was abundant exudation in bags A, B and D.

Primordia: On day 69 there were 3 primordia on substrate A and one small one on substrate C. On day 106 however primordia of bag A still had not started to differentiate into fruit bodies, the plastic was eliminated to allow better aeration. There were 3 primordia 3 to 4cm in diameter on substrate C on which the filter was also removed. Four months after inoculation substrate C had differentiated into a fruit body while A only had produced a stipe-like structure which was rejected because of contamination. B and D did not form any tissue.

Fruit bodies: the only fruit body obtained on C substrate was misshapen, having a big stipe with a small cap (fig. 4.5).

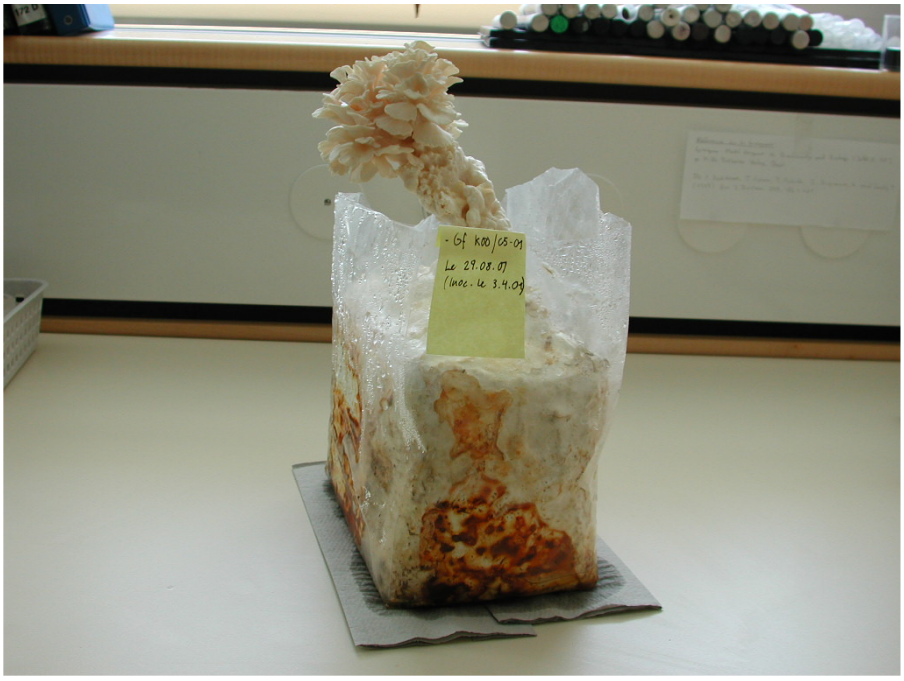

Figure 4-5: GfK00 strain of G. frondosa growing on C substrate in industrial bag

Discussion: Our results were based on only few replicates, however they seem to confirm that fruiting capacity varied between strains, like previously observed (Job and Schiff-Giovannini, 2004; Shen and Royse, 2002; Stamets, 1993, 2000). Thus the "in vitro" fruiting success strongly depends on the choice of strain used. This should motivate the cultivation testing of various strains. Adaptation of different strains to various substrates is demonstrated by the variaty of substrate material used in *G. frondosa* cultures: oak in the US and Japan (Lee, 1994), beech (Kirchhoff, 1996) and larch in Japan (Stamets, 2000), cottonseed hulls in China (Zhao et al., 1983) with various nutrient supplements such as wheat (Mayuzumi and Mizuno, 1997) or rice bran (Takama et al., 1981), corn meal (Kirchhoff, 1996) or soybean cake (Mizuno and Zhuang, 1995). Generally no C/N values were given, however, it might be the C/N coefficient which is important rather than the lignocellulositic material used (Job and Schiff Giovannini, 2004).

Yields obtained for the four tested strains on the various substrates are presented in figure 4.6. B substrate was especially designed for commercial GfNE strain fruiting, explaining the high yield obtained. GfB strain also formed primordia on substrate A. Yield was variable between strains and substrates; however these data on fructifying capacities and yield were collected on few replicates, thus giving an indication, but being insignificant. Yields obtained in our tests are reasonably good if compared to Kirchhoff (1996) who obtained fruiting with a yield of about 17.5% and 7.5% for the two tested strains on one of the three tested substrates.

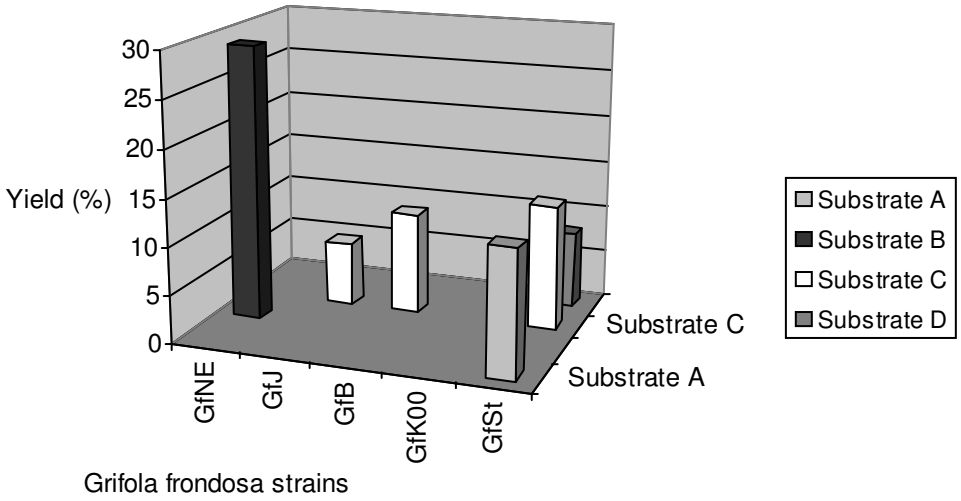

Figure 4-6: yield obtained for each of the tested species of G. frondosa on four substrates

In our experiments, features of the fruit body obtained in the same environmental conditions did show some variations. For instance GfSt showed great variations in colour and shape between substrates: on A the fruit body was much darker than on the other substrates and had a tiny stipe while on D it had a big stipe with the leaves turning up. We would have expected the fruit body growing in the closed A bag to have a big stipe, as in the experience aquired on GfNE strain cultivation, CO^2 level has shown to favour stipe growth (Job, pers. obs.) GfB growing on C also had a tiny stipe and a dark colour. GfSt strain had a strong forest smell while GfB on B had a very strong hazelnut smell, much stronger than the GfNE strain. Shen and Royse (2001) have also previously demonstrated that type and quality of nutrient supplement influence yield and basidiome quality.

Vegetative phase showed differences between strains and substrates. While in previous studies, one strain formed a fleshy tissue layer only on nitrogen rich substrate (Job and Schiff-Giovannini, 2004), here we have observed that only two out of the 4 tested strains formed such layer, moreover on 3 of 4 substrate types. Moreover resistance to fungal contamination also varied. Generally the *G. frondosa* strains tested did not resist well to *T. viride*, the main fungal contaminant. However GfSt behaved a somewhat like GfNE: the mycelium could resist *T. viride* on A substrate, but not the fruit body. In comparison GfNE biomass was more important on that substrate and was not contaminated when the sack was open, but fructifications aborted by fungal and bacterial contamination (Job and Schiff-Giovannini, 2004). The capacity of a strain to invade and defend a substrate is one of the most important steps in the selection of a future industrial strain for edible species (Terashima et al., 2002). Thus the search continues for new strains in order to hopefully discover a particularly resistant strain.

4.1.3.10 Laetiporus sulfureus

Experiments development: 2 strains N°41 and the newly isolated I1 were tested on the four substrates A, B, C and D in industrial bags. Strain N°41 was tested twice, and strain No I1 once on each of the substrates and then in two other A substrate bags. Only primordia could be obtained on A substrate with the I1 strain.

Strain No41

Culture conditions: incubation was performed in standard conditions. In a first experiment the bags were transferred to the fructification room at standard temperature after 25 days. In a second experiment they were transferred to the fructification room at lower temperature than usual (16-17°C) after 17 days with their filter removed. The bags were placed vertically as the fruit bodies normally arise from vertical surfaces. The bag of substrate B was again placed horizontally on day 42 as the mycelium layer on the surface was breaking.

Spawn run: substrate colonisation was rapid; it was performed in less than 25 days in all bags. In the first experiment, mycelium on substrate A and D had a strong orange colour, a yellowish one on B and was more or less white on the C substrate. No tissue differentiation was observed in any of the bags. In the second experiment after 42 days all the substrates had formed a thick salmon colour fleshy tissue layer with orange spots on the surface. It had a creamy consistence, except on substrate A where it was more cotton-like. Substrate B was less coloured than the others.

Contamination: In the first experiment substrate C was rejected on day 82 because of total contamination by *T. viride*, B on day 96 and A and D on day 113. In the second experiment, all the bag were rejected after 80 days of experiment because of *T. viride* contamination in bags B, C and D and of an undetermined mould in bag A.

Strain I1

Experiments development: A first experiment with the four substrates was performed and primordia was obtained on A substrate through the filter. Thus two other A substrate bags were inoculated in a second experiment.

Culture conditions: In the second experiment the plastic of the bag was pinned against the surface just after inoculation to avoid mycelium growth in the total free volume like in the first one. The aim was to try to obtain a fruit body through the filter without breaking the mycelium, because it had formed primordia that way in the previous experiment. Incubation was performed at room temperature. 63 days after the start of the experiment filters were removed from bags C and D and bags A and B were transferred into the fructification room. In the second experiment bag A2 was transferred to the fructification room on day 39 to try to induce fruiting phase. However on day 80 as the primordia had blackened, the bags were transferred to room conditions.

Spawn run: In the first experiment, substrates B and C were almost totally colonised only 8 days after inoculation and A and D after 15, however with a sparse mycelium except on the top surface. Later in all bags mycelium became beige with a woolly aspect, especially on the top surface where there was a very thick soft fleshy layer. When the substrates started to subside, they broke. The creamy-powdery yellow-beige mycelium invaded all the free space of the bags and smelled of rotten eggs, producing a black liquid (pH 3) during all the rest of the experiment. On day 110 the mycelium was in general very crumbly like sand, with the substrate soft, like cream so that all bags were rejected because of the condition of the substrate, even if there was no contamination. In the second experiment both bags were totally invaded by a dense, yellow powdery mycelium13 days after the inoculation. The substrate just under the filter was not invaded by the mycelium at all and there was some flesh all around the filter limit. On day 32 both bags had a black matter dried on the filter and a zone of flesh just under the filter.

Primordia: after 63 days of the first experiment a hard black primordia had formed on the filter of substrate A bag. Fourteen days later it was contaminated, but another, orange-yellow one had formed through the plastic soldering. The same day soft orange-yellow primordia was visible on substrate C just under the hole left by the removed filter. On day 110 the primordia had broken naturally because the mycelium was so soft.

In the second experiment a primordia was visible on the filter of bag A1 only 38 days after the start of the experiment. On day 50 the primordia had grown and showed signs of differentiation: the half superior part was orange and the half inferior part yellowish. 7 days later however it had stopped growing (6cm x 5.5cm x 4cm), but 2 had appeared on the filter of bag A2. On day 64 the primordia of A1 was soft, black and stinking but two others were forming over and under it. After 80 days of experiment all the primordia were black, soft and stinking like rotten eggs. They were removed from both bags, but afterwards no other primordia appeared.

It is interesting to note that even after staying in laboratory conditions; there was still no trace of contamination on day 125. The bags were finally thrown away, even if not contaminated.

Discussion: These results seem to indicate a different behaviour between strains as strain 41 was just able to form a kind of hard tissue on A and D substrates, while strain I1 was able to form primordia on A and C substrates. It seems that it is the enhanced aeration induced by the removal of the filter which was responsible for tissue differentiation. For further research on this species, it might be interesting to isolate other strains of this quite frequent mushroom, and test them on different substrates with no empty spaces in the bag A and with sufficient aeration which has shown to be an important environmental factor for differentiated tissue formation. An interesting fact about strain I1 is that none of the substrates were contaminated while all colonised by strain 41 were. It could be correlated with the production of a black liquid, pH 3, which had not been observed with strain 41. This liquid might inhibit fungal contaminant growth by its acidity or eventually by the presence of an active compound.

4.1.3.11 Laricifomes officinalis

Experiments development: Two strains, 70 from the mycotheca and I1, isolated during the present work were tested. The first strain was tested on the four usual substrates with no satisfying results and later on LO substrate in polypropylene pot. Strain I1 was tested on Lo1 to Lo9 and LO and LO2 substrates specially designed for this species and contained in polypropylene pots. No fruiting induction could be observed.

Strain 70

Culture conditions: incubation was performed in standard conditions, but with the bags and two LO substrate pots placed vertically, because *L. officinalis* fruit bodies naturally arise from vertical surfaces.

On day 40 the two LO pots were transferred to the fructification room. On day 56, B substrate was breaking, the bag was therefore again placed horizontally. The same day, bag of substrate D was transferred to the fructification room (primordia) at 17°C-18°c, a lower temperature than usual as this mushroom grows in altitude where temperatures are normally lower. Four days later the filter was removed from that bag. On day 66 the plastic was cut all around the very breakable D substrate to try to induce an evolution of the primordia. Bag of substrate A

was finally transferred into the fridge at 6°C on day 151 and bags B and D on day 207, to see if a drop of temperature could influence a fruiting induction.

Spawn run: It took 56 days for a sparse mycelium to colonise substrates B, C and D, and 74 days to colonise substrate A with formation of cords on the plastic of this bag. The mycelium did not become any denser during the several months long experiment. After 98 days of experiment fleshy tissue was visible were the plastic was touching the substrate on a side of the bag.

Primordia: After 56 days of experiment small yellowish exudating primordia were visible on substrate D (transfer to FR). Ten days later pores had formed on the primordia which were exudating abundant liquid even with the plastic removed. The primordia aborted at that stage. A very small primordia was visible on one of the LO substrate after 140 days of experiment. However as the substrate was invaded by mites we can not know if it would have evolved further.

Contamination: one of the LO pots was totally contaminated by *T. viride* after 70 days of experiment and the other one was full of mites after 140 days.

Strain I1

In a first experiment this strain was inoculated on the nine substrates Lo1 to Lo9. About 800g of each substrate was contained in polypropylene pots. Lo9 is an nitrogen enriched D substrate as the best results obtained with strain 70 was with this substrate.

Culture conditions: incubation was performed in standard conditions. After 23 days of experiment the Lo1 to Lo9 pots were placed vertically, and after 9 days in the second experiment with LO substrate. After 40 days of incubation both LO pots were transferred to the fructification room. 24 days later one of the pots was transferred to the cooler at 9°C to try to induce fruiting. On day 98 it was transferred again to the fructification room.

Spawn run: 10 days after inoculation mycelium had only grown on Lo3 and on Lo9, where the best growth was observed. On LO substrate strain I1 was growing better with a denser mycelium compared to strain 70.

Contamination: 10 days after inoculation Lo1 was slightly contaminated by *T. viride*. On day 32 Lo2 was also contaminated, while in Lo1 there was abundant exudation liquid. All the Lo1 to Lo9 pots were contaminated in the next months with no trace of tissue formation on any of the substrates. On day 64 both LO pots presented some *T. viride* contamination spots. 13 days later the pot which had stayed in the fructification room was rejected because of total contamination.

Discussion: This species seems to be very hard to please. Mycelium didn't even grow well on normal birdseed inoculum until asparagin was added. Thus this amino acid seems essential. Mycelium also grew very sparsely on the four basic substrates tested. Thus new substrates were designed to try to first enhance mycelium density and growth rate, and encourage fruiting by adding among other things, asparagin. Asparagin visibly favoured mycelial growth and allowed the formation of differentiated tissue for the strain 70. It would be particularly interesting to achieve the cultivation of this species because it has a great number of medicinal properties claimed in various popular medicines. However as the two tested strains

were growing slowly we could test more strains on LO substrate which was the best and then further modify its composition.

4.1.3.12 Lentinus conchatus

Experiment development: Strain K98, isolated during the present work was tested on the four basic substrates A, B, C and D as well as on a Dcaf substrate.

Culture conditions: The bags were incubated in standard conditions. Bag of substrate C was transferred to the fructification room after 37 days, bag of substrate Dcaf after 38 days and substrate A and D after 50 days (primordia). Substrate D was transferred despite the fact that the substrate was not fully colonised. The filter was removed from bag B after 63 days and it was also transferred to the fructification room.

Spawn run: substrate C was colonised by the mycelium in 37 days, substrates A in 50 days and substrate B in 63 days. The mycelium never totally colonised substrate D.

Primordia: some primordia were visible on substrates A and D 50 days after inoculation and on B 63 days after inoculation.

Fruit bodies: Substrate A allowed collecting a total of 271.9g in 3 flushes: 153.2g on day 68, 68.7g on day 86 and 50g on day 113.

Substrate B allowed collecting a total of 54.5 g in 2 flushes: 44,7g on day 78 and 19.8g (rotten) on day 86.

Substrate C allowed collecting 77g on day 63.

Substrate D allowed collecting 36g on day 78. Substrate Dcaf allowed to collect 203.8g on day 51.

Contamination: Substrate Dcaf was contaminated by *T. viride* after 58 days of experiment, substrate C after 63 days, bags B and D on day 93 and bag A on day 131.

Discussion: This strain seems to have little trophic requirements as it fructified on all the substrates with great yield variations (fig. 4.7). It is interesting to note that all substrates were contaminated only after fruiting. Thus *L. conchatus* might loose its resistance ability after fructifying as observed with other species in this work.

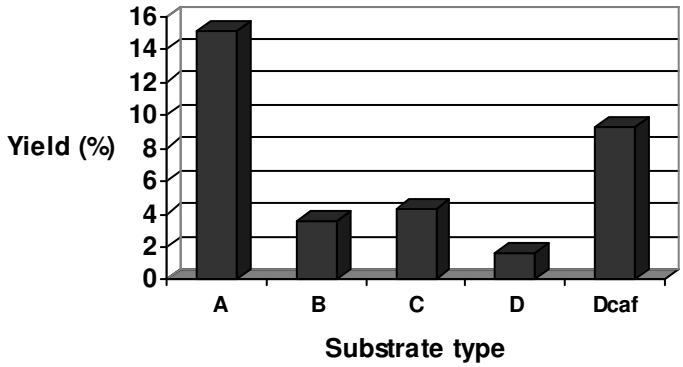

Figure 4-7:L. conchatus yield obtained on 5 substrates

4.1.3.13 Meripilus giganteus

Experiment development: strain K1 was tested on the four standard substrates. If fruiting could be induced on all of them, only A substrate allowed to complete the reproduction cycle.

Culture conditions: incubation was performed in standard conditions with the bags in a vertical position as fruit bodies naturally grow from vertical surfaces. The A and C bags were transferred to the fructification room 80 days after the start of the experiment, and B and D bags after 91 days. On day 91 the filter was removed from bag A to enhance aeration because of the presence of one big primordia. On day 105 the whole top plastic of this bag was removed to try to induce the differentiation of the primordia into a fruit body, while bag C was left at room temperature and in the light because of the *T. viride* contamination progression. On day 166 the filters of bags B and D were taken off to see if it would induce primordia differentiation into fruit bodies, without success.

Spawn run: The mycelium took 22 days to colonise B and C substrates and 35 days for A and D substrates. The mycelium had an unusual aspect on the four substrates: it was like a succession of folds. Mycelium grew denser on A and C substrates than on the two others. Later (day 49) the mycelium got discoloured: orange-fawn colour zones on the top of substrates A, B and C and beige on D. On day 180 we could see some melanised rhizomorph-like structures everywhere on the sides and bottom of B and D substrates.

Primordia: Primordia were visible after 22 days on B and C substrates on the birdseed inoculum only, and after 35 days on A and D. Thus this strain formed primordia on all substrates when they were just fully colonised. All these first primordia aborted early, however new primordia appeared on the non contaminated side of substrate C on day 35, a shrivelled primordia on substrate D and some primordia on A and C on day 80. During 2 more months primordia appeared on both B and D substrates but always aborted.

Only A substrate allowed evolution of the primordia which had a different aspect than the others observed until then: it was yellow and white. It continued growing until the whole of the plastic top was removed on day 105, allowing differentiation into a fruit body.
Fruit bodies: on day 131 the humidity dropped to 70% because of a humidifier problem in the fructification room and the fruit body stopped growing. The 184g fruit body was cut and frozen.

Contamination: On day 22 there were some *T. viride* contamination spots on the plastic but not on the mycelium growing on C substrate which presented some orange-brown mycelium zones. Thirteen days later small contamination spots also appeared on the upper surface of this same substrate**.** *M. giganteus* mycelium had become brown and hard at its contact, the rest being yellow-orange on the upper surface and normal white elsewhere. On day 105 contamination on substrate C had overgrown the mycelium and even attacked the primordia. The mycelium of about half of the bag had become rusty-brown with orange or even red spots, while exsudation droplets were orange. On day 112 the bag of substrate C was rejected because of total contamination. Some contamination spots were present on substrate A on day 80 but could not progress further on the mycelium.

Discussion: Substrate colonisation by *M. giganteus* mycelium was quite rapid. It always formed primordia as soon as the substrate was totally invaded by the mycelium, however only substrate A allowed to complete fruiting cycle. It is after the filter was removed from bag A that small primordia started to grow and only when the plastic was removed that the primordia evolved to a fruit body. Thus it might be necessary to enhance aeration for fruiting process. The yield was 10.2%, however it could have been probably higher as the fruit body stopped growing only when the relative humidity dropped to 70% due to the fore mentioned problem. Thus this species certainly need a constant elevated air humidity to develop well.

Like observed in several other species *M. giganteus* mycelium on C formed a brown and hard mycelium in response to *T. viride* contamination. *M. giganteus* shows a quite good resistance to that invasive pathogen especially on substrate A where it could not progress at all.

4.1.3.14 Perenniporia fraxinea

Experiment development: strain K1 was tested on the four standard substrates. Only primordia could be obtained on A, C and D substrates, but none evoluted to a normal fruit body, only forming pores.

Culture conditions: incubation was performed in standard conditions. Bags of substrates A and D were put vertically in the fructification room on day 49. A week later the filter was removed from bag D to enhance gas exchanges. On day 71 bag of substrate C, the filter removed, as it was slightly contaminated by *T. viride,* was transferred into a desiccator's vessel with incoming air.

Spawn run: On day 22 substrates A and C were totally colonised by the mycelium, very dense on A substrate, especially on top were it formed a approx. 3cm high hard fleshy tissue layer. On substrate D some spots of substrate were not at all colonised by the mycelium and later on day 35, formation of black layers beside the non-invaded spots became visible. On day 180, strong melanisation lines appeared on the sides of that same substrate.

Primordia: after only 15 days of incubation and before total colonisation of the substrate by the mycelium there were primordia visible on substrate A, while some fleshy pore-bearing tissue was visible on substrate D and C. Primordia on A aborted rapidly, but new ones were visible on day 35. On day 49, pores had also formed on substrate A, while 2 primordia had formed on C. On day 71 the primordia on substrate C were grey and yellow, bearing pores in formation, however the substrate was contaminated.

Contamination: substrate B was contaminated by *T. viride* and rejected only 15 days after the start of the experiment, while substrate C had some brown exudation drops on the surface near a small contamination spot. On day 71 the contamination had extended slightly on the last substrate while some small contamination spots had appeared on A on day 92. On day 124 substrate C was totally contaminated with the exception of the primordia. The bag was rejected. On day 134, bag A was rejected because it was totally contaminated. On day 313 substrate D was rejected because it was totally invaded with flies.

Discussion: Substrate colonisation by the mycelium was very rapid. *P. fraxinea* formed primordia as well as pores on all of the 3 non contaminated substrates but none evolved further. The only interesting fact is that this species seems quite resistant to *T. viride* parasitism. It took more than a month for *Trichoderma* to overgrow mycelium on A substrate, 3 ½ months on C. On D there were strong melanised lines formed around non-invaded substrate spots which never got contaminated by the pathogen, showing a resistance mechanism which resembles that observed in *Lentinus edodes* cultures (Tokimoto, 1980).

4.1.3.15 Phellinus ignarius

Experiments development: two culture collection strains were tested, MUCL 39333 and MUCL 30499 on the four standard substrates. Fruiting was induced on B, C and D substrates for strain MUCL 39333, but could never be completed.

Strain MUCL 39333

Culture conditions: Incubation was performed in standard conditions. Bag C was transferred into the fructification room after removal of the filter on day 42 to try to induce the differentiation of the primordia into a fruit body.

Spawn run: It took about 38 days for the mycelium to colonise B, C and D substrates and 74 days for A substrate, which is the only one which presented quite hard flesh layer in front of the bag. The mycelium had a yellow-rusty colour white only at the margin of growth on all substrates. Mycelium, growing on substrates C and D, exudated brown liquid.

Primordia: After 38 days of incubation small primordia were visible on B, C and D substrate, however only the one on C grew to a several centimetres wide primordia. All primordia aborted.

Contamination: on day 32 substrate A was slightly contaminated by *T. viride*, however on day 74 the mycelium had overgrown the parasite. Substrate C was thrown away as it was invaded by mites 81 days after inoculation and substrate D 167 days after inoculation. The bag of substrate A was thrown away because of *T. viride* contamination 176 days after inoculation. Some contamination appeared on substrate B on day 188, but 12 days later it had not further evolved. On day 256, new contamination occurred, but a black crust had formed, which seems to have stopped the pathogens' progression. Finally a mite invasion brought an end to the experiment with bag B on day 285.

Strain MUCL 30499

Culture conditions: Incubation was performed in standard conditions. Bags C and D were transferred to room conditions after 21 days of incubation because of the presence of a *T. viride* contaminant.

Contamination: After 8 days on incubation, all the substrates were partly contaminated by *T. viride*. On day 21, bags A and B were rejected because they were too contaminated but bag C and D were left at room temperature and in the light because the mycelium of *P. igniarius* progressed despite the contamination. On day 33 bags C and D were rejected because of contamination.

Discussion: Two strains of this species were tested on the 4 basic substrates A, B, C and D, however strain MUCL 30499 did not give any results as all the bags became contaminated by *T. viride,* with a better ability to resist on the part of C and D, which are the lower N content substrates, than on A and B substrates with a higher N content. Thus this strain might behave like *L. edodes* against *Trichoderma* spp. according to Tokimoto and Komatsu (1979).

Moreover strain MUCL 39333 grew better on substrates C and D than on A and B, forming primordia on substrates B, C and D. These two observations seem to indicate that this species might be more adapted to subtrates with lower nitrogen content.

4.1.3.16 Pleurotus tuber-regium

Experiments development: It is the only sclerotia forming mushroom tested during the present work. The strain was first tested on the four standard substrates. All substrates allowed sclerotia formation. Sclerotia were submitted to different treatments to induce fruiting, which succeeded for sclerotia formed on A substrate. The first generation F1 was isolated and used for the following experiments realised only on A substrate. All the sclerotia obtained allowed the production of fruit bodies.

Screening of different substrates for sclerotia obtention with NEU-M strain
In a first experiment, strain NEU-M of *Pleurotus tuber-regium* was inoculated on the 4 basic substrates. At room conditions it took about 34 days to totally invade substrate B and C, while it took about 57 days to invade substrate A and D. Mycelium aspect was different depending of the substrate on which it grew: mycelium grew denser on the A substrate than on the inoculated birdseed, on the contrary to the three other substrate types. Moreover, mycelium on D substrate was sparser than the others but also forming a lot of cords. Sclerotia were collected 5 ½ months after inoculation on substrate C with a yield of 14.6% (2 sclerotia) and 9 months after inoculation on A substrate with a 16.5% yield (3 sclerotia), and on B with a 15.8% yield (2 sclerotia). Yield could not be measured in substrate D as the sclerotia were left in the substrate (see below) because it was the only substrate which was not at all contaminated *by Trichoderma viride***.** When sclerotia were collected, the substrate had shrunk considerably, showing probably a higher substrate utilisation than in *Pleurotus ostreatus* culture for instance (pers. obs.). The remaining substrate was not weighed. The sclerotia produced seemed harder than those produced on other substrates.

Sclerotia fruiting of NEU- M strain
The whole substrate D containing sclerotia was buried in humid compost and placed at room temperature. Sclerotia from substrate C were dried before further treatment. All the sclerotia from substrates A, B and C were buried in humid compost with a pH between 6.5 and 7.0 in polypropylene round Japanese pots covered with parafilm and were either placed in a fructification room at 19°C with artificial daylight, or at a room temperature of 20°C-22°C with daylight, or at 29°C with daylight. Only sclerotia from substrate A at 29°C fruited, when compost was humid enough, taking only a few days to start fruiting. Up to 3 flushes could be obtained for bigger sclerotia (241.3 g for a 8.3 cm x 7.5 cm size), but were only induced by a

shock of high CO_2 level (covering of the pot with parafilm). As soon as fruiting started, aeration was enhanced to lower CO_2 level. A sclerotia from substrate B was placed at 29°C but fruiting could not be induced. Smaller sclerotia (19.2g, 3.5 diameter size) could not fruit in the same conditions.

Yield between sclerotia and fruit bodies were quite high ranging from 48.9% to 53.9% in up to 3 flushes. Time between the first flush and the third flush took an average of 27 days. The first flush was the one with the most fructifications. There are generally more fruit bodies (Fig. 4.8) however the stipes are empty so the weight is lower than in other *Pleurotus* spp.

Yield between fruit bodies and substrate range from 4.6% to 6.55% with A substrate if we only consider real fruit bodies obtained, but goes up to 8.1% and 8.5% if we consider the theoretical yield in function of the sclerotia weight obtained by substrate bag.

The first generation obtained in our laboratory was isolated from a piece of clean carpophore and named NEU-MF1. This generation was used for the next experiments.

Figure 4-8. Pleurotus tuber-regium emerging from sclerotia buried in compost.

Sclerotia production and fruiting of NEU-MF1 on A substrate
Further experiments realised with strain NEU-MF1 generation was performed only on A substrate as it is the only substrate which produced fruiting sclerotia. The bags were incubated at 25°C in the dark. Invasion time was reduced to 26 days on A substrate. After total invasion of the substrates, bags were transferred to room conditions. Four bags were transferred at different times to 29°C (48 days and 81 days after inoculation). Sclerotia induction was shown to be enhanced at 29°C compared to at room temperature, about a week after the transfer, sclerotia were visible emerging from the substrates. Sclerotia were collected 7 months after inoculation with a yield ranging from 12.5% to 17.3 %. (225 g to 311 g). The sclerotia were generally heavier than in the previous experiment. They were all buried in humid compost as previously and placed at 29°C. They produced 3 flushes formed of 3 to 16 fruit bodies each with a average total weight of 172g (yield 9,55%)

Pests encountered during the research

Two kinds of pests have caused problems in our culture experiments. *T. viride* was capable of invading the substrate in the first days following inoculation with *P. tuber-regium* and caused a few losses in the pasteurised substrates, however, it was overgrown by *P. tuber-regium* when the mycelium had grown partly on the substrate. It is notably the case with bags of substrate A inoculated with NEU-M strain. *T. viride* caused contamination in old substrates (A and B) inoculated several months before and having formed sclerotia, but never in fruiting experiments.

Flies were a problem in the fruiting experiments as a few sclerotia were totally invaded by larvae. Care had to be taken as these pests seem to be attracted by the sclerotia. No problem was encountered with the fruit bodies.

Discussion

In the first experiment good sclerotia yield was obtained. Biological efficiency (BE) was estimated to 64.9% (min: 61.3%, max: 68.9%) in substrate A, to 61.9% (min:58.5%, max: 65.8%) in substrate B and to 47.8% (min 45.5%, max 50.2%) in substrate C. Interestingly higher yields corresponds to lower C/N values, which means, with a higher nitrogen content. Our results are quite good if compared to published data: Fasidi & Ekuere (1993) have obtained 18.58% to 30.11% BE depending of the substrate. Chen (2001) cites that Huang (1997) obtained a 30-40% BE on a substrate composed of 78% broad-leaf trees sawdust, 20% wheat bran, 1% sucrose and 1% calcium carbonate. The longer time taken in our experiment probably allowed a more efficient use of the substrate by the fungus. Chen (2001) cites that Huang (1997) was for instance harvesting sclerotia after 1-2 months or longer in function of the used strain, substrate formulation and growth parameter. Contrary to Okhuoya & Okogbo (1991) we obtained sclerotia in all tested substrates. It corresponds with the declaration of Isikhuemhen *et al.* (2000) that African isolates always form sclerotia before fruiting.

Compost was chosen for planting sclerotia, because Okhuoya & Etugo (1993) had stated that loam was the best type of soil for planting sclerotia, apparently because of its water-holding capacity. This choice revealed to be adequate, however not all sclerotia produced fruit bodies. Only sclerotia produced in A substrate and placed at 29°C could initiate fruiting. A minimal size seems to also be needed for fruiting induction.

These values are possible considering that the fruit bodies contain about 16.5% dry matter (Ogundana & Fagade, 1981). Conditions for fruiting in our experiment fits previous results obtained by Stamets (2000) which mentions a fruiting temperature between 24 and 32°C or 30 to 35°C depending of the strain and by Oei (1996) who mentions a 30°C temperature for fruiting.

Further experiments were focused only on A substrate as yield of sclerotia was the highest and as only sclerotia from this substrate produced fruit bodies. Reduction of invasion time on substrate A by NEU-MF1 strain is probably due to the higher incubation temperature. Temperature seems also to influence primordia initiation and maturation as in all bags (8) sclerotia were in general heavier with a similar yield as in the previous experiment (12.5% to 17.3 % versus 16.5%), but in two months less incubation time. It represents a BE of 49% to 67.7%, which is in the range of results obtained first. Fruiting was easily achieved by heat and CO_2 level shocks allowing an estimated BE of 111 to 153%.

To conclude we can state that the formation of the sclerotia seems not to be dependent of the substrate type since they where produced in all tested ones, independently of their C/N values. Initiation seems to be stimulated by temperature. Yield of the fruit bodies depend of the sclerotia weight with probably a minimal sclerotia size for initiation. Yields between substrate and fruit bodies are interesting but could be optimised with further trophic research.

4.1.3.17 Polyporus brumalis

Experiment development: strain 22 was inoculated on A, B and C substrates in polypropylene pots (about 400g) and on D in industrial bag.

Culture conditions: incubation was performed in standard conditions. Pots A and B were transferred to the fructification room after 34 days and pot C after 58 days. The bag of substrate D was placed in a cooler at 5°C on day 58 to see if a drop of temperature could influence fruiting induction as this species naturally fructifies in the winter. On day 81, holes were made in the plastic near nascent primordia on D substrate. The cover of pot C was removed and the pot was recovered with parafilm and also placed in the cooler at 5°C to see if the cold temperature could also induce fruiting in that substrate, without success.

Fruit body: only 3.5g fruit bodies could be collected from D substrate after 99 days of experiment.

Contamination: substrate B was totally contaminated by *T. viride* after 49 days of experiment and substrate A was invaded by flies after 92 days.

Discussion: Only a very low yield of 0.2% was obtained on D substrate in cold temperature. In this experiment, we tried to place one of the substrate in the cooler at 5°C because it naturally fructifies in the winter (Courtecuisse, 2000). Thus temperature might have an influence on fruiting induction; however it was not confirmed by C substrate placed in the same conditions. No further experiments were realised on that species as it took more than 3 months for the first one and as it was not a prioritary species. However if further experiments are realised the strain should probably be tested again on all the substrates in industrial bag to have more substrate and to better conserve the humidity and the bags should be transferred to low temperature after spawn run to try to induce fruiting.

4.1.3.18 Polyporus ciliatus

Experiment development: strain neu270 was tested once on substrate A and C and twice on substrate B and D in polypropylene pots (about 400g). Fruiting cycle was achieved on all substrates.

Culture conditions: incubation was performed as usual. All the pots were transferred to the fructification room after 23 days.

Spawn run: it took less than 23 days for the mycelium to colonise all substrates.

Fruit bodies: primordia were not observed before the collection of the fruit bodies: on substrate A: 23g on day 47. On substrate B1: 46,5g on day 66. On substrate B2: 9g on day 47 and 18g on day 66. On substrate C1: 26, 8g on day 33 and 18,5g on day 79. On substrate D1:

19g on day 97. On substrate D2: 15,4g on day 63 and 10.3g (with some kinds of warts on the fruit bodies) on day 69.

Contamination: substrates A, B1 and B2, and C were thrown away after the fruit bodies collection because of *T. viride* contamination.

Discussion: This species fructified quite easily on the four substrate types tested with reasonably good results as shown by figure 4.9. All fruit bodies' collection was done between 1 month and $2^{1/2}$ months after inoculation. This species seemed to be very sensitive to CO_2 level as a fruit body formed a loop with its very long stipe in one of the D substrate.

A, B and C substrates were only contaminated after collection of the fruit bodies, while D was free of contamination. It might show a lowering of the resistance capacity of this strain after achieving the reproduction phase, excepted on D substrate which seems to favour this strain.

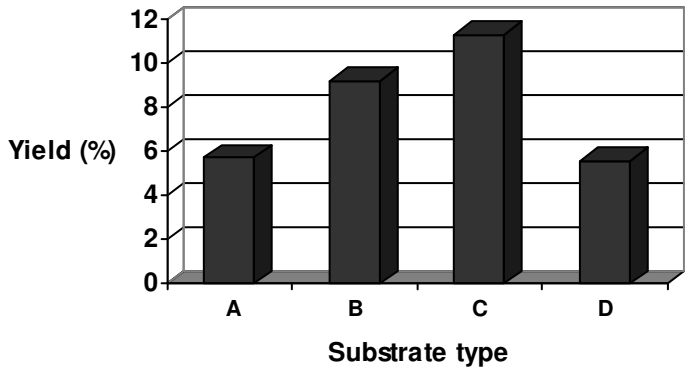

Figure 4-9: P. ciliatus yield on the four standard substrates in polypropylene pots

4.1.3.19 Polyporus squamosus

Experiment development: the Neu F271 strain was inoculated once on A substrate and twice on the three others. Fruit bodies were obtained on 3 substrates A, B and C, while on D there was not even tissue formation.

Culture conditions: the bags were incubated in room conditions. Bag B2 was transferred to the fructification room after 69 days of incubation. Bag C2 was transferred on day 79 to see if the primordia would also evolve to a fruit body in these conditions.

Spawn run: the mycelium growing on B1 substrate at room condition had a cucumber smell.

Primordia: there were visible primordia on substrate C2, 79 days after the start of the experiment.

Fruit bodies: The following fruit bodies could be collected. Substrate A: 353g on day 59; Substrate B1: 170.5g 64 days after the start of the experiment, and another 51.6g one on day 126, while it was still at room conditions. Substrate B2: 150g on day 79. Substrate C2: 252.3g of yellow fruit bodies with sclerified borders and a cucumber smell on day 87.

Contamination: Substrate B1 was contaminated by *T. viride* quite early after inoculation; however the mycelium kept on growing and overgrew the pest. The bag of substrate A was thrown away 5 days after fruit body collection on day 64, because it was totally contaminated by *T. viride*, while there was no trace of the pathogen at the time of collection.

Discussion: This species seems not to be exigent as it has fructified on three of the four substrates tested. Yield was good in this small assay as shown by figure 4.10. If further fruit bodies are needed it would be better to cultivate *P. squamosus* on A substrate where best yield was obtained in the shortest time. The mycelium on substrate B and the fruit bodies collected on C had a cucumber smell which was undetectable elsewhere. This strange odour does not fit descriptions of a honey smell given for *P. squamosus* (Maublanc, 1995), but is strangely similar to the one detected after fly larvae invasion in *G. applanatum* stain 87 culture Thus a trophic factor might influence secondary metabolism of this strain. This would be very interesting to compare chromatographic profiles of the different extracts.

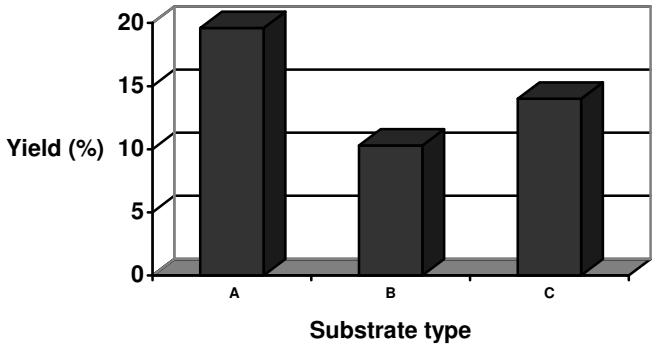

Figure 4-10: P. squamosus yield on 3 of the 4 basic substrates tested.

4.1.3.20 Polyporus tuberaster

Experiment development: strain 53 was inoculated on the four standard substrates (about 400g) in polypropylene pots. Fruit bodies could be obtained on substrates A, B and C.

Culture conditions: incubation was performed in standard conditions. Pots A, B and D were transferred to the fructification room after 23 days and pot C after 28 days.

Spawn run: Substrate A, B and D were totally colonised by the mycelium after 23 days and substrate C after 28 days.

Fruit bodies: the following fruit bodies were collected. Substrate A: 65g on day 56, 16.5g on day 82, 9.2g on day 87, 33.5g on day 102, 11.8g on day 116 and 3g on day 144. Substrate B: 38g on day 56 and 30g on day 66. Substrate C: 22g on day 56 and 68,5g on day 73

Discussion: This species is one of the rare ones tested which grew perfectly in polypropylene pots. Of the 4 substrates tested, only D did not allow a fruiting cycle. Yield obtained was high for a first experiment as shown in figure 4.11. In the nature this species grows mainly on the ground near ligneous residues, however Courtecuisse (2000) mentions that it forms a sclerotia, which we have not observed in our culture experiment. It formed the carpophore directly. When we had enough fruit bodies for the extraction, the experiments were not

continued, but it seems that cultivating this species in pots with the cap on is fine especially on substrate A.

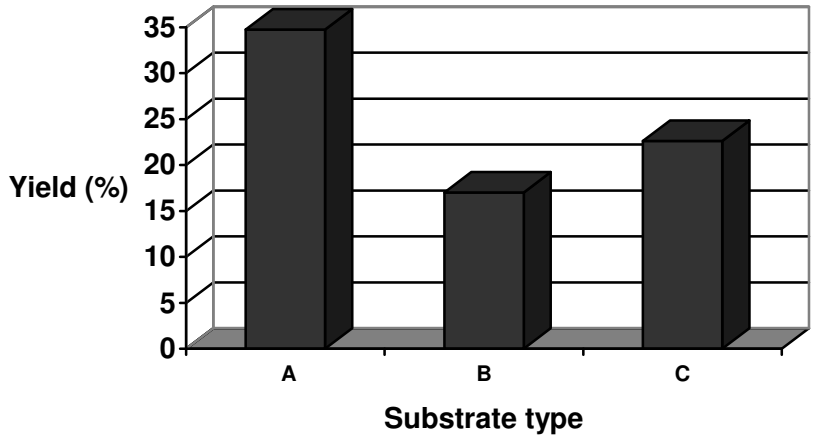

Figure 4-11: P. tuberaster yield obtained on 3 of the 4 tested substrates.

4.1.3.21 Pycnoporus cinnabarinus

Experiments development: strain 71 was inoculated on the four standard substrates. Primordia were visible on all substrates, however only malformed fruit bodies were obtained on A, B and C substrates.

Culture conditions: incubation was performed in standard conditions. After 31 days of incubation the bags were placed vertically with their filter removed in the fructification room at 16°C-17°C, a lower temperature than usual. One of the two A substrate bag was only transferred on day 39. On day 63 the plastic was removed from bag A to try to induce the formation of a fruit body instead of pores. It was also removed from the top of the 3 other bags.

Spawn run: total colonisation of the substrates by an orange mycelium was observed after 31 days. Pores were visible on the surface of substrate A, B and D. On day 56 there was a thick orange fleshy tissue layer with sporulating hymrnium on all substrates. On day 63 when plastics were removed from the bags, different odors arising from the bags could be detected: there was almost no smell on A and B, while a typical wood mushroom smell was detected on C and a strong sweet smell on D.

Primordia: first primordia were observed on substrates A, B and D 56 days after the start of the experiment, at that stage they all aborted. On day 66 another primordia (about 2 cm of diameter) was visible on the side of substrate D. A hole was made in the plastic beside it. It formed a pored structure which had to be rejected because of contamination. The 3 other substrates also allowed formation of pored structures. Indeed 111 days after inoculation 212g were harvested on substrate A1, 58g (very dry) on B and 61g of dry ones on C. However they were not used for biological tests as they did not have a normal form, but looked more like "bumpy" pored crusts.

Contamination: One of the two A substrate bags presented an undetermined fungal contaminant after 13 days of incubation, however it was overgrown by *P. cinnabarinus*

mycelium everywhere except on the birdseed inoculum on day 32. It was finally thrown away on day 68 because of the presence of mites in the bag. There were the same bumpy pored structures like those mentioned above, however they were not weighted so as not to propagate the pest. Substrate D was thrown away because of *T. viride* contamination on day 89 and substrate A on day 129.

Discussion: We have not succeeded in fructifying this mushroom normally, but it formed organised structures with sporulating hymenium. Substrate C is the one where the structure was the closest to a normal fruit body. According to Courtecuisse (2000) the cap is 3-13cm, quite thin, or thick (up to 4cm), hemispheric or larger than long (2-7cm), irregularly bumpy. In our case we had what looked like the start of the fruit body without the hemispheric part. The factor(s) influencing evolution of primordia to normal fruit bodies were visibly lacking. It could be a trophic factor or the influence of an environmental factor like the presence of natural light.

Two interesting observations, first, *P. cynnabarinus* resisted and even overgrew a contamination on the A substrate but not on the birdseed inoculum, thus giving an example of the influence of trophic factors in the resistance which has already been demonstrated for *L. edodes* for instance (Savoie et al., 2000; Goltapeh and Danesh, 2000; Tokimoto and Komatsu, 1995; Badham, 1991; Tokimoto and Komatsu, 1979). Second, the smell was different in different bags. The only one which smelled almost normal was the bag of substrate C. All these observations seem to indicate that this species is highly modulable.

4.1.3.22 Schizophyllum commune

Experiment development: strain neu F14 was inoculated on 2 bags of each of the industrial substrates B, C and D and on one bag of substrate A. Fruit bodies could be obtained on all substrate types, however with a low yield.

Culture conditions: Incubation was performed in standard conditions. On day 14, all the bags were transferred into the fructification room, as the substrates were totally colonised.

Fruit bodies: they were collected quite rapidly after inoculation. Substrate A: 19,2g on day 33 and 13,4g on day 37. Substrate B1: 20,8g on day 33 and 11,4g on day 37. Substrate C1: 7,2g on days 33 and 8g on day 78. Substrate C2: 14g on day 51. Substrate D2: 24.8g on day 51.

Contamination: Bags A1 and D2 were rejected because of *T. viride* contamination.

Discussion: Of all substrates tested we could collect fruit bodies - sometimes malformed ones- rapidly after inoculation showing that this species can grow on a variety of substrates. Yield achieved was very low as shown by figure 4.12. Thus, if further culture is to be developed, substrates A or B should be modified to enhance yield or other strains should be tested on these same substrates. It is without interest for us to develop the culture of this species as it is already done in many countries, principally for medicine. As we had enough crude material for our extraction, no further experiments were realised.

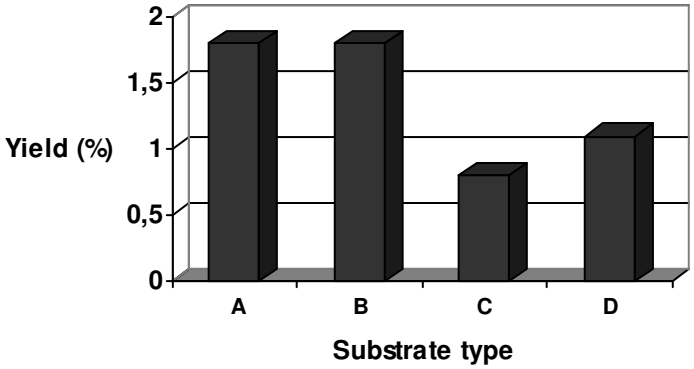

Figure 4-12: S. commune yield obtained on the four basic substrates tested.

4.1.3.23 Stropharia rugosa-annulata

Experiments development: FTA strain was inoculated on Bm (1x), Jm (2x) and LO (2x) substrates in polypropylene pots (about 400g). Kit strain (isolated from a commercialised kit) was inoculated once on the four standard substrates in industrial bags as well as 3x on two specially designed substrates RC and RCM in polypropylene pots. A, B and D were inoculated with the original straw inoculum, and the others with a normal birdseed inoculum.

FTA strain

Culture conditions: incubation was performed as usual. Both LO pots were transferred to the fructification room after 26 days. Jm pots were transferred after 31 days and Bm pot after 42 days. On day 80, Jm substrate pots were transferred to the cooler at 9°C, however on day 103, as there was no fruiting induction, they were transferred to the fructification room again.

The RC and RCM pots were all transferred into the fructification room on day 53.

Spawn run: 26 days after inoculation both LO pots were fully colonised by a mycelium resembling a brown spider web with round black structures. The microscope showed very thick hyphae with many septae. The round structures had no visible components, thus they could have been chlamydospores or asexual fruit bodies. Jm substrate was fully colonised by the normal white mycelium after 31 days and Bm after 42.

Contamination: On day 31, Jm2 substrate had *Penicillium* spp contamination spots. On day 80 there was some contamination on the birdseed inoculum which was removed in all pots. The contamination did not evolve further. On day 93, LO substrates had to be thrown away because of fly larvae invasion.

Kit strain

Culture conditions: incubation was performed as usual. On day 34 bag D was transferred to the fructification room. On day 54 as there had been no modification on that substrate, the plastic was opened and the bag placed at room temperature. On day 117 as there had been still no changes mycelium on the surface of D, the substrate was damaged to see if it would induce a reaction.

Spawn run: On day 28 all the RC and RCM substrates were totally colonised by *S. Rugosa-annulata* mycelium

Contamination: 6 days after inoculation there was a undetermined fungal contaminant growing on the straw inoculum on A, B and D substrates while *T. viride* was present on substrate C. Five days later *S. rugosa-annulata* had reacted by forming melanised lines on substrate C. Ten days after inoculation, RC1 and RCM3 were also contaminated, but the fungal pathogen was then totally overgrown in the following 18 days. On day 18, on substrates A and B the contaminant had the advantage everywhere except on the inoculum, which the mushroom species had totally colonised. The two bags were rejected while *S. rugosa-annulata* was overgrowing the mould on D substrate. It is interesting to note that the morphology of the mycelium overgrowing the contaminant, by forming some kinds of cord, was different than the one growing on the straw substrate. On day 19, C substrate was totally invaded by *T. viride* and had to be rejected. On substrate D however the mycelium kept progressing slowly until it recovered totally the contaminant.

Discussion: no differentiated tissue was formed by the two tested strains on any of the tested substrates. Neither staying at room conditions, in the fructification room, in the cooler at 9°C, nor the voluntary destruction of the top mycelium (on D) induced fruiting. We could have tried casing with a slightly acid ground like mentioned in the literature, however the kit from which the second strain was isolated instructed only to place the inoculum in a heap of straw and to keep it humid. So it should fructify without casing. The only conclusion we can draw from this experiment is that of the four basic substrates, D was the only one were *S. rugosa-annulata* resisted a fungal contamination and even overgrew it and this with a different mycelial morphology than usual. The initial kit inoculum clearly allowed higher resistance capacity of this kit strain against fungal contamination. This was demonstrated by the fact that on contaminated substrates, mycelium growing on that inoculum could resist the pathogen. Thus we can demonstrate here that resistance and agressivity is not only dependent of a strain but also of a substrate composition. If further work is to be realised, a substrate based on reed straw with casing should be tried or work on a higher quantity of substrate.

4.1.3.24 Trametes gibbosa

Experiments development: the four substrates A (4x), B, C and D (1x) were inoculated with strain 28. Fruit bodies could be collected on all substrates.

Culture conditions: incubation was performed in standard conditions. Three A bags (A1 to A3) were moved to the fructification room after 29 days of incubation. The filters were removed from the other bags; they were then placed in the fructification room on day 34. All were placed vertically as fruit bodies naturally arise from vertical surfaces. On day 91 the plastic was cut all around the top surface of substrate B and D to enhance gas exchanges during the fruit body formation. The same thing was done to bag C on day 127 and to bag A4 on day 139.

Spawn run: One bag of substrate A (A4) and the 3 substrates B, C and D were totally colonised by the mycelium after 20 days and the 3 other A substrates after 29 days. When the filters were removed on day 34 there was a strange smell of organic solvent or vinyl emanating from all the bags which faded slightly when the fruit bodies were growing.

Primordia: One A4 substrate and the 3 substrates B, C and D allowed the formation of primordia after 20 days of experiment. On day 34 there were 6 primordia on substrate A4, 2 on B, a big white one on C and a line of small ones on D. On day 91 the primordia on A4 had

aborted and a new one had appeared on C substrate. On day 120, a kind of primordia was growing on the old one on A substrate.

Fruit bodies: they were collected on the following substrates.

Substrate A1: 15g on day 91. Substrate A2: 31.7g on day 91. Substrate A3: 8.7g on day 99 and 22.2g on day 152. Substrate A4: 32.5g on day 212 (because of the presence or *T. viride* contaminant it could not be used for extraction) On day 139 there was a malformed fruit body with closed pores over the cap and not under it as normal.

Substrate B: 91.4g on day 127 (they had a depression in the centre which was not observed in the others).

Substrate C: 35g on day 212 (because of the presence or *T. viride* contaminant it could not be used for extraction).

Substrate D: 70.6g on day 127

Contamination: Three A bags (A1 to A3) were thrown away about 4 months after the start of the experiment as they were invaded by fly larvae. It is interesting to note that many species were in the fructification room when a fly invasion occurred but it was mainly the *Trametes* spp. which was attacked.

T. viride contamination was visible on the mycelium growing on substrate D on day 110 and on the flesh along the plastic of substrate C on day 120. On day 127 bag D was thrown away because it was all contaminated. On day 146 in bag B, only a small fruit body was not contaminated by *Trichoderma*. On day 169, contamination also started on the mycelium of substrate A but not on the growing fruit body. On day 178, contamination on B was steady, but conidia were yellow instead of the normal dark green colour. On day 187, contamination was starting on the fruit body of substrate B which had to be thrown away on day 207. A few days after the fruit body collection on day 212, bags A4 and C became totally contaminated by *Trichoderma* and had to be thrown away.

Discussion: We could obtain fruit bodies on all tested substrates. Yield was quite low except on B substrate (fig 4.12). This species seems easy to cultivate but further studies should be realised to enhance the yield in the first flush and to lower crop cycle time if it is found interesting as a product source.

Sufficient aeration seems to be essential for normal fruit body production. The strange smell of solvent or vinyl detected in our experiment does not fit the description of Breitenbach and Kränzlin (1986) who mention that the smell of this species is about like the one of *Heterobasidion annosum*. This latest species' smell is itself described as a "strong mushroom smell". However the smell described in literature always corresponds to the fruit body, and the solvent or vinyl smell was detected in the mycelium phase then faded when the fruit body was growing.

We could observe that often there was a contamination of the mycelium by *T. viride* after collecting the fruit body as if *T. Gibbosa* was loosing its resistance ability. Both substrate B and D got contaminated some times after first flush collection, except the new growth of fruit

bodies on B. They resisted about 40 days before being in their turn, contaminated. It would be very interesting to elucidate resistance mechanism of *T. Gibbosa*.

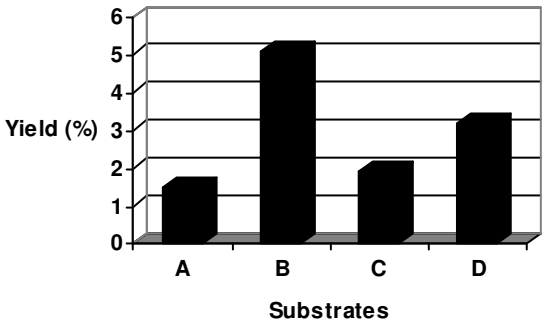

Figure 4-13: T.gibbosa yield on the four tested substrates

4.1.3.25 Trametes hirsuta

Experiment development: Strain 11 was first inoculated in polypropylene pots filled with 400g of each of the substrates A, B, C and D. Later it was also inoculated on B, C and D substrates in industrial bags. Fruit bodies could be collected on B, C and D substrates both in industrial bags and polypropylene pots.

Culture conditions: Incubation was performed at room conditions. After 23 days of incubation, all the polypropylene pots were transferred to the fructification room. On day 71 bag C was transferred to the fructification room. On day 130 the bag of substrate D was placed into the cooler at 9°C.

Spawn run: the substrates in polypropylene pots were fully colonised by the mycelium after 23 days.

Fruit bodies: The following fruit bodies were collected:

Substrate B in polypropylene pot: 3.8g in 16 months. Substrate B in industrial bag: 55.3g were picked up on day 172; however a part of them (23,5g) had to be thrown away because of moulds and fly larvae.

Substrate C in industrial bag: 90.9g on day 172. Substrate D in polypropylene pot: 16.5g after11 months.

Substrate D in industrial bag: 56.8g on day 172.

Contamination: On day 120 there were many dead flies on the surface of substrate C in industrial bag, while all other species present in the fructification room and with their bags open were totally invaded by fly larvae. The 3 bags were thrown away a few days after fruit body collection (day 172) because they were invaded by fly larvae and moulds. After almost 5 months of experiment, substrates A and C in pots were thrown away, because of *T. viride* contamination.

Discussion: This species seems not to be bound to a specific substrate as it fructified on 3 different substrates. Crop time cycle was long as it took almost 6 months on all substrates of the second experiment but 11 months and 16 months in the first. Interestingly, the 3 substrates of the second experiment fructified at the same time, even if one was in the fructification room (C), one at room temperature (B) and one in the cooler at 9°C (D). It seems thus that temperature is not so important. This species could have another signal to induce fruiting. Yield varied greatly between substrates, but even more for a given substrate in different container types (fig 4.13).

Another interesting observation is that when the fructification room was invaded by flies 4 months after inoculation (second experiment) the mycelium on C substrate seemed to have an insecticide action, because all the flies were dead at the surface, while it was not the case in any of the other bags of other species present at the same time in the fructification room.

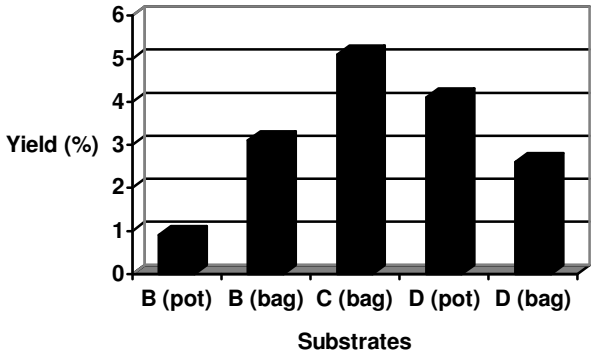

Figure 4-14: T. hirsuta yield obtained on 3 of the 4 tested substrates in both containers type.

4.1.3.26 Trametes suaveolens

Experiments development: strain 73 was inoculated on substrates A (2x), B (1x), C (2x) and D (3x). Fruiting occured on the four substrates tested.

Culture conditions: incubation was performed in standard conditions. Bags C1, D1 and D2 were transferred to the fructification room 23 days after inoculation. On day 31 the bags of substrate B, C2 and D3 were transferred after removal of the filter to 16°C-17°C, a lower fruiting temperature than usual. Bag A2 was transferred to the fructification room after 42 days of incubation. On day 59 the plastic was removed all around the vertical surface of bag B, C2 and D3. Bag A1 which had no primordia was transferred into the cooler at 10°C. On day 76, the plastic of bag D3 was opened to allow better aeration for the growing fruit bodies. On day 82, bag C1 was transferred to the cooler at 9°C to try to induce a tissue formation by a drop of temperature.

Spawn run: After 23 days of incubation B, C and D substrates were fully colonised by the mycelium. The filters were removed, revealing a strong anise smell in the three substrates. Substrate A2 was fully colonised after 42 days of incubation

Primordia: First primordia were observed on substrate A2 42 days after inoculation which differentiated into fruit bodies 10 days later. The plastic was removed from around them.

After 50 days of experiment a primordia was visible on C2. A hole was made in the plastic near the primordia to enhance gas exchanges. On day 56 it started to form the hymenium. The same day primordia up to 5 cm in diameter were visible on substrate B with one also starting to form the hymenium. Substrate D3 also presented primordia with one differentiating into a fruit body. A hole was made in the plastic to avoid contact and enhance gas exchanges. New primordia kept on forming on the substrates in the following weeks.

Fruit bodies: On substrate A2 growing fruit bodies were visible on day 69 but were collected on day 97 (52g). They were not used for extraction as they were slightly contaminated. On day 66 substrates B, C2 and D3 all presented growing fruit bodies. The anise smell was still very strong in all the substrates, however progressively it seemed to diminish with fructification growth. The fruit bodies were collected on day 111 on these 3 substrates. However they all had some contamination spots so that they could not be used for extraction: 63g on substrate B, 111g on substrate C2 and 82g on substrate D3. Three fruit bodies were produced on substrate C1 in the cooler at 9°C after 4 months of experiment, however as the whole bag was contaminated by *T. viride*, they were thrown away without being weighed.

Contamination: On day 62 bag A1 was thrown away because of *T. viride* contamination. On On day 69 there were two types of undetermined mould in bag A2: one type where the straw was emerging from the mycelium and the other on a small primordia, had spread to 2 small fruit bodies on day 80. On day 82 bags D1 and D2 also were contaminated by *T. viride.* On day 94 *T. viride* had invaded the mycelium and primordia of *T. suaveolens* but not at all the fruit bodies of bag D3. It was finally rejected 35 days later. On day 115 bag A2 was also thrown away because of contamination.

Discussion: This species seems not to be very specific as it fructified on the four substrates tested. Yield obtained are presented in figure 4.14.

In each case primordia appeared between 42 and 56 days after inoculation and fruit bodies observed between 52 and 56 days after inoculation, thus indicating perhaps an influence of the age of the mycelium on fruit body production.

They needed a lower temperature than we usually work at to induce their fruiting, as shown in the first experiment, *T. Suaveolens* fructified at 9°C on C, and at 16°C for the others. Aeration seems also to be important. In bag C2, a primordia evolved to a fruit body only near a hole done in the plastic, then all the fruit bodies formed when the whole plastic was removed.

If more fruiting experiments are needed in the future we should try to ameliorate C substrate composition to enhance yield. The newly invaded substrate should be placed straight away in the fructification room at 16°C or at a lower temperature with the filter removed. Moreover when primordia are formed, the whole plastic should be eliminated.

It is interesting to point out that even when the mycelium and the primordia were totally infected by *T. viride*, none of the mature fruit bodies were attacked, at least for a certain time. It could be due to a different composition of the cell walls, to lower water content or because of the presence of an antimycotic in the mature fruit bodies.

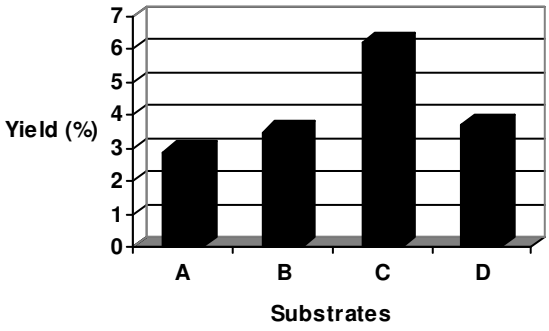

Figure 4-15: T. suaveolens yield on the 4 tested substrates

4.1.3.27 Trametes versicolor

Experiments development: Strain 2 was inoculated twice into each of the A, B, C and D substrates in polypropylene pots (400g) and Chine strain once on each of the A, B and C substrates in industrial bags. Fruiting cycle was achieved for strain 2 on B and C substrates in polypropylene pots and for Chine strain on substrates A and B in industrial bags.

Strain 2

Culture conditions: the pots were transferred to the fructification room when mycelium had totally colonised the substrates.

Fruit bodies: The following ones were collected. Substrate B2: 1.3g on day 63 and 11,5g on day 106. Substrate C2: 8.4g on day 63, 1g on day 71 and 5,5g on day 106.

Contamination: both A and D pots, as well as B1 were contaminated by *T. viride* after about 2 months of experiment without forming any tissue. The other pots all got contaminated after about 4 months of experiment.

Chine strain

Culture conditions: after 53 days of incubation in standard conditions, all the bags were transferred to the fructification room. Bag C was transferred to room light and temperature on day 56 and bag A on day 77. On day 97 the filter was taken off from bag B and some holes were opened near some primordia to try to induce differentiation into fruit bodies. On day 103 the filter was also taken off of bag A and the bag was transferred to the fructification room again. On day 109 the whole plastic was removed from bag B and bag A which presented deformed fruit bodies, probably by lack of aeration.

Spawn run: 14 days after inoculation substrates A and B were totally colonised by the mycelium.

Primordia: On day 35 there were plenty of small primordia on the top and on the sides where the plastic wasn't touching substrate A. They had aborted but had formed new ones on day 53. Primordia were observed on day 67 on substrate B and on day 77 on substrate C. In this last bag they were mostly formed on the brown zones, with even one in a contaminated zone.

Fruit bodies: the first ones were observed on day 77 on substrate B, and then on day 97 on substrate A placed at room condition. On day 109 there were fruit bodies everywhere under

the holes in the plastic of bag B. On day 132 they had stopped growing, but others were growing. Finally they rotted and could not be collected. On substrate A, 21.9g were collected on day 123 and 25.5g on day 160.

Contamination: 14 days after inoculation about 50% of the lower part of substrate C was contaminated by *T. viride* visibly because of a bad suture of the plastic bag. On day 35 there were tiny spots of contamination on B substrate, whereas *T. versicolor* had overgrown slightly the pathogen on substrate C. Probably bound to the contamination, *T. versicolor* also exuded liquid and formed some brown zones delimited by a black line. On day 53 substrate B took about the same appearance as substrate C with brown zones, dried black drops and black lines separating zones of white and grey mycelium. However there was a proliferation of mites and bag C had to be thrown away on day 77. On day 109 there were also a few little *Trichoderma* contamination spots and pigmentation of the mycelium about everywhere except where it was touching the plastic of bag A. It is interesting to note that on day 123 fruit bodies had formed only on the melanised mycelium, but not where the mycelium had previously touched the plastic.

Discussion: *T. versicolor* could induce fruiting on various substrates however with a low yield (figure 4.16) which might be due to inadequate environmental conditions. Chine strain also fructified on B and formed primordia on C, which may have evolved to fruit bodies if the bag had not been invaded by mites 2 ½ months after inoculation. *T. versicolor* seems to prefer room conditions to the fructification room for fruiting. It remains to determine if it is because of the higher temperature or of the wave length of the natural light. Aeration also seems important for the evolution of primordia to fruit body.

Like for *Ganoderma* spp., it is clear that brown pigmentation is correlated to resistance toward *T. viride*. Indeed about 50% of substrate C was invaded by the mycoparasite, however it formed brown zones delimited by a black line, which completely stopped the pest progression. *T. versicolor* overgrew it slightly and even formed primordia, mostly on the brown zones. When substrate B got contaminated the same reaction was observed. In both cases there was abundant exudation. *Trichoderma* invasion was not responsible for the end of the experiment in any of the samples, which is quite unusual in our culture experiments.

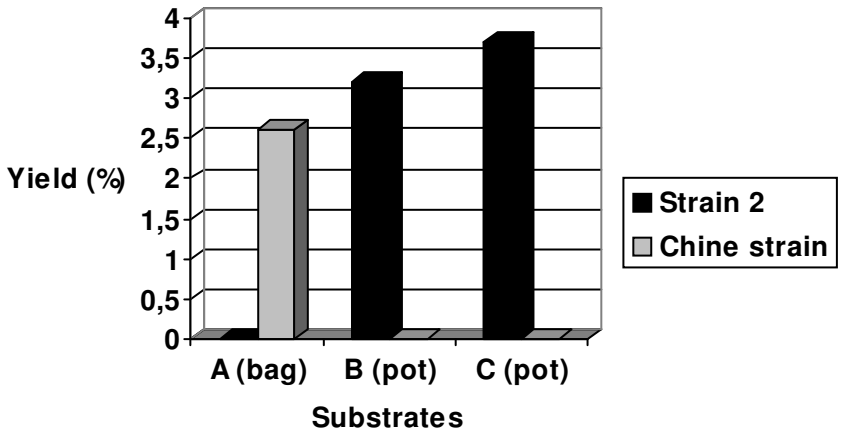

Figure 4-16: Yield obtained for T. Versicolor strain 2 and Chine strain

4.1.4 Discussion and conclusions

During this part of the work 14 species have been successfully cultivated according to techniques previously developed in the laboratory. Moreover 37 new strains from 27 species have been tested for their fruiting ability on different substrates in industrial bags or in polypropylene pots. In most experiments the four substrates A, B, C and D varying in their C:N ratio were used to determine influence of this factor on fruiting success.

Of the 37 strains and 27 species tested 59.5% and 63% respectively could be fructified successfully. Table 4.7 shows whether the fruiting cycle was completed, whether it was stopped at the primordia stage or whether the fruiting process was not induced on the four standard substrates (excepted *A. brasiliensis* and *P. tuber-regium*).

Family	**Species**	**Strain code**	**Substrate type**			
			A (C:N=75)	B (C:N=90)	C (C:N=125)	D (C:N=265)
Polyporaceae (S. stricto)	*Lentinus conchatus*	NEU-K98	F	F	F	F
Polyporaceae (S. lato)	*Dendropolyporus umbellatus*	NEU-M	P	P	-	P
	Fomes fomentarius	MUCL 35117	F	F	P	-
	Fomitopsis pinicola	MUCL 30677 MUCL 30544 NEU-K2	F - F	P - -	F P F	F F P
	Gloeophyllum trabeum	NEU-K00	P	-	-	P
	Grifola frondosa	GfSt GfJ GfB GfK00	F - P	F - -	F F F	F - -
	Laetiporus sulphureus	NEU-41 NEU-I1	- P	- -	- -	- -
	Lariciformes officinalis	NEU-70	-	-	-	P
	Meripilus giganteus	NEU-K1	F	P	P	P
	Perenniporia fraxinea	NEU-K1	P	0	P	-
	Polyporus brumalis	NEU-22	-	-	-	P
	Polyporus ciliatus	NEU-270	F	F	F	F
	Polyporus squamosus	NEU F271	F	F	F	-
	Polyporus tuberaster	NEU-53	F	F	F	-
	Pycnoporus cinnabarinus	NEU-71	P	P	P	P
	Trametes gibbosa	NEU-28	F	F	F	F
	Trametes hirsuta	NEU-11	-	P	P	P
	Trametes suaveolens	NEU-73	F	F	F	F
	Trametes versicolor	NEU-2 NEU-M	- F	F F	F P	0 0

Family	Species	Strain code	Substrate type			
Ganodermataceae	*Ganoderma applanatum*	NEU- 78 NEU-87	F -	F P	F 0	0 0
	Ganoderma resinaceum	NEU-K1	P	0	F	F
	Ganoderma tsugae	NEU-M	F	0	P	P
Schizophyllaceae	*Schizophyllum commune*	Neu F14	F	F	F	F
Hymenochaetaceae	*Phellinus igniarius*	MUCL 39333	-	P	P	P
Strophariaceae	*Stropharia rugosa-annulata*	Kit strain	-	-	-	-

Table 4-7: stages achieved by the tested strains in the cultivation tests. -: no fruiting induction, P: primordia, F: fruit body, 0= not determined.

Table 4.8 shows that the number of species which could induce reproduction process was quite similar on all substrate types, with only a slight constant diminution from A to D substrate. However the number of species able to complete their reproduction cycles is clearly higher on A substrate, followed by B, C and finally D. This means that the lower the C:N ratio is (= the higher nitrogen content), the more a species is able to fruit. When considering strains, the same tendency is observed concerning the complete reproduction cycle, except for substrate C which has the higher fructifying success. This is due to the fact that the four tested *G. frondosa* strains were able to fructify on substrate C. Interestingly the industrial strain developed in our laboratory (GfNE) could only fruit well on B substrate. If we also consider the 14 species each one cultivated on a given substrate the results obtained are 53.8 % of fructifying species (total of 39 species) on A (vs. 56% with the 27 species), 33.3% on B (vs. 50%), 38.5% on C (vs. 48%) and 30.8% on D (vs. 34.8%). The tendency is quite similar as A substrate still allowed more species to fruit.

	Number of species starting reproduction phase (%)	**Number of successfully fruiting species (%)**	**Number of strains starting reproduction phase (%)**	**Number of successfully fruiting strains (%)**
Substrate A	80	56	69.7	45
Substrate B	77.3	50	63.3	40
Substrate C	76	48	78.1	50
Substrate D	73.9	34.8	65.5	31

Table 4-8: percentage of species and strains starting reproduction phase and successfully completing reproductive phase on the 4 substrates A, B, C and D.

In the same manner *G. frondosa* GFNE strain shows to have a better capacity to complete life cycle with a higher nitrogen complementation, however showing a strong inhibition of the fruiting phase in nitrogen rich substrates (C:N 60) (Job and Schiff Giovannini, 2004). Royse (1996) also observes that a nitrogen supplementation in *Lentinula edodes* (Berk.) Sing. substrate increases biological efficiency of the mushroom, however no data on C:N ratio are given. Chang (1993) cites that a 20 to 25:1 carbon to nitrogen ratio is required for *L. edodes*, a lower ratio than that tested in our study. Optimum C:N ratio for mushroom species other than Aphyllophorales is about 40 to 60:1 for *Volvariella volvacea* (Bull.: Fr.) Sing., 17 to 18:1 for *Agaricus bisporus* (Lange) Imbach according to Chang (1993), but 3:1 for *V. volvacea* and 4:1 for *A. bisporus* according to Janardhan and Hussain (1978). Kalberer (2000) however indicates that if C:N ratio was too low *Lentinus tigrinus* (Bull.: Fr.) Fr. fruit bodies were inhibited or deformed. Croan and Kim (1997) show that nitrogen concentration suppressed fruiting for *T. versicolor*, which does not correspond to our results as fruiting was also obtained for this species in the substrate with a higher nitrogen content, however nitrogen concentration is unknown. *S. commune* (Croan and Kim, 1997), *Phanerochaete chrysosporium* Burds. (Gold and Chang, 1979), *P. cinnanbarinus* (Bjurman 1988) and *Gloeophyllum sepiarium* (Wulf. Fr.) Karst (Bjurman, 1984) are reported to be controlled both by glucose and nitrogen limitation, however C:N ratio values are not given either. For ascocarp induction too, Moore-Landecker (1992) had already stated that not only the absolute concentration of carbon and nitrogen is important but also the C:N ratio. Fungi typically require a much larger carbon than nitrogen concentration to promote ascocarp induction. For instance optimal requirement of *Morchella* sp. is a 5:1 ratio (Janhardan and Hussain, 1978).

Some species have very little trophic specificity since they fructified on all four tested substrates: *L. conchatus, P. ciliatus*, *T. gibbosa*, *T. suaveolens* and *S. commune*. Some species were clearly able to fructify successfully only in substrates with a lower C:N ratio (with higher nitrogen content): *F. fomentarius, M. giganteus* and *T. versicolor* strain NEU-M and *G. tsugae*. Two species reproduction cycles were only inhibited with higher C:N ratio: *P. squamosus* and *P. tuberaster*. On the contrary *G. resinaceum* and *F. pinicola* strain MUCL 30544 seem to be favoured on this ratio extremity.

Results also show that strains seem to be adapted to different substrates. Our results on 3 different European *F. pinicola* strains originating from Belgium and Switzerland show that they behaved differently. Strain MUCL 30677 was able to complete its reproduction cycle on A, C and D substrates, strain NEU-K2 on A and C and strain MUCL 30544 only on D. It shows that a substrate designed for one strain would not necessarily be suitable for another, even with the same geographical origin. For this species, we can not link fruiting with C:N ratio. For *T. versicolor* we can observe a slight difference in the best C:N ratio needed for fruiting success for the Swiss strain (Neu-2)and the Chinese strain (Neu-M). The two tested strains of *G. applanatum* were also different, one could not be fructified at all while the other one could on three tested substrates A, B and C. Shen (2001) already showed that out of 23 strains of *G. frondosa*, 5 could not even form primordia, 4 aborted at the primordia stage, 5 produced mushrooms unconsistently and 9 were able to fruit consistently. Miles and Chang (1997) also mention the importance of testing the ability of a culture to form fruit bodies upon the substrate selected.

Spawn run, time taken for the primordia to grow and time taken to form the fruit bodies of most of the tested stains on the four industrial substrates A, B, C and D are presented respectively in figures 4.17, 4.18, 4.19 and 4.20. *A. brasiliensis* and *P. tuber-regium* are not presented on these graphs as the first one was tested on other substrates and the second

fructified from the obtained sclerotia. The separation between primordia and fruit body stages was arbitrarily fixed to the apparition the hymenium on well formed fruiting structures. In the case of *G. tsugae* these primordia were more than 10 cm long. It was more like stipes which could not open onto a cap. In the case of *Pycnoporus cinnabarinus* pores were formed on a malformed fruit body. It was therefore; not taken into account as a fruit body.

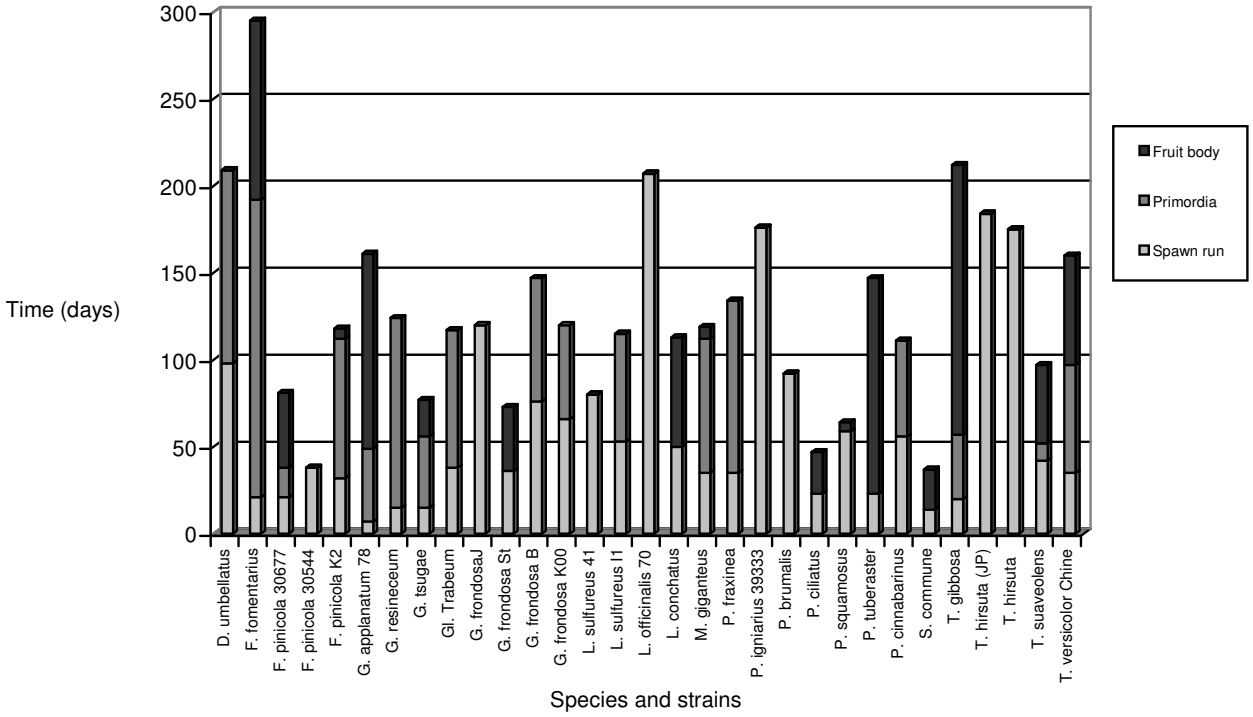

Figure 4-17: spawn run time, primordia formation time and fruit body growth time until collection in days of various Basidiomycetes species on substrate A.

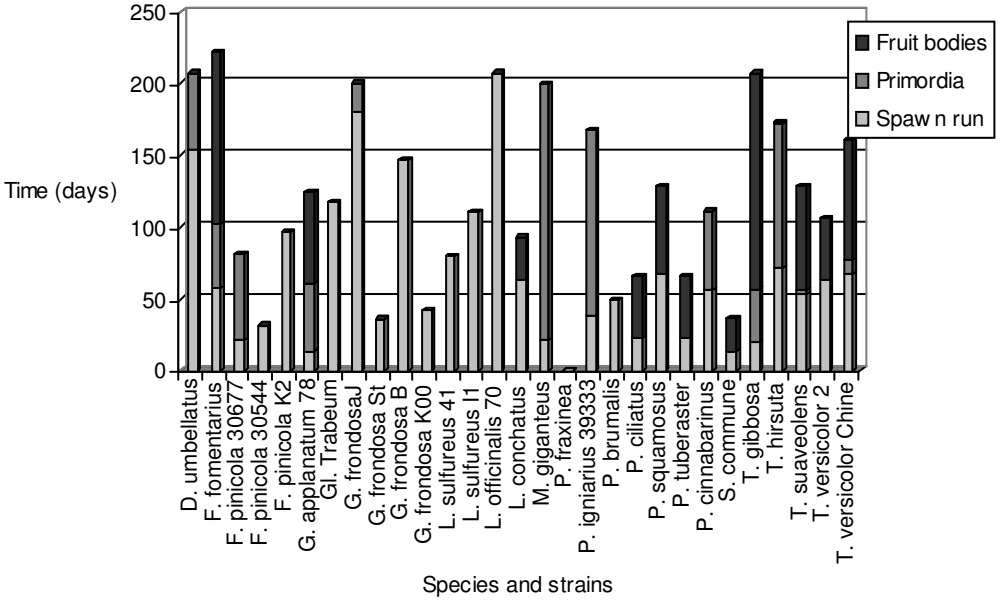

Figure 4-18 : spawn run time, primordia formation time and fruit body growth time until collection in days of various Basidiomycetes species on substrate B

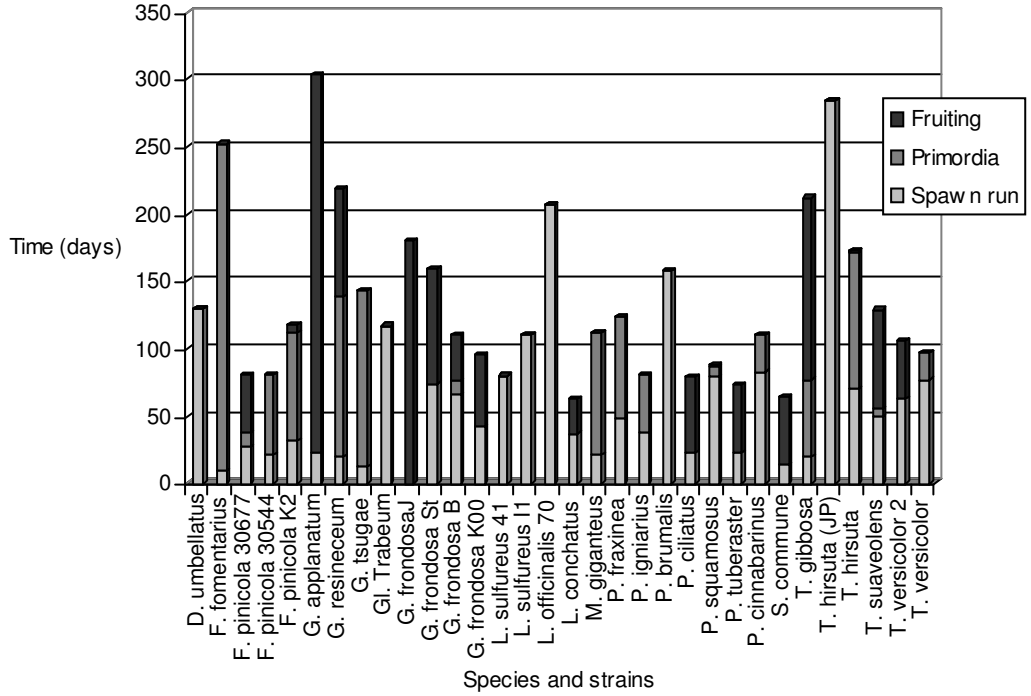

Figure 4-19: spawn run time, primordia formation time and fruit body growth time until collection in days of various Basidiomycetes species on substrate C

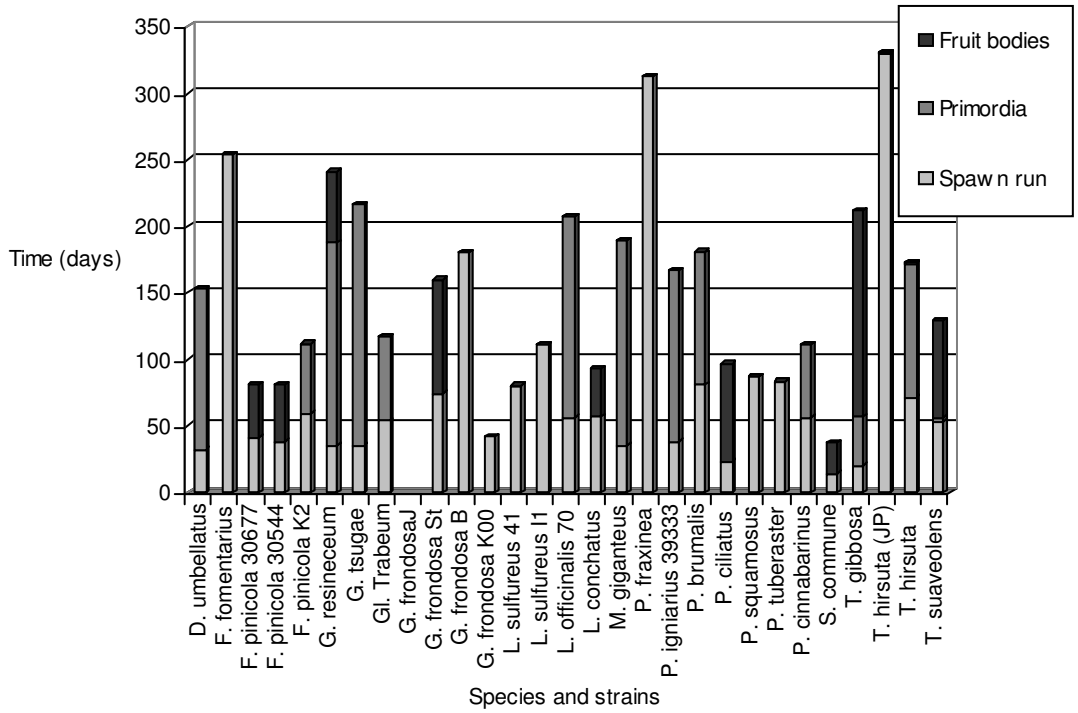

Figure 4-20: spawn run time, primordia formation time and fruit body growth time until collection in days of various Basidiomycetes species on substrate D

The mycelium in some species colonised different substrates at about the same speed, however others grew very differently on the different substrates as shown in figure 4.21. *P. igniarius* for instance, took more than double the time to invade A substrate compared to C and D substrates. If we consider *P. squamosus* we see that growth is the best on D, decreasing continuously on C, B and A. This was expected as it was already demonstrated several times. For instance growth of the mycelia of 30 mushroom species and strains showed to be different depending of the type of agar media and pH (Bilay et al., 2000).

There are also important differences in colonisation between strains of a same species. If we consider the three strains of *F. pinicola* we observe that if the a given strain has about the same invasion rate on different substrates tested, one of the strains (K2) takes almost 10 more days to totally invade the substrates compared to the two others. Substrate colonisation rate however was not necessarily connected with fruiting capacities.

Comparison of yield (figure 4.22) of a same stain on different fruiting substrates also shows this influence of trophic specificity. For instance *P. tuberaster* produced only about half of fruit bodies' weight on B substrate compared to A substrate. The same can be observed for *L. conchatus* which fructified on the four substrates but with a yield on A substrate being about 7 fold that on D substrate. This is only indicative as yields were calculated on only a few replicates, thus not being significant.

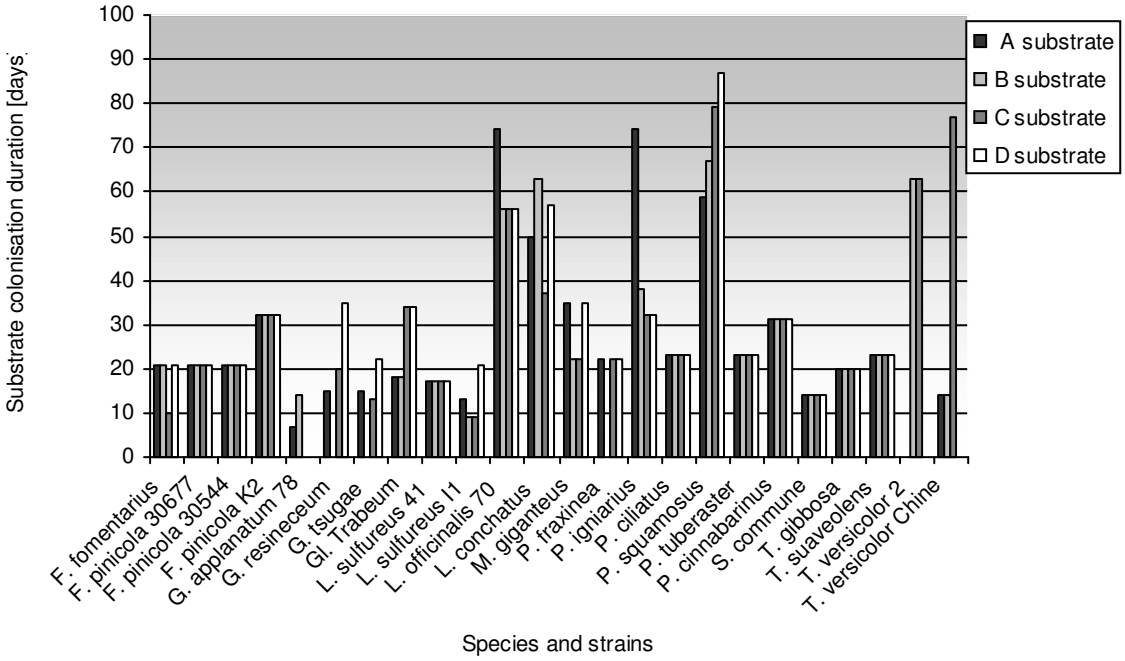

Figure 4-21: Time taken (days) by the mycelium of various Basidiomycetes species and strains to colonise different substrates

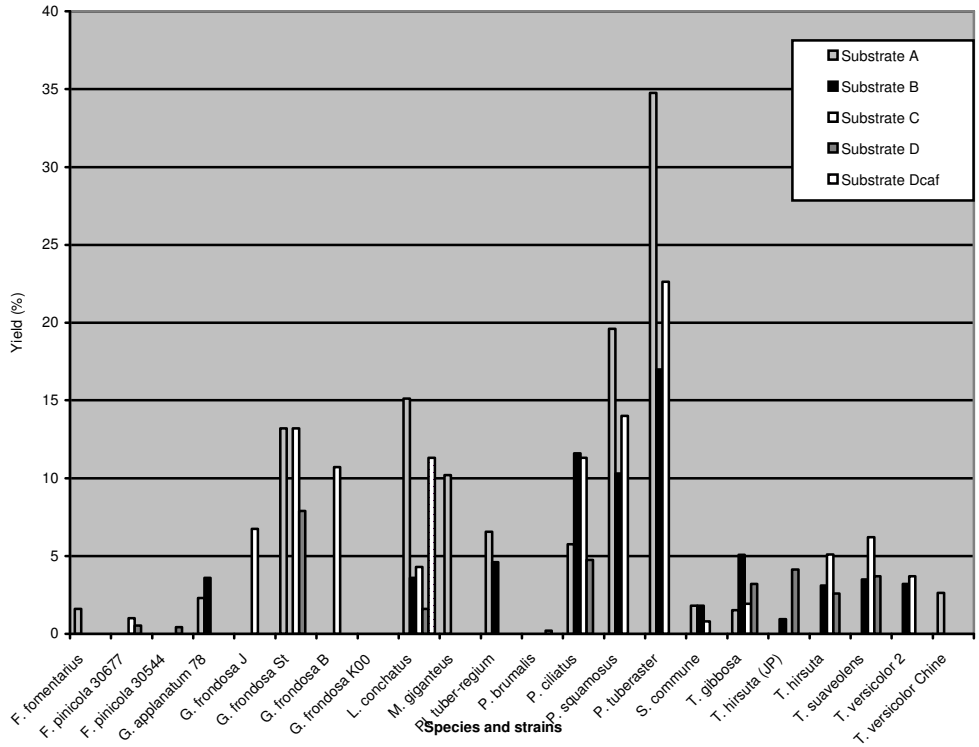

Figure 4-22: yields obtained on the four substrates A, B, C and D and Dcaf of the fructified species

Our results fit with the published studies on the effect of genotype and substrate formulation for other edible Aphyllophorales species: *Grifola frondosa* (Shen and Royse, 2001; Kirshoff, 1996), *Lentinus edodes* (Royse and Bahler, 1986).

For most of the species which succeeded in fruiting, artificial light of the fructification room was sufficient (standard environmental conditions). However some of the *Ganoderma* species needed a natural light source. In our experiments, *G. resinaceum* could only form primordia in natural light, while fruit bodies also developed better in this condition than in artificial light. *G. tsugae* also needed natural light for all of the reproduction phase, but *G. applanatum* could fructify with artificial light.

External biological or technical factors have induced problems in mushroom culture by provoking important losses of the studied material. These losses sometimes delayed results for several months. During the vegetative phase, the most important problem lied in contamination with the fungus *Trichoderma viride* and invasion by mites or flies. During the fruiting phase, biological causes where the same as during the vegetative phase. A lowered humidity sometimes had important consequences by aborting the primordia or the forming fruit body. These technical problems were mainly due to humidifier break-down or lack of water in the reservoir.

In our study we have noticed that a certain number of species are able to resist the strong mycoparasite *Trichoderma viride*. In most cases, reaction was visible by a brown-black discolouration of the mycelium (lines or zones). Indeed *F. fomentarius*, the ganodermataceae species, *M. giganteus*, *P. fraxinea*, *P. igniarius* and *T. versicolor* all showed this type of reaction and were fairly resistant. The two strains of *G. applanatum* however behaved differently as the fructifying strain was much more resistant to fungal contamination. This was linked with very early brown line formation by the fruiting strain compared to the other which showed only late, thin lines. Resistance in function of the substrate is observed with *P. fraxinea* which only produced a brown line on D substrate where it was much more resistant than on the others. Brown lines were reported in studies on the interaction between *L. edodes* and *Trichoderma* spp. stopping the progression of *Trichoderma* spp if resource colonised by *L. edodes* is significant. The browning of the mycelium gives a protective coating by crosslinking preformed phenols and cell-wall proteins associated with oxidative mechanisms (Savoie et al., 2000) and antifungal compounds production (Tokimoto and Komatsu, 1995). The reactions observed during our experiments could therefore be similar to those encountered between *L. edodes* and *Trichoderma* spp. Other resistance mechanisms like the orange discolouration of *L. sulfureus* mycelium are known. For this subject see further chapter 4.2.

To conclude, species have different reactions but it is evident that genotype and substrat formulation has an effect on the fruiting process of the tested Badidiomycetes species. Fruiting success was generally bound to C:N ratio: in general the higher the nitrogen content in the substrate, the more strains were able to fruit on it. However some species seem to be inhibited by too high nitrogen content.

4.2 SELECTION OF GRIFOLA FRONDOSA AND FOMITOPSIS PINICOLA STRAINS RESISTANT TO TRICHODERMA VIRIDE IN TEFLON TUBES CONFRONTATION METHOD

4.2.1 Somatic incompatibility tests

Somatic incompatibility between all the *G. frondosa* and *F. pinicola* strains have been proved in dual confrontation by the presence of a thick line at the meeting of the two colonies often with orange or brown discoloration.

4.2.2 Growth measurements on 2% AM medium

Results of the 2% AM medium growth measurements (fig.4.23) clearly show that all strains of *G. frondosa* grow much better at 25°C and are strongly inhibited at 30°C. Both *F. pinicola* strains and *Trichoderma viride* grew slightly better at 30°C. However, 25°C is the standard incubation temperature where most of *T. viride* infection problems occurred in our research programs. Thus, the temperature for all the experiments was fixed at 25°C.

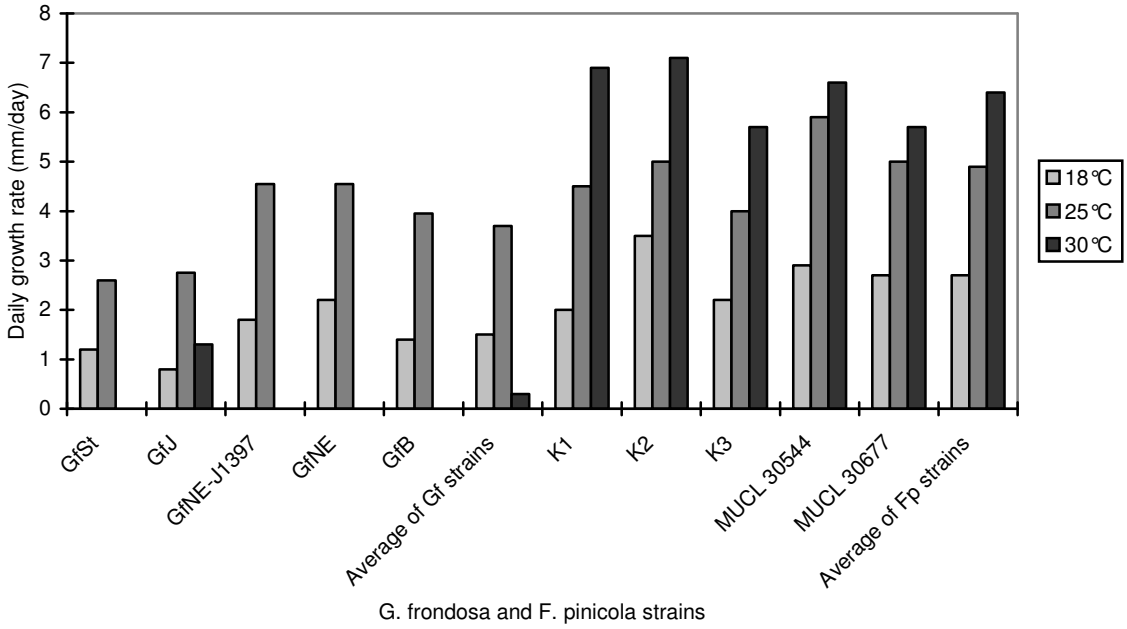

Figure 4-23: daily growth rate (mm/day) of G. frondosa *and* F. pinicola *strains on 2% AM medium at 18°C, 25°C and 30°C.*

4.2.3 Vegetative resistance on 2% AM medium

In all experiments on 2% agar-malt medium with the 5 *G. frondosa* strains, *T. viride* was able to continue its development over *G. frondosa* mycelium without any growth rate decrease. The 5 *F. pinicola* strains however were able to stop the progression of *T. viride* during several days in the mycelium contact zone before to be overgrown, and the resistance duration was inversely proportional to the *Fomitopsis* colony diameter as show in table 4.9. The resistance of the colony's 3 diameter groups were significantly different (P = 0.03).

Strains	Resistance duration (days) at 2 cm diameter	Resistance duration (days) at 4 cm diameter	Resistance duration (days) at 6 cm diameter
Fpk1	17	13	9
Fpk2	22	8	4
Fpk3	8	4	0
Fp 30544	12	11	3
Fp 30677	11	6	2

Table 4-9: Time of resistance (in days) of the F. pinicola *strains at the contact zone with* T. viride *with different colony diameters.*

4.2.4 Experimental design

For selecting fast growing strains with a good aptitude to competition toward *T. viride*, our choice was directed toward transparent tubes. They would allow visualising substrate colonisation by the mycelium as well as interactions between the two fungi. Influence of substrate type and quantity, strain, colony size had to be analysed.

4.2.4.1 Experiment 1: evaluation of the method in tubes: influence of substrate type on the resistance of the 5 *G. frondosa* strains

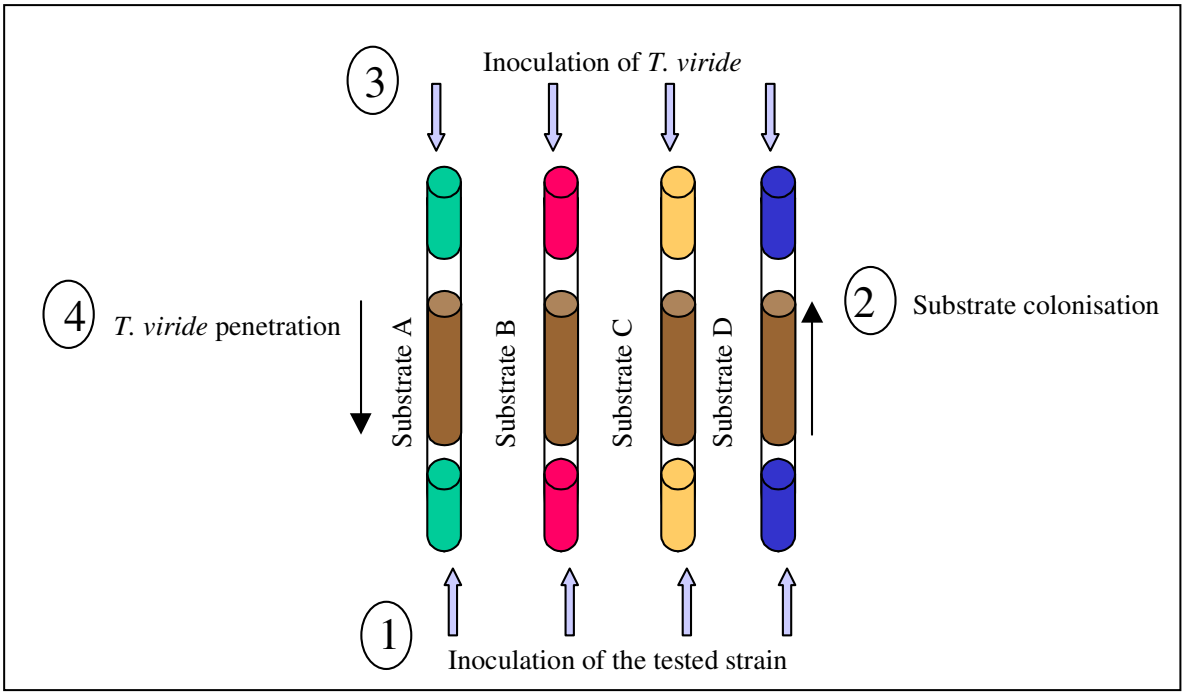

Figure 4-24: experiment 1 design

Figure 4.24 shows the experiment design. Colonisation of the substrates by the different strains varied with time like shown in table 4.10. The reason for colonisation rate variations between measures is unknown. Compression of the substrate or oxygenation might vary along the tube. However the mycelium was very sparse which made the observation of the colony front quite difficult with the naked eye, especially on substrate B, and on D substrate for

GfNE strain. Moreover the mycelium front was uneven, especially in the first days of substrate invasion. Mean colonisation rate of each strain was compared to growth on 2% AM medium (figure 4.25). Colonisation rate clearly varied between substrates with D substrate allowing the best growth.

Substrate type		**A**	**B**	**C**	**D**
Day of measure		**13 24**	**13 24**	**13 24**	**13 24**
Daily invasion rate (mm)	**GfSt**	3.2 ↓ 1.7	2.4 ↑ 3.2	2.8 ↑ 3	3.2 ↓ 2.9
	GfJ	2.7 ↓ 2.25	1.5 ↑ 3.3	2.1 ↑ 3.3	2 ↑ 3.6
	GfNE-J1397	3.7 ↓ 0.8	3.8 ↓ 1.8	3 ↓ 2.7	3.1 ↑ 3.3
	GfNE	3.8 ↓ 0.8	2 ↑ 2.2	2.1 ↑ 3	2.6 ↑ 3
	GfB	3.4 ↓ 1.6	2.7 ↓ 2.3	2.9 ↓ 2.7	2.8 ↑ 3.4

Table 4-10: daily colonisation rate (mm) of the 5 G. frondosa strains on substrates A, B, C and D between the start of the experiment and day 13, and between day 13 and day 21.

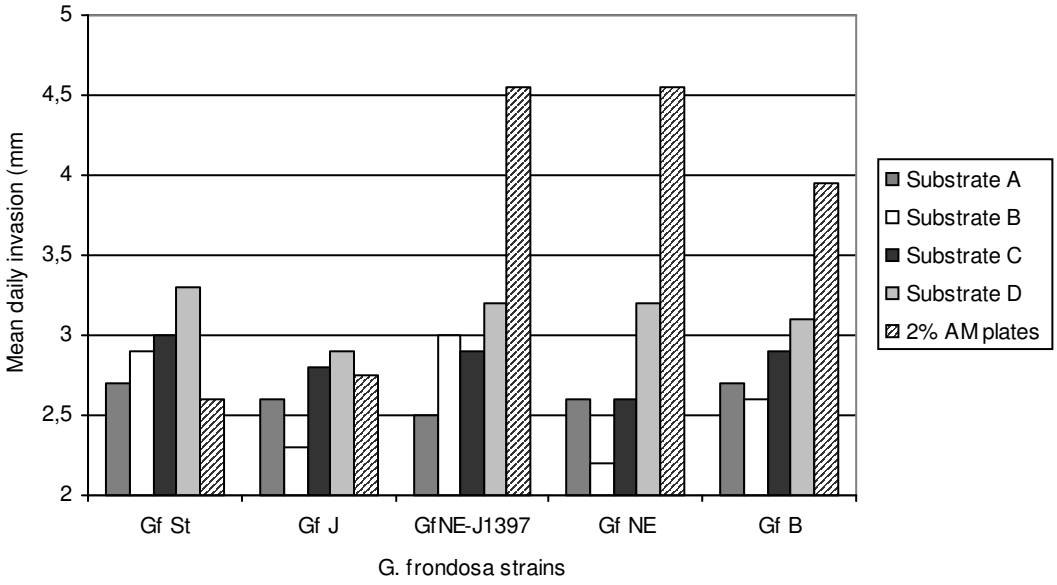

Figure 4-25: daily invasion rate of the five G. frondosa strain on substrates A, B, C and D in tubes and 2% AM medium in Petri plate in mm.

When *T. viride* was inoculated, all the tubes were totally colonised by the tested strains or had stopped growing. Six days after *T. viride* inoculation, resistance observed on substrate D was the best for all strains except GfJ which did not stop *Trichoderma* progression for a while as the other strains had. The maximum penetration of *T. viride* in the colonised substrates is shown in figure 4.26. GfNE was totally parasited by *T. viride* on substrate D, thus showing different behaviour in function of the time of the observation. Replicates were in general reproducible whether all the substrate was invaded by *Trichoderma* or not at all, but invasion percentage in the partially invaded tubes changed.

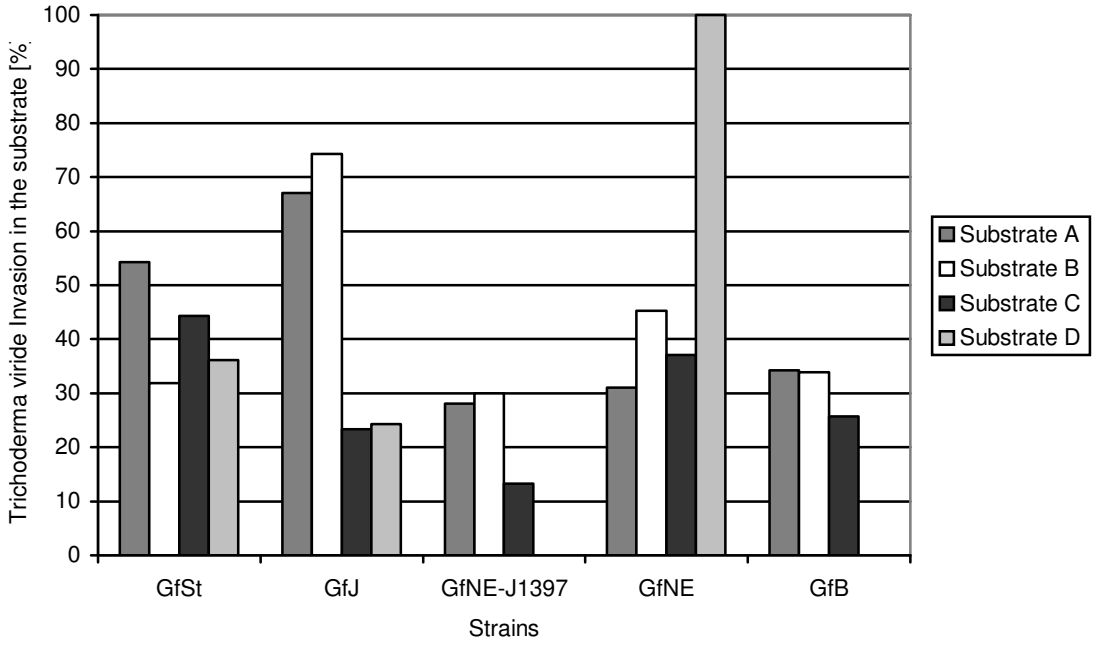

Figure 4-26: T. viride penetration (%) in the substrates invaded by the different G. frondosa strains.

Discussion: This method in tubes seems to be adequate to visualise mycelium front advance. However the mycelium front was uneven. Thus in further experiments, calibrated lines should be drawn along the tubes before starting the experiments, so the measures could not be influenced by the experimenter. Colonisation rate was also varyed with time, between strains and substrate types. This behaviour is unexplained. When considering mean colonisation rate however, all strains colonised D substrate (lower nitrogen content) faster than the others. It is already clear that rapidity of colonisation depends not only of the strain but also of the substrate type. There is no correlation between growth on 2% AM medium and substrate colonisation rate. Thus selection of fast growing strains in AM medium seems to be inadequate. This should be confirmed in further experiments.

T. viride progression in the invaded substrate was clearly visible by the observation of green conidiation all along the contaminated zones. Thus, the tube method also seems adequate to visualise the interaction between the two fungi. This experiment shows that resistance is variable between strains and for a same strain on different substrates. GfJ-1397 and GfB are for instance totally resistant on D substrates but not on the others, while it is the only substrate where GfNE was not at all resistant. The others strains stopped the progression of *Trichoderma* after a while. In this experiment resistance is not correlated to substrate colonisation rate. As *T. viride* visible penetration was not the same on all sides of the tubes, it should also be measured on the pre-drawn line.

4.2.4.2 Experiment 2: influence of A substrate quantity on *F. pinicola* FpK2 strain resistance

This small experiment (figure 4.27) was realised in parallel to the first one to see if the quantity of substrate was important for strain behaviour toward resistance. Figure 4.28 shows that measures varied with the time, as observed for *G. frondosa,* figure 4.29 shows that colonisation varies in function of substrate quantities. Contrary to observations with *G. frondosa*, this strain had an even colony front. *F. pinicola* mycelium stopped growing before total invasion of the substrate in both 1g substrate tubes and tube 1 from 3g substrate probably because the substrate was too dry.

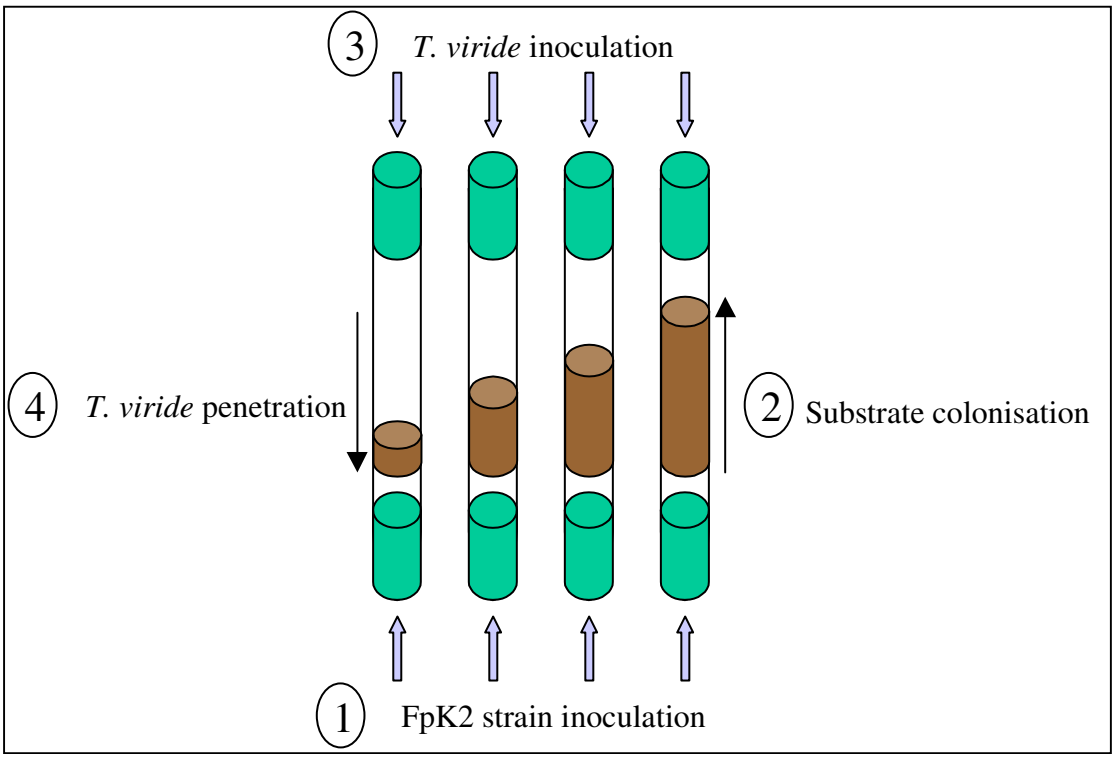

Figure 4-27: Experiment 2 design.

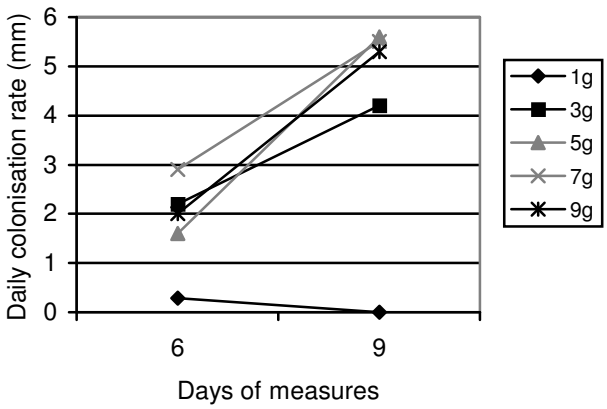

Figure 4-28: 1st and 2nd measures of colonisation rate of F. pinicola K2 on different quantities of A substrate.

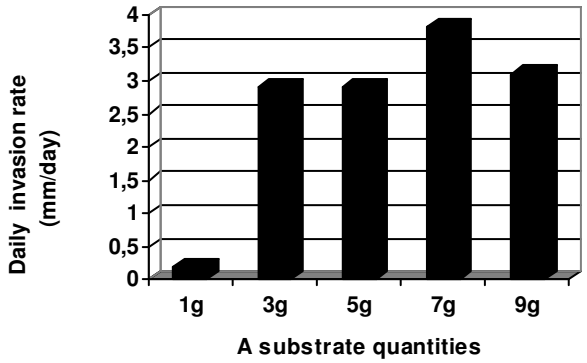

Figure 4-29: mean daily colonisation rate of F. pinicola strain FpK2 for each substrate quantities

Twenty-one days after *T. viride* inoculation, it hadn't penetrated any of the tubes. To try to strengthen *Trichoderma* inoculum, a conidial suspension was added in the tubes. Figure 4.30 shows *Trichoderma* contamination 6 days later. In both tubes of 7g and 9g substrate, *F. pinicola* has formed a brown pigmentation.

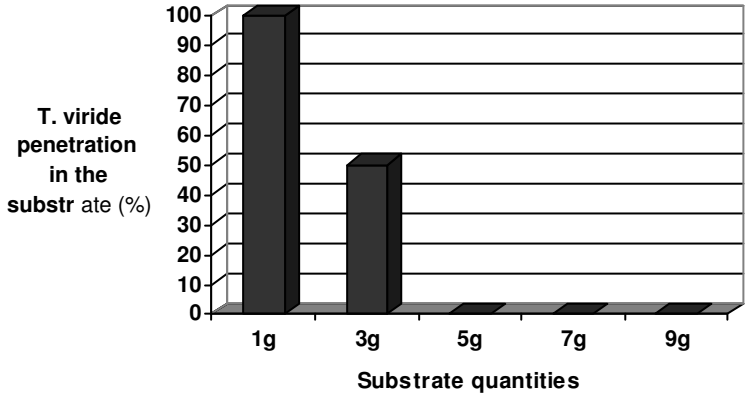

Figure 4-30: T. viride penetration in % in different substrate quantities colonised by F.pinicola strain FpK2.

Discussion: We can observe great variation in the first range of substrate colonisation measures between tubes, probably due to the time taken for the mycelium to start growing from the broken hyphae of the agar plugs. In the second range however we can observe that colonisation rate values are quite close for tubes with 5g, 7g and 9g substrates but not for tubes with lesser amounts of substrate. This could be due to lower water content due to a higher coefficient between volume and surface. Moreover the reproducibility between daily invasion values in both tubes was better with higher substrate quantity. Thus in further experiments the amount of substrate should not be too small.

The results of this experiment seem to indicate that conidial suspension of *Trichoderma* has a better power of contamination than a mycelium plug. This should be tested again in a further experiment.

This strain of *F. pinicola* had a visible reaction to the mycoparasite as it produced brown pigmentation, however only when the colony had reached a certain size. This pigmentation might play a role in the resistance like previously described in the interaction between *Lentinus edodes* and *Trichoderma* spp. (see paragraph 4.3.4), but other mechanism are probably involved in resistance as one of the 3g tube and both 5g tubes also resisted contamination without any visible reactions.

As substrate quantity showed to be important for resistance in this experiment, the influence of substrate quantities as well as colony size on resistance will be further studied for each of the tested strains. Moreover we should use *Trichoderma* conidial suspension for inoculation of the mycoparasite in the tubes.

4.2.4.3 Experiment 3: influence of Bm substrate quantities on *G. frondosa* 5 strains resistance

Experimental design is shown in figure 4.31. Figure 4.32 shows colonisation rates for each strain on the different substrate quantities but the data obtained for the different substrate quantities are not significantly different (P = 0.03).

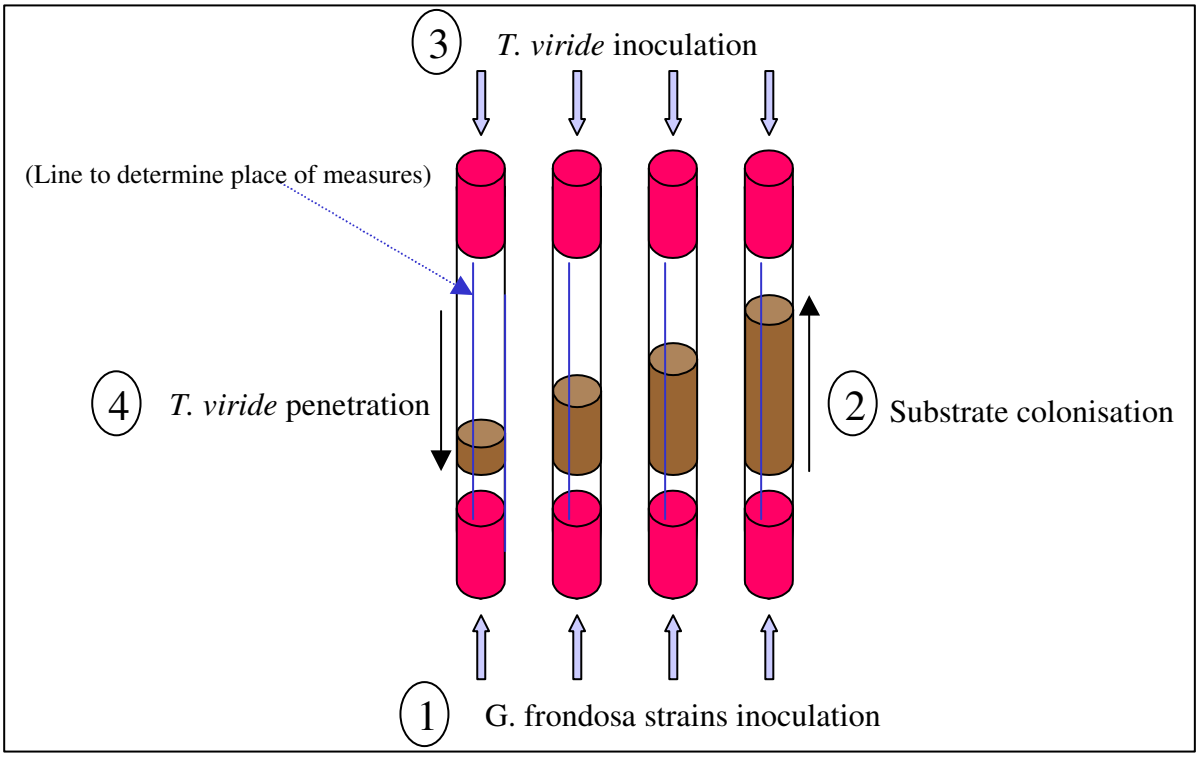

Figure 4-31: experiment 3and 4 design

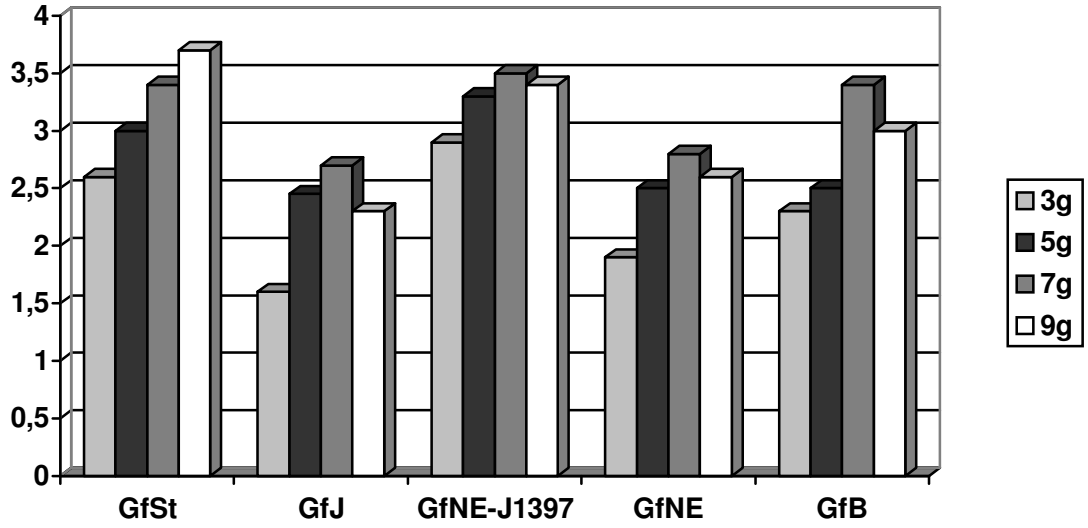

Figure 4-32: substrate colonisation rate (mm) of G. frondosa *strains on different substrates quantities.*

As in other experiments with *G. frondosa*, the mycelium was very sparse and the margin very difficult to see. Moreover even if not shown here, there were variations with time, the invasion rate often increasing or decreasing. *G. frondosa* made some kind of long hyphae which went much further than the other hyphae of the margin, thus rending the measure difficult. The measures were taken at the extremities of these long hyphae.

GfSt had the denser mycelium with a gradient of density from the smaller to the higher substrate quantities. One of GfJ's and GfB and both of the blank 7g tubes were accidentally contaminated with *Trichoderma*. One of the 7g tubes of GfJ as well as one of the 9g tube of GfB had stopped growing before total invasion. All the tubes of GfNE formed very thick mycelium layers on the top of the tubes.

Figure 4.33 shows *Trichoderma* penetration in the substrate after 69 days of experiment. At that stage, GfJ was the less resistant strain and was the only which did not produce a thick tissue layer on top of the tubes. As almost no contamination had occurred, 3ml of a conidia suspension was added on day 70. Figure 4.34 shows penetration after 103 days of experiment. Results obtained with *G. frondosa* strains however are unexplained and are not statistically significant.

Small primordia were visible on all the 7g and 9g substrate tubes invaded by GfSt strain.

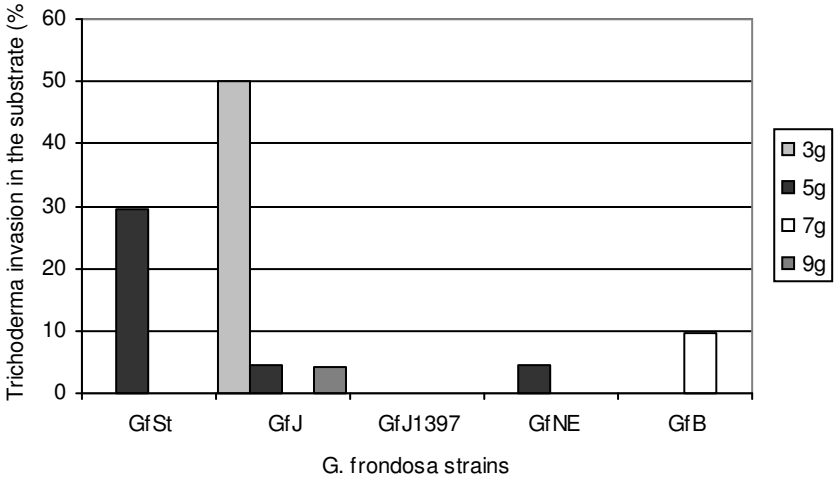

Figure 4-33: Trichoderma penetration in the substrate 28 days after the first inoculation

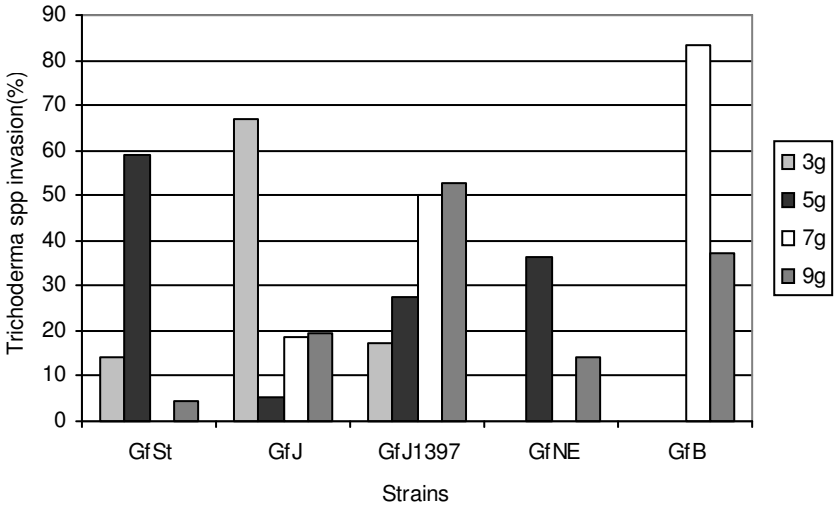

Figure 4-34: Trichoderma viride penetration 33 days after a second inoculation

Melanised lines (ML) and zones of yellow mycelium (ZYM) which could be linked to a reaction to the antagonistic fungus were observed in some tubes after *T. viride* inoculation (table 4-11). This type of reaction was not correlated with resistance toward the pathogen.

	GfSt	GfJ	GfNE	GfNE-J1397	GfB
3g	-		ML	ML	ML + ZYM
5g	Light ML one side	ML	ML	ML + ZYM	ML + ZYM
7g	ML +ZYM	ML	ML	ML + ZYM	ML + ZYM
9g	ML + ZYM	ML	ML + ZYM	ML + ZYM	ML + ZYM

Table 4-11: visible reactions of the Gf strains after Trichoderma viride inoculation. ML= melanised lines: ZYM= zones of yellow mycelium.

One of the problems in this experiment was that reproducibility between the 2 tubes was quite low when considering the percentage of *Trichoderma* penetration in the substrates invaded by the tested strains.

Discussion: Substrate colonisation rates measured showed that GfNE-J1397 was the faster growing strain followed by GfSt, GfB, GfNE and GfJ. It is quite similar to the order measured in experiment 1 on substrate B, except that the places of GfNE and GfJ are inverted, even if they are close. In every case, first measurements had the lowest values like expected as the broken mycelium of the agar plug has to start growing on a different medium.

Like in the two previous experiments *Trichoderma* penetration in the invaded substrate was clearly visible by its green conidiation. We expected that the strains would be more resistant with a higher quantity of substrate, but it was not the case for any of the strains. For GfNE-J1397 for instance it was even the reverse: *Trichoderma* progression was increasing with substrate quantities. For the others we have no explications for the differences between substrate quantities. Some strains resisted with certain substrate quantities after the first *Trichoderma* inoculation but were contaminated when a second conidia suspension with higher quantities was added. For instance GfSt first resisted in all tubes but after the second inoculation all the tubes were partially invaded. The reasons could be either the increased amount of infecting conidia or the adjunction of water. In all cases however the strains were able to stop *Trichoderma* progression after a while as in the previous experiment realised with this species. The substrate of 3g tubes of GfSt was dryer than the others, this being a potential explication for the reaction in these tubes.

The same conclusions were reached as in experiment 1 concerning a strains' capacity to stop the progression of *Trichoderma* (resistant strain), or not (non resistant strain) on substrate B. The percentage of *Trichoderma* invasion does not always correspond.

4.2.4.4 Experiment 4: influence of Bm substrate quantities on *Fomitopsis pinicola* 5 strains resistance

Experimental design is the same as experiment 3, except that *G. frondosa* is replaced by *F. pinicola*. Figure 4.35 shows substrate colonisation rate for each strain on different substrate quantities but the data obtained for the different quantities is not significantly different (P = 0.03). Mycelium of all the strains was very sparse on the substrate which does not seem to correspond to this species. Like for *G. frondosa* measures varied slightly with time (not shown).

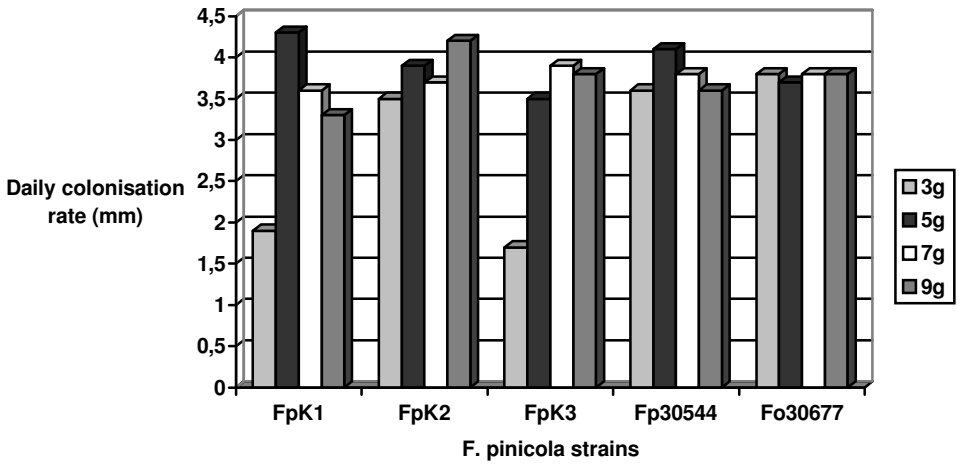

Figure 4-35 Substrate Bm colonisation rate of the 5 strains for each substrate quantity

Figure 4.36 shows *Trichoderma* penetration in the *F. pinicola* colonies in all cases whether the tubes were totally colonised by *Trichoderma*, not at all or only on the surface of inoculation. The 50% value of strain Fp30677 was due to the fact that one of the tube was totally invaded by *Trichoderma* and the other not at all, which is the only of the replicates not alike. It clearly shows that *F. pinicola* strains become resistant on a substrate only when at least 5 g substrate has been colonised.

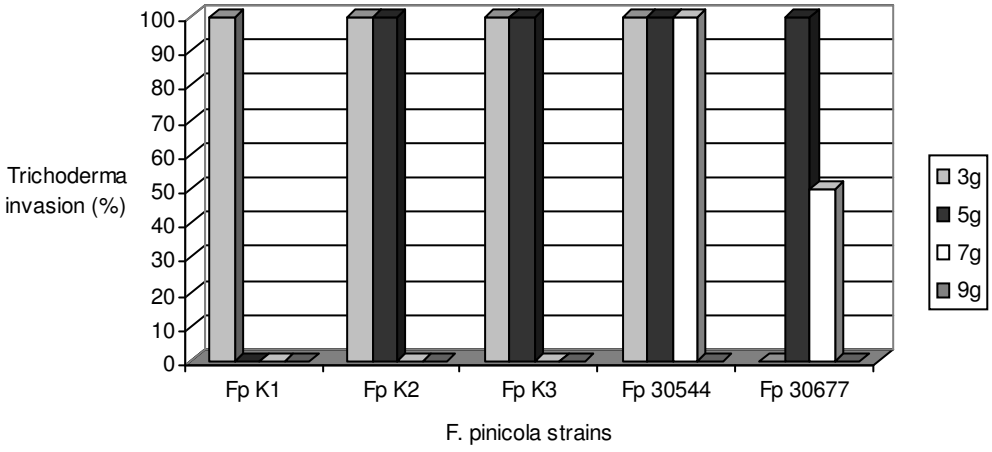

Figure 4-36: final Trichoderma penetration (%) in different quantities of substrate Bm colonised by the 5 strains of F. pinicola.

Discussion: Results of this experiment clearly show that *F. pinicola* strains are more resistant with a higher quantity of substrate. The only exception is Fp 30677, their 3g tubes were not colonised by *Trichoderma* while 5g tubes as well of one of the 7g tube were. This might be due to the fact that tubes with only 3g were a bit dryer which could have hindered *Trichoderma* progression. Thus in further experiments at least 7g of substrate will be used to avoid such problems and the tubes placed in an environment with a higher relative humidity.

Substrate colonisation rate was not correlated to resistance, as FpK1 was one of the slowest strains to colonise the substrate but to the strain which needed the least amount of substrate to resist. The behaviour of this species corresponds to the description for *L. edodes*. This species could indeed resist *Trichoderma* when a sufficient substrate zone was colonised (Savoie et al. 2000). However on the contrary to what was observed in the experiment on A substrate quantities inoculated with FpK2 strain there was no visible reaction of the strains on Bm substrate. The mycelium was also much sparser than in the experiment with A substrate.

4.2.4.5 Experiment 5: influence of the colony size on *G. frondosa* strains

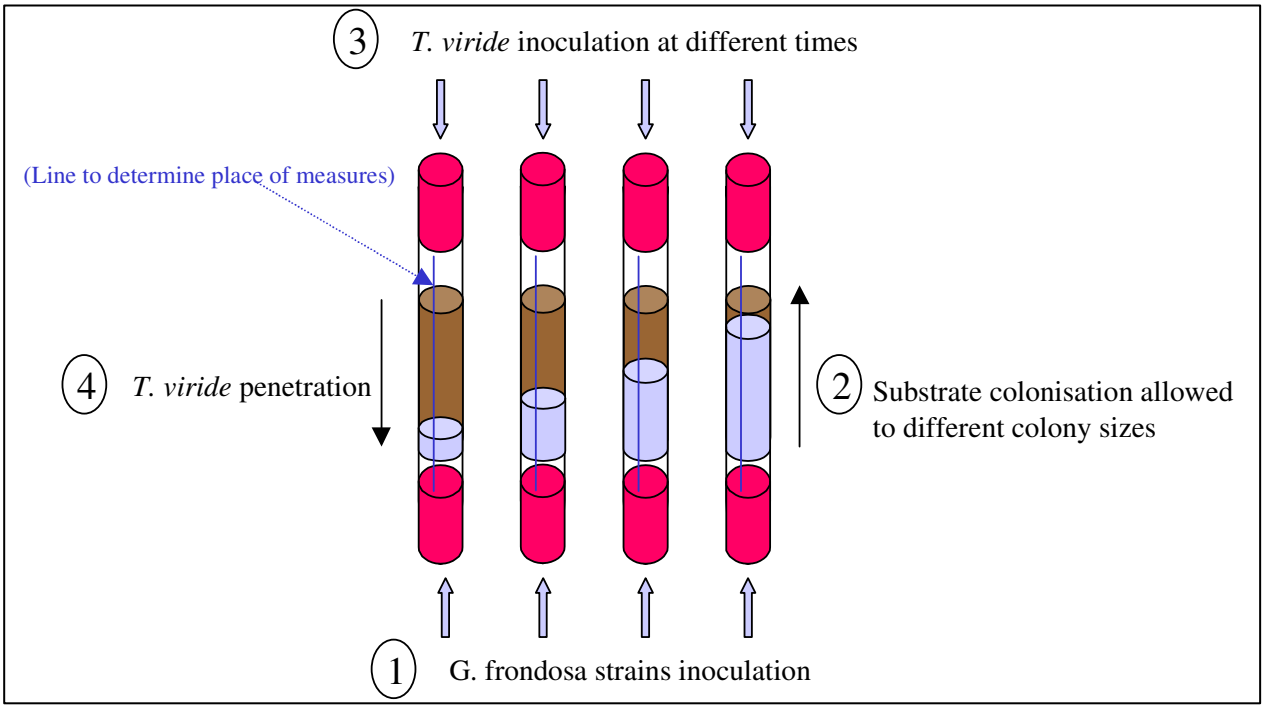

Figure 4-37: experiment 5 design.

To evaluate the influence of colony size at the time of inoculation of *T. viride*, two strains were inoculated on 7g B substrate (figure 4.37). The two chosen strains were GfJ1397 b, the fastest to colonise Bm substrate and GfJ, the slowest. Mycelium was allowed to grow to different lengths before inoculation of the pathogen (Table 4.12).

	Tubes a-b	Tubes c-d	Tubes e-f	Tubes g-h	Tubes i-j
***G. frondosa* GfJ**	0	32	60.6	67	67
***G. frondosa* GfSt**	0	26.5	52.3	67	67

Table 4-12 Mean colony length (mm) in the substrate of G. frondosa GfJ and GfSt strains at the inoculation of T. viride .The total length of the substrate was about 67 mm.

In some tubes of *G. frondosa* during invasion phase, there was a sudden rapid advance of a few hyphae while there was a normal slower growing but denser mycelium front. In this experiment, growth was only measured at the denser colony front. Even with 6 measures per tube and several tubes, invasion rate was not constant along time even if always incubated in the same conditions, thus showing an unknown factor influencing mycelial elongation. Interestingly variations followed by the two strains are quite similar, exception being between day 12 and 14 after inoculation, growth increased for GfSt but lowered for GfJ.

Final *T. viride* penetration in the 2 strains' colonies is shown in figure 4.38. In substrates colonised by GfJ *Trichoderma* conidiation was delayed in comparison to substrates colonised by GfSt. For the 2 tested strains, colony size at the moment of confrontation with *T. viride* had an influence on their resistance capacity. The results of the ANOVA statistical test evidence the influence of the colony size in *G. frondosa* (F: 46, probability of null hypothesis 0.000) *T. viride* resistance.

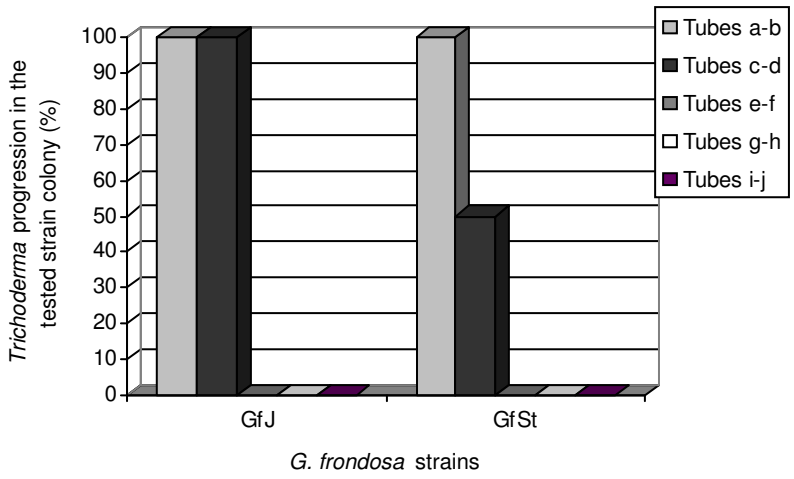

Figure 4-38 T. viride *penetration (%) in the substrate previously invaded with different colony sizes of G.* frondosa *GfJ and GfSt strains.*

Like in experiment 3, melanised lines (ML) and thick mycelium layers on top of the tubes (TML) were observed after *T. viride* inoculation (table 4-13). A minimum colony size was necessary for such reactions. This type of reaction seems correlated to resistance toward the pathogen; the four tubes which did not present any visible reaction did not show *T. viride* inhibition.

Considering *Trichoderma* penetration, the 2 tubes submitted to the same treatment were identical, except for tubes e and f of GfSt, one was partially invaded and the other not at all.

	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*
GfJ	-	-	-	-	*TML*	*ML*	*ML+TML*	*ML*	*ML+TML*	*ML+TML*
GfSt	-	-	-	-	*TML*	*TML*		*ML*	*ML*	

Table 4-13: visible reactions of the GfJ and GfSt strains after Trichoderma viride inoculation. ML= melanised lines. TML= thick mycelium layer.

Discussion: Results show that quantity of substrate is important for testing in tubes. Indeed the *G. frondosa* colony needed a certain size before it was able to resist *Trichoderma*. Moreover, like in the experiment with substrate quantities, reactions were visible only with a minimum colony size, which probably indicate an size influence on the interaction of the two fungi.

4.2.4.6 Experiment 6: influence of the colony size on *F. pinicola* strains resistance

Experimental design is the same as in the previous experiment. FpK1 and FpK2 strains were chosen, because in a previous experiment with that species FpK1 was one of the slowest on Bm substrate and FpK2 the fastest. Mycelium was allowed to grow to different extents before the pathogen inoculation (Table 4.14).

	Tubes a-b	**Tubes c-d**	**Tubes e-f**	**Tubes g-h**	**Tubes i-j**
***F. pinicola* FpK1**	17.5	25	36	57.8	67
***F. pinicola* FpK2**	19	27.8	42.1	65.9	67

Table 4-14. Mean colony length (mm) in the substrate of F. pinicola *FpK1 and FpK2 strains at the inoculation of* T. viride *.The total length of the substrate was about 67 mm.*

The day after *Trichoderma* inoculation, FpK1 mycelium grew faster than previously and then diminished drastically 2 days after inoculation, even before being in contact with the pathogen (sometimes several centimetres away). The same happened to FpK2 tubes a and b, while in the others, colonisation rate was already lower the next day. This visibly indicates an early detection of *T. viride* at a distance by the *F. pinicola* strains tested, followed by an inhibition probably also caused by antibiotic production by *T. viride* at a distance. *Final* T. viride *penetration in the 2 strains' colonies is shown in figure 4.39. The results of the ANOVA statistical test evidence the influence of the colony size in* F. pinicola *(F: 8.5¸ probability of null hypothesis 0.003)* T. viride *resistance.*

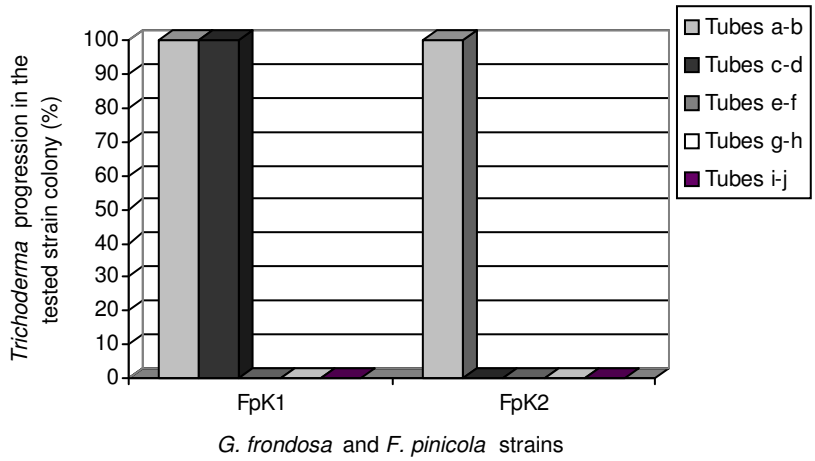

Figure 4-39 T. viride *penetration (%) in the substrate previously invaded with different colony sizes of* F. pinicola *FpK1 and FpK2 strains.*

Discussion: The results clearly demonstrate that colony size of *F. pinicola* is important in the resistance ability as observed for *G. frondosa* in the previous experiment. In the experiment with various substrate quantities the results led to the same conclusions, except FpK1 showed resistance with lower substrate quantities, than FpK2, the strain which resisted with the smallest colony size in this experiment. Interestingly it is the reverse that was observed with these strains in Petri dishes against bacteria: *F. pinicola* was producing a bacteriostatic and bactericide substance in the first days following inoculation in an AM medium but not later, allowing resistance and even agressivity (overgrowing) toward Gram+ and Gram – bacteria only at an early stage (this part was studied by Abdelaaziz Hmamda in his work on "Méchanismes de défense in vitro de la phase végétative de *F. pinicola* et *G. frondosa* (Aphyllophorales) vis-à-vis de *Trichoderma viride*, *Bacillus subtilis*, *Escherichi coli* et *Pseudomonas fluorescens*")

The reaction of *F. pinicola* after *T. viride* inoculation is interesting. Indeed *Trichoderma* spp. have already shown to produce more than 70 inhibitory substances (Sivasithamparam and Ghisalberti, 1998) with antimicrobial and antifungal activity (Godfredsen and Vangedal, 1965; Ooka et al. 1966; Meyer and Reusser, 1967; Pyke and Diets, 1966). Trichopolins have for instance, been shown to inhibit the respiration and growth of *L. edodes* (Tokimoto 1985). This can explain the distant growth inhibition, however, no data was found about the faster growth of the distant antagonist fungus when *Trichoderma* spp. is inoculated. It was found in our culture experiments that strain K2 could inhibit the growth of a *Penicillium* species contaminant up to 3.5cm away from the mycelium front, showing a diffusion in the medium of antifungal compounds which seem to be inactive against *T. viride*, this was further confirmed on AM plate confrontation tests.

This and the previous experiment on substrate quantity seems to indicate that in culture bags these strains would not resist if *Trichoderma* contamination happened at an early stage of incubation, but might resist when the *F. pinicola* has colonised more substrate.

4.2.4.7 Experiment 7: influence of substrate type on resistance of the *G. frondosa* and *F. pinicola*'s strains and evaluation of the validity of the method

Experimental design is shown in figure 4.40. Rates of substrate colonisation are shown in figure 4.41, where results obtained on 9 g B substrate in the first experiment were added for comparison between all substrates. All *G. frondosa* strains grew better on A substrate which contains the higher nitrogen content, while *F. pinicola* strains all invaded the C substrates faster than the others. After total colonisation of the substrate, *T. viride* was inoculated. Final penetration of *T. viride* in the colony of the tested strains is shown in figure 4.42

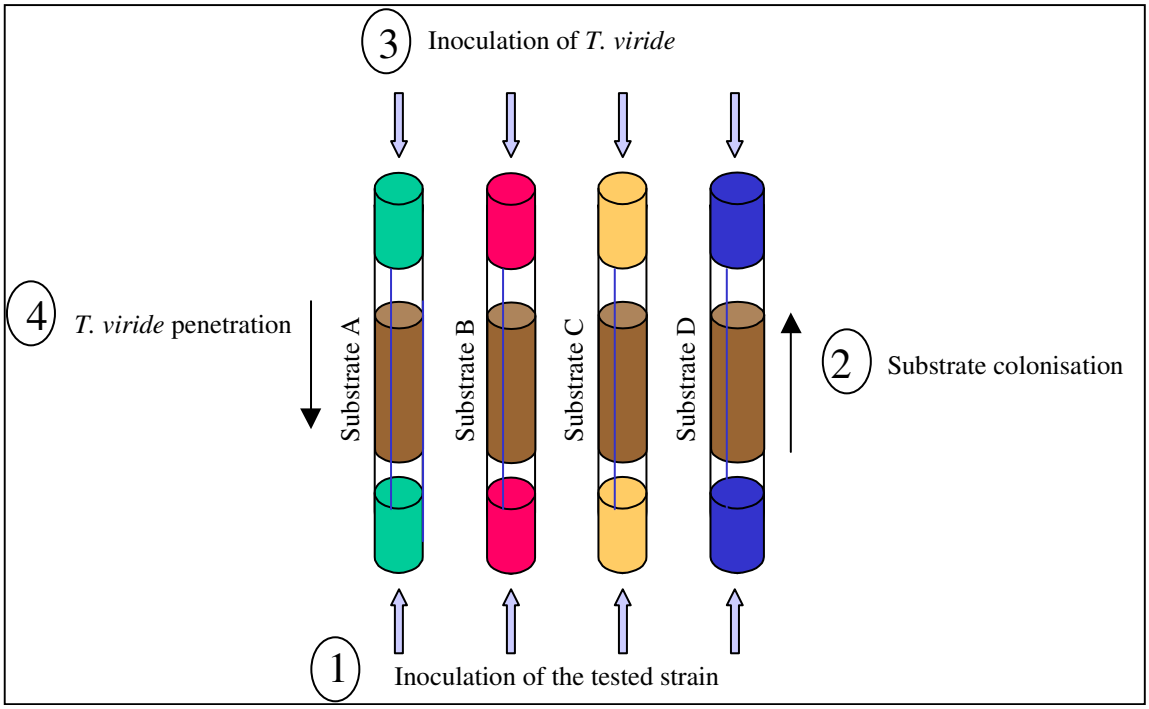

Figure 4-40: experiment 7 design

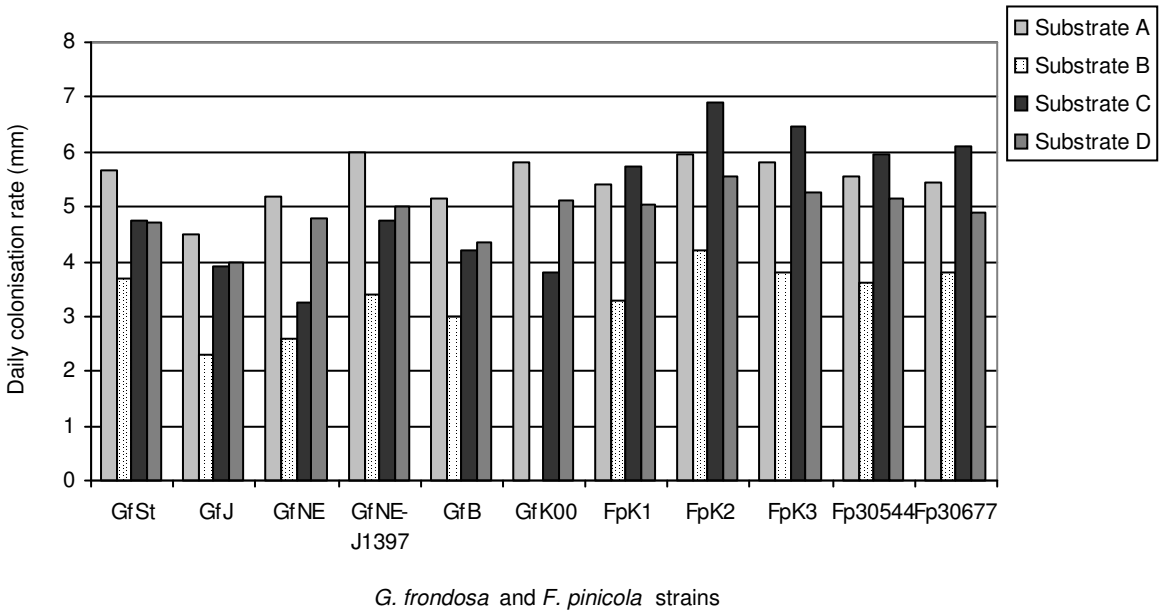

Figure 4-41: daily colonisation rate (mm) of the G. frondosa and F. pinicola strains on 4 substrates

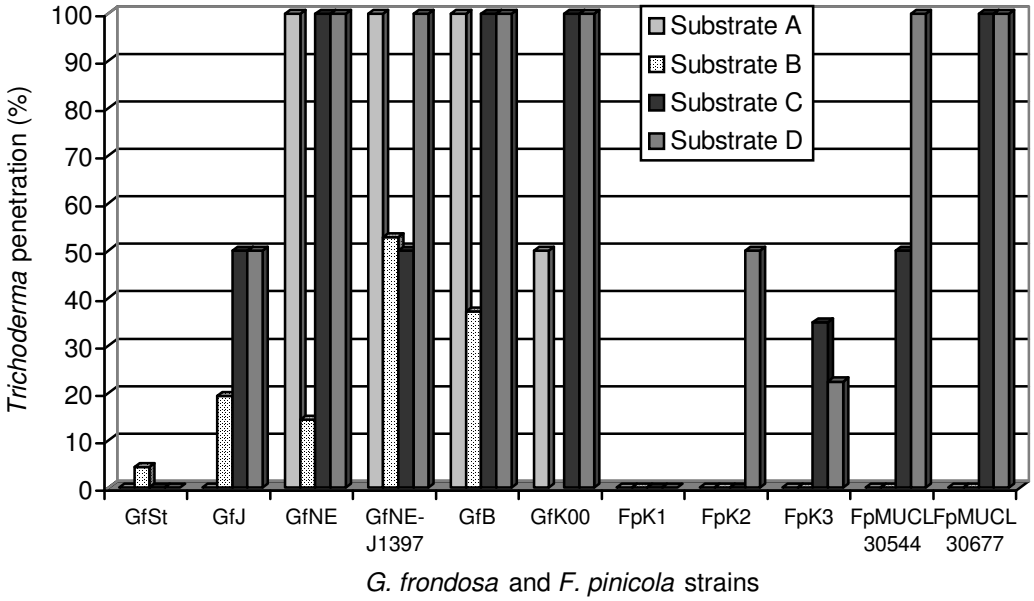

Figure 4-42: Percentage of T. viride *penetration (%) in A, B, C and D substrates previously colonised by* G. frondosa *or* F. pinicola *strains.*

The results of the ANOVA statistical test evidence the influence of the substrate type in the invasion rate of *G. frondosa* (F: 23.73, probability of null hypothesis 0.000) and *F. pinicola* (F: 53.27¸ probability of null hypothesis 0.000). The results of the ANOVA statistical test evidence for *G. frondosa* the influence of the strain in the resistance to *T. viride* (F: 13.39, probability of null hypothesis 0.000) but not for *F. pinicola* (F: 1.272, probability of null hypothesis 0.324). The Student's-T test show for *G. frondosa* that in the B substrate the resistance was significantly different (P = 0.03) compare with the others and for *F. pinicola* the resistance were significantly different (P = 0.03) in the A and B substrate compared to the C and D.

Discussion: The colonisation rate of the different substrates varied for both *G. frondosa* and *F. pinicola* strains. Interestingly all *G. frondosa* strains grew better on A substrates and the least on B substrate, while all *F. pinicola* strains grew better on C substrate and the least on B. *F. pinicola* had an even mycelium front and a faster growth, while *G. frondosa* presented uneven mycelial front and a slower growth.

For both species' capacity to resist *Trichoderma* progression was generally not correlated to substrate colonisation rate. Colonisation rate however brings an advantage to the studied species because in natural culture conditions it would colonise the substrate faster.

4.2.5 Discussion and conclusions

The system in Teflon® tubes seems to be adequate for testing both substrate colonisation and competition ability of strains on different substrates in a relatively short term experiment. It allows a good visibility of the interactions because of transparency of the tubes and because of the green sporulation of the pathogen tested. This kind of system was previously used to determine *Lentinula edodes* competition ability against *Trichoderma* spp. by strains selection

in glass tubes (Tokimoto and Komatsu, 1995) and by environmental and cultural conditions analysis in drinking straws (Badham, 1991). If in our experiments, only substrate composition and genetic variations between strains were considered, other factors like temperature could also be analyzed. Indeed the competition mechanism clearly depends, among other factors, on the temperature, shown to influence *Trichoderma* mycelial growth (Samuels, 1996) and competitive ability (Badham, 1991).

For both species and all strains tested, it is clear that not only are there different behaviors between strains but also for a given strain grown on different substrates. Our results agree with those obtained by Savoie et al. (2000) in the study of the interaction between *L. edodes* and *Trichoderma* spp, where it was observed that there are great differences between two modified substrates for each strain. In the same manner Tokimoto and Komatsu (1979) have observed that a C rich medium favours *L. edodes* while a N rich medium favours *Trichoderma* and damage could be reduced by controlling nutritional conditions. In our experiment it seems that the opposite is observed for *F. pinicola*: the substrate with the lower nitrogen content was favourable to *Trichoderma*. Goltapeh and Danesh (2000), Tokimoto and Komatsu (1995), Badham (1991) have also demonstrated that substrate composition has an influence on the interaction between two fungi.

Results showed that growth rate on AM medium could not be extrapolated to substrate colonisation capacity of the different strains. Thus these tests in tubes allow a better selection of a fast colonising strain able to compete with *T. viride* for nutrient use on chosen substrates. Pattern of hyphae branching is, however, not taken into account in the mycelial elongation measures so that the final fungal biomass and substrate utilisation could differ between substrates for a given strain. Substrate colonisation rate, competition ability and fructification capacities were not correlated either. For instance *F. pinicola* strain MUCL 30677 fructified on C and D substrates (see chapter 4.1) on which it could not resist at all against *T. viride* contrary to the other two substrates where no primordia were even formed. GfSt was the slowest strain on 2%AM medium and also the only *G. frondosa* strain never totally colonized by *T. viride*, except with small substrate quantities or small colony size. This is contrary to what was observed by Tokimoto et al. (1994) that *L. edodes* damage by *T. harzianum* in the substrate was predicted by the damage level in sawdust medium and growth level of the mycelium on Agar medium. Our results show the importance of testing 3 characteristics; growth rapidity, *Trichoderma* resistance ability and fruiting ability of different strains in order to select the best strain, which could be recombined to obtain the desired final characteristics.

Interaction between the species studied and *T. viride* varied between strains and substrate formulations. However, selection of fast growing strains, resistant on given substrates is possible using Teflon® tubes. As colony size of the strains tested has also shown to influence resistance ability, *T. viride* should be inoculated a few days after the tested strains in one range of tubes and in another range of tubes when their entire substrate has been invaded by the tested strain, to select the strains able to resist the earliest in the colonisation stage.

Interaction interface in the tubes between the two antagonistic fungi is restricted compared to real cultivation conditions in bag. Thus the results obtained are only indicative for selecting strains, substrates and conditions. Improving the competition ability of a cultivated mushroom is important, but does not dispense with good hygiene around the cultivation bag.

This method was used by Magali Kocher in the course of her diploma work. She tested tens of species and extracted the mycelium and culture medium of resistant strains to be tested in a

biological assay which was developed during the present work (see chapter 4.3). She isolated an antifungal compound against the pathogen in a very aggressive species (able to resist *T. viride* then to overgrow it when the pest was inoculated before total invasion of the substrate by the tested strain). Her results prove the potential of this method not only for culture development but also for pharmaceutical research.

4.3 SEARCH OF BIOLOGICALLY ACTIVE MOLECULES

4.3.1 Biological tests

The biological tests realised are presented in chapter 2.3.6. Detailed description of the test system is presented in the experimental section, paragraph 4.3.3. The biotests against the filamentous fungus *Trichoderma viride* were adapted from existing tests during the present work as described below.

Biotests were realised in two phases: 8 mushrooms were first analysed at the Institute of Pharmacognosy and Phytochemistry of the University of Geneva (previously in Lausanne) led by Dr. K. Hostettmann. Active molecules were partly isolated. Then cultivation experiments were realised in our laboratory followed by a second testing phase, however without molecule isolation.

4.3.1.1 Development of a biotest against *T. viride*

Agar diffusion method in which the active substance is transferred directly from the filter paper to an inoculated agar plate through a diffusion process gave unsatisfactory results in preliminary experiments. Thus agar-overlay technique was chosen and adapted from published protocols.

The procedure adopted was to pour 2%AM medium inoculated with conidia on the developed chromatogram. After solidification of the medium it was incubated in a humid atmosphere allowing the growth of the fungi (fig. 4.43). As a positive control, the DU-SIN surface disinfectant detergent (DUDLER, Switzerland) diluted to 1% and applied at 20 µl was used. Glass-backed solid TLC plates were better than the aluminium-backed plates. To maintain the glass-backed TLC plates at 35°C on a heating plate, allowed the application of a thinner medium layer. To know what agar concentration was the best for a thin layer on TLC, 3 AM medium containing respectively 5g/l, 10g/L and 15g/l agar were poured on TLC. All 3 were about the same, thus showing that that factor was not important. Various incubation conditions were tested. Plates were incubated either at 25°C, 30°C or 37°C with or without light. The best incubation condition was at 25°C with natural light. Light did not enhance growth rate but did conidiation, thus allowing a better visualisation of the inhibition spots.

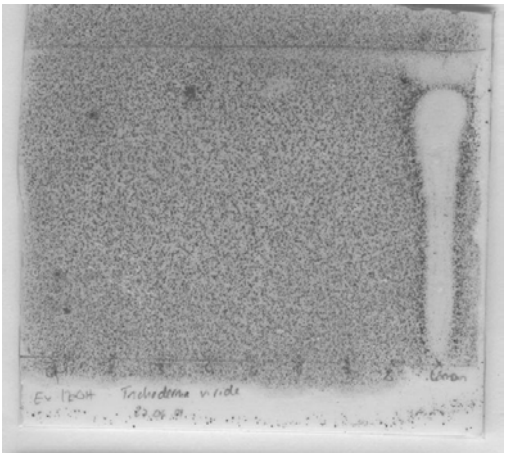

Figure 4-43: TLC of the G. frondosa *MeOH extract Gf1 to Gf8 with the positive control on the right tested against* Trichoderma viride.

The final technique was as follows: A spore solution was prepared a few days before the tests by putting a quantity of young cultured conidia in sterile water. After shaking to separate the conidia, a dilution technique was used to count spores number by ml. TLC plates were prepared in the same manner as for the bacteria, except the extract concentration was 200µg. Two plates were realised in parallel. 10 ml of a approximately 10^{-6}/ml conidia suspension obtained by dilution of the first solution and 2mg MTT are added in 90ml of 2% AM medium at 60°C, the MTT being swinnexed through a 0.2µ filter. The medium was spread directly in a thin layer over one of the TLC plates maintained at 35°C, while the other was observed under UV light at 366nm and 257nm then revealed with Godin reagent. The incubation conditions of the plates for the biological test were as mentioned above. The fungus was then killed by spraying with H_2SO_4 or with a 94% ethanolic solution.

4.3.1.2 Biotest results

Table 4.15 shows results obtained in the different biological tests.

Species	**Extract**	***B. subtilis* 20µg**	***C. albicans* 100µg**	***Cl. cucumerinum* 100µg**	***A. aegyptii* LC100 (500µg/ml)**	***B. glabrata* LC 100 (400µg/ml)**	**Anti-radicalar DPPH 100µg**	***T. viride* 200µg**
**A. aegerita*	DCM	+	-	+	-	+	+	
(air dried)	MeOH	-	-	-	-	-	+	
A. aegerita	DCM:MeOH							-
	H_2O							-
F. hepatica	H_2O							+
F. velutipes	DCM							+
	MeOH							-
F. pinicola	DCM							-
	MeOH							-
**G. lucidum*	DCM	+	-	-	-	+	+	+
(air dried)	MeOH	-	-	-	-	-	+	
**G. frondosa*	DCM	+	-	+	-°	+	+	
(dried stipe)	MeOH	-	-	-	-	-	-	
**G. frondosa*	DCM	+	-	+	-°	+	+	
(dried cap)	MeOH	-	-	-	-	-	-	
G. frondosa 1	DCM	+						-
	MeOH	-						-
G. frondosa 2	DCM	+						-
	MeOH	-						-
G. frondosa 3	DCM	+						-
	MeOH	-						-
G. frondosa 4	DCM	+						-
	MeOH	-						-
G. frondosa 5	DCM	+						-
	MeOH	-						-
G. frondosa 6	DCM	+						-
	MeOH	-						-
G. frondosa 7	DCM	+						-
	MeOH	-						-

Species	**Extract**	***B. subtilis* 20µg**	***C.* albicans 100µg**	***Cl. cucumerinum* 100µg**	***A. aegyptii* LC100 (500µg/ml)**	***B. glabrata* LC 100 (400µg/ml)**	**Anti-radicalar DPPH 100µg**	***T. viride* 200µg**
G. frondosa 8	DCM	+						-
	MeOH	-						-
G. frondosa 9	DCM	+						-
	MeOH	-						-
G. frondosa 10	DCM	+						-
	MeOH	-						-
G. frondosa 11	DCM	+						-
	MeOH	-						-
G. frondosa 12	DCM	+						-
	MeOH	-						-
G. frondosa 13	DCM	-						+
	MeOH	-						-
G. frondosa 14	DCM	-						-
	MeOH	-						-
G. frondosa 15	DCM	-						-
	MeOH	-						-
G. frondosa strain B	DCM							-
	MeOH							-
H. erinaceium	DCM:MEOH							-
L. conchatus	DCM	-						-
	MeOH	-						-
**L. edodes* (air dried)	DCM	+	-	-	-	+	+	
	MeOH	-	-	-	-	-	-	
L. edodes	DCM:MeOH							-
**L. nuda*	DCM	+	-	-	-	+	+	
	MeOH	-	-	-	-	-	-	
M. giganteus	DCM							-
	MeOH							-
P. citrinopileatus	DCM:MeOH							-
**P. eous* (air dried)	DCM	+	-	-	-	+	+	
	MeOH	-	-	-	-	-	-	
P. eous	DCM :MeOH							-
	H_2O							-
**P. eryngii (air dried)*	DCM	+	-	-	-	+	+	
	MeOH	-	-	-	-	-	-	
P. eryngii	DCM							-
	MeOH							-
**P. ostreatus* (air dried)	DCM	+	-	-	-	+	+	
	MeOH	-	-	-	-	-	-	
P. ostreatus	DCM:MeOH							-
P. squamosus	DCM							-
	MeOH							-
P. tuberaster	DCM							-
	MeOH							-
S. commune 80	DCM	+						+
	MeOH	-						-
S. crispa	DCM:MeOH							-
	H2O							-

Species	**Extract**	***B. subtilis* 20µg**	***C.* albicans 100µg**	***Cl. cucumerinum* 100µg**	***A. aegyptii* LC100 (500µg/ml)**	***B. glabrata* LC 100 (400µg/ml)**	**Anti-radicalar DPPH 100µg**	***T. viride* 200µg**
S. laminosa	DCM							-
	MeOH							-

*Table 4-15: results obtained in the different biological tests. += Positive result, -= negative result, °= 50% dead larvae; * extracts tested in the first stage, thus not tested against* T. Viride *as the test was not yet developed.*

Few statements could be drawn:

- Test in the IPP: DCM extracts of *A. aegerita, G. lucidum, G. frondosa stipe* and *cap, L. edodes, L. nuda, P. eous, P. eryingii* and *P. ostreatus* presented an activity spot against *B. subtilis* all at the same RF value. Test in the laboratory of Microbiology: the DCM extracts GF1 and Gf2 were active against *B. subtilis* all along the migration line with a more defined spot at a RF=0.26. Gf6 had the same activity and Gf10 had an activity at RF=0.29. However the latest was done on another TLC plate. Gf3 to Gf8 had a double line of activity, which is found also in Gf9 to Gf12 and in Gf16, but much less active. Gf17 and Gf18 had a slight activity at the point of application of the extract. The DCM extract of *S. commune* showed an activity at a RF value of 0.52.

- DCM extracts of *G. frondosa* stipe and cap had an antifungal activity against *C. cucumerinum*. The activity in the stipe was higher than in the cap.

- DCM extracts of *G. frondosa* stipe and cap were not officially active, but nevertheless, induced a 50% death in *A. aegyptii* larvae. This species might have a substance with an ecological role to defend itself against flies or predators.

- All the DCM extracts tested in the mollusquicide test were active: *A. aegerita, G. lucidum, G. frondosa stipe* and *cap, L. edodes, L. nuda, P. eous, P. eryingii* and *P. ostreatus* all induced the death of the snails within 24 hours in an aqueous solution containing 400ppm extract.

- All the DCM extracts of the mushrooms tested (*A. aegerita, G. lucidum, G. frondosa stipe* and *cap, L. edodes, L. nuda, P. eous, P. eryingii* and *P. ostreatus)* were slightly active in the antiradicalar test with the same RF value, however *P. eryngii's* activity was much higher at this same RF value. Thus it is probable the same substance is present in a much higher quantity in this latest species. MeOH extracts were less homogeneous: *A. aegerita, G. lucidum* and *L. nuda* extracts presented a weak activity at the base line. The *G. lucidum* extract also presented two other more important activities at two other RF values.

- *F. hepatica* DCM:MeOH extract showed an inhibition spot against *T. viride* at Rf 0.89 corresponding to red discoloration by Godin Reagent and to fluorescence extinction under UV light at 254 nm and fluorescence under UV light at 366nm. *F. velutipes* DCM extract was active at Rf 0.21 corresponding to fluorescence extinction under UV light at 254 nm. *G. lucidum* DCM extract presented two inhibition activities: at Rf 0.09 corresponding to fluorescence extinction under UV light at 254 nm and Rf 0.68 corresponding to both fluorescence extinction and a brown red spot revealed by Godin

reagent. Of all of them the strongest activity was found in *F. hepatica*. *G. frondosa* Gf13 extract and *S. commune* DCM extracts inhibit *T. viride*. For the MeOH extracts it is interesting to note that the Tetrazolium reacted for Gf1, Gf2 and Gf3 at a RF of 0.6, by a darker round spot. It seems that there are some molecules not present in the other extracts of *G. frondosa* which have a reducing power.

4.3.2 Activities against *B. subtilis*

The ubiquitous active substance in the 8 species tested (figure 4.44) was not UV-active and was not revealed by Godin's reagent. The Rf value was 0.5 on a silicium TLC plate in the ligroïne/EtOAc 1:1elution system.

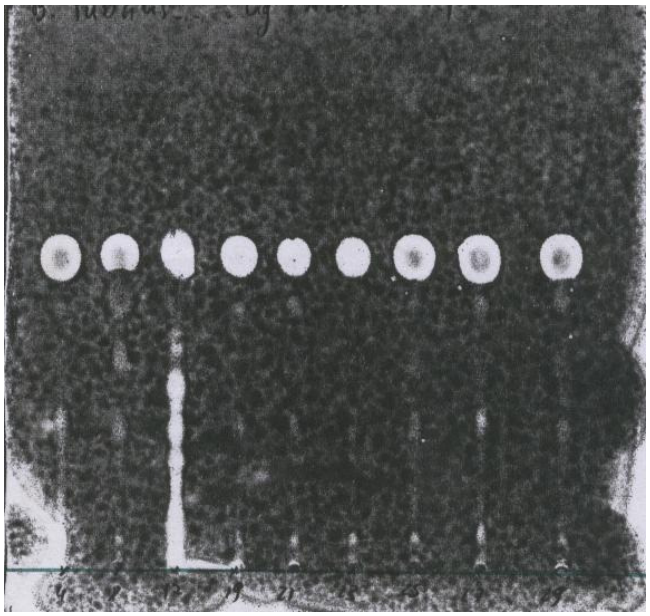

Figure 4-44: bioautogramm of the activity test against B. subtilis *of the DCM extracts of* P. ostreatus*,* L. nuda*,* G. lucidum*,* L. edodes*,* A. aegerita*,* G. frondosa *stipe 2nd extract,* G. frondosa *primordia,* P. eous *and* P. eryngyi.

Keller (1997, thesis) has demonstrated –with the same technique- activity against *B. subtilis* and/or *E.coli* in three quarters of the DCM extracts realised out of 57 species. Activity was variable, only *F. pinicola* DCM extract showed strong antibacterial activity. Isolation of active compounds showed they were only active in autobiographic assays but not in classic dilution tests.

As there was the same activity in all our extracts, the spots were probably due to a ubiquitous molecule with low activity but in high concentration. Moreover the activity was low compared to the reference compound chloremphenicol (0.01µg). Thus it was decided not to isolate the molecule responsible for this activity.

Gf1 and Gf2 DCM extracts showed an inhibition zone against *B. subtilis* at a different Rf value, we thus decided to try to isolated the active molecule: 20.6mg of the Gf2 DCM extract was separated on a silicium reverse phase C18 column (superco, supelclean, LC18. SPE tubes, 3ml). A gradient of solvent from 100% water to 100% MeOH, followed by DCM and MeOH with 1 $^{0}/_{00}$ TFA were used as eluant (figure 4.54).

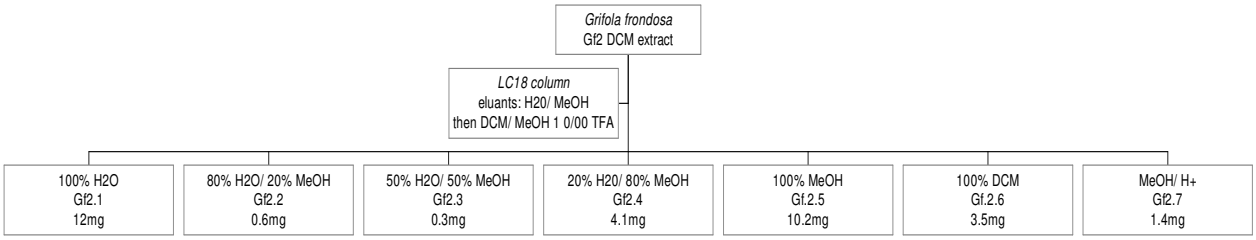

Figure 4-45: sheme of separation of G. frondosa *Gf2 DCM extract active against* B. subtilis.

After separation, the initial extract and the fractions were tested against *B. subtilis*. Gf1 was still active, as well as fractions Gf1.1, Gf1.4 and Gf1.5. Gf1.4 seemed pure by TLC. Thus it was analysed by MS and RMN showing the presence of a fatty acid. As there are many fatty acids showing a slight activity against *B. subtilis*, we decided not to determine the structure of the molecule.

4.3.3 Activities against *C. cucumerinum*

DCM extracts of *G. frodosa* stipe and cap had an activity at the same RF value, but it was higher in the stipe extract than in the cap extract (Figure 4.45). A yellow spot was revealed with Godin's reagent corresponding to the activity. The positive control was propiconazole (0.01g).

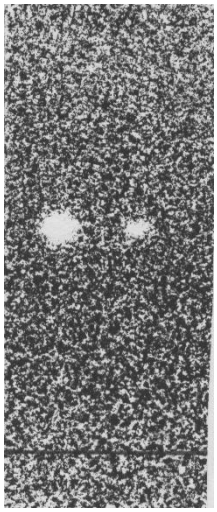

Figure 4-46: Bioautogramm of the activity test against C. cucumerinum *of the DCM extracts of* G. frondosa *stipe and cap respectively.*

Grifola frondosa is an Aphyllophorales. Thus carpophore with or without stipe evolve from undifferentiated primordia, contrary to the Agaricales where primordia are minute fruit bodies with differentiated stipe and cap. Our hypothesis was that primordia was producing defence substance early in the stage of fruiting cycle to protect itself and this substance was then diluted in the entire fruit body during the growth of the stipe and then of the cap.

To confirm that this substance was not previously present in the substrate or in the mycelium, substrate, substrate invaded by mycelia, primordia and stipe and cap of *Grifola frondosa* fruit bodies was again extracted and tested against *C. cucumerinum* in the same manner as before.

However there was no activity at the normal tested extract quantity (100µg). The yellow spot corresponding to the activity found in the first extracts were not present in these.

This result could be explained by 2 different factors: first the extracts with activity were done out of mushrooms cultivated in the laboratory, air dried immediately after collection, while the inactive extracts were realised with mushrooms cultivated with a higher CO_2 level at Fermenta SA for a bigger stipe obtention than in the laboratory. Moreover, the fruit bodies were frozen before being air dried. Thus this unknown substance might have deteriorated.

It was thus decided to choose *G. frondosa* as a model to study the influence of strain, substrate and technical treatment on the variation of chemical compounds (see chapter 4.4).

As further *G. frondosa* extracts were inactive, we tried to isolate the substance from the two mixed active extracts. The total extract weight was 1.7g. The isolation schema is shown in figure 4.46.

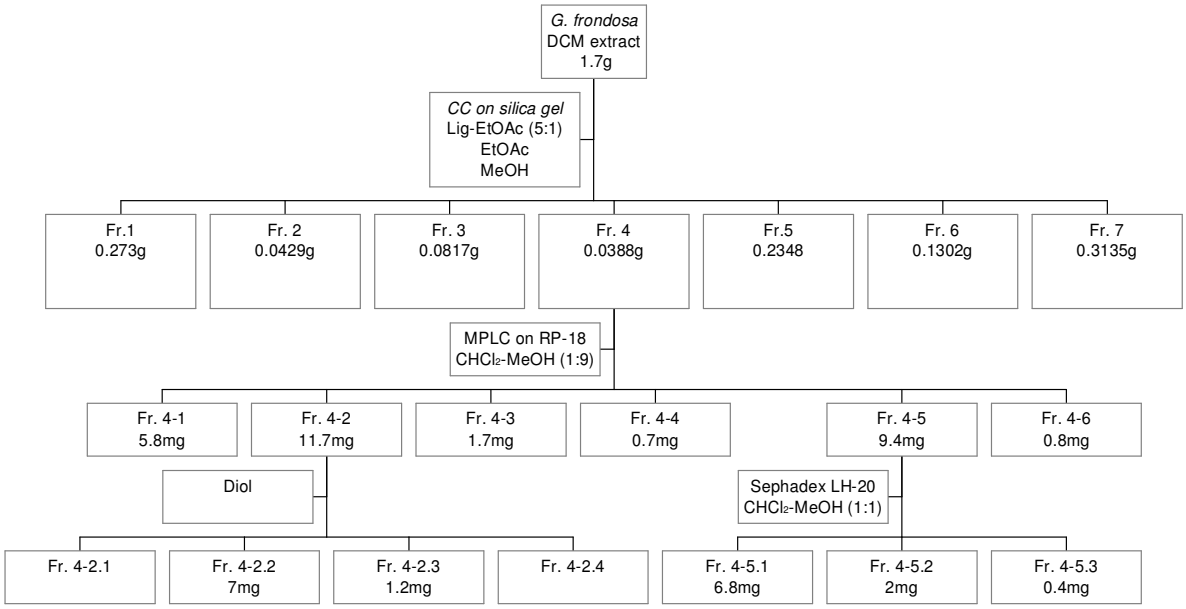

Figure 4-47: isolation scheme of DCM extract of G. frondosa

The extract was fractioned by CC on silica gel to yield 7 fractions representing 63.52 % of the initial fraction. Each fraction was tested on TLC against *C. cucumerinum* to find the active compound in fraction IV weighting 0.0388g. It was fractioned by CC on RP-18 lobar to obtain 6 fractions (4-1 to 4-6) representing a 77.84% recovery of fraction 4. Biotest against *C. cucumerinum* showed 2 active fractions: 4-1 and 4-5, however the activity of 4-1 fraction was weak and had not the same Rf value as the first observed activity. This activity was not observed before probably because it was due to a molecule with very weak activity. As the fraction was not pure and the quantity was low, we did not try to isolate this molecule. Fraction 4-5 had the same Rf value as the activity studied, but did not correspond to the yellow spot revealed by the Godin's reagent at the same RF value as the activity spot. This yellow spot was present in the inactive 4-2 fraction. The 9.4mg 4-5 fraction was further separated by exclusion chromatography on a Sephadex column. Three fractions were obtained

and were tested against the target fungus. However the substances seemed to be degraded as there were only long trails on the TLC plate and there was no more activity visible.

Biotests on *C. cucumerinum* of the substrate with or without mycelium were negative at the normal 100µg extract normally used for the test.

4.3.4 Activity against *Biomphalaria glabrata*

All the DCM extracts tested in the mollusquicide test were active: *A. aegerita, G. lucidum, G. frondosa stipe* and *cap, L. edodes, L. nuda, P. eous, P. eryingii* and *P. ostreatus* all induced the death of the snails within 24 hours in an aqueous solution containing 400ppm extract. It is probably a unique substance present in all the species tested. These results might be due to defensive substances present in mushrooms to protect themselves against predators like snails. To confirm this hypothesis the *G. frondosa* substrate invaded by the mycelium was when tested was negative. It seems to confirm that this substance is present only in the carpophores and not the mycelium. The active compound against this snail was obtained during fractionation of *G. frondosa* extract (see figure 4.46 above). It was obtained in the pure 4-2.2 fraction as revealed by the activity test.

This substance was not UV active but was revealed by Godin reagent. The structure elucidation was elaborated by MS and NMR spectrometry. The sample was passed in the MS in TSP mode (figure 4.47) and gave a mass spectrum with a molecular pick at m/z 280 [M], which indicates a mass of 280uma.

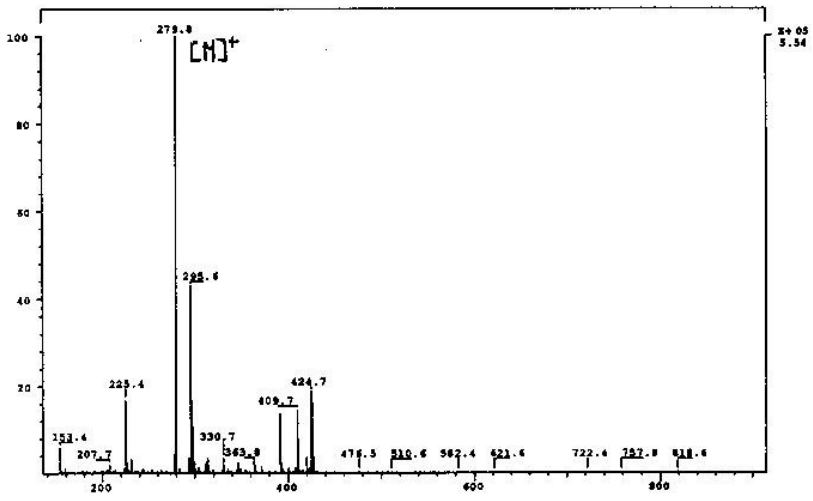

Figure 4-48 : Mass spectrum in TSP mode of the active molecule against B. glabrata

^{1}H NMR brings only little information: there is an important aliphatic part. This spectrum resembles those of fatty acids (figure 4.48).

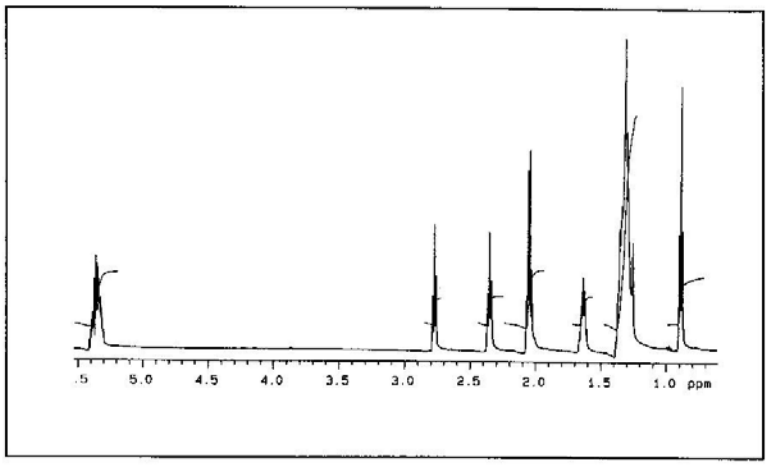

Figure 4-49: 1H spectrum of the active molecule against B. glabrata

^{13}C NMR spectrum presents signals corresponding to a non substituted carbonyle group at δ 179.94 and at two double bonds appearing by four –CH group at δ 128.59, δ128.76, δ130.71 and 130.91 in the low field region. The fact that the compound is not UV active indicates that both double bonds are not conjugated. Moreover twelve $–CH_2$ signals and one $–CH_3$ signal appear in the high field zone. There are 18 signals altogether. The under-spectrum DEPT (figure 4.49) confirms these pieces of information. The raw chemical formula proposed is $C_{18}H_{32}0_2$.

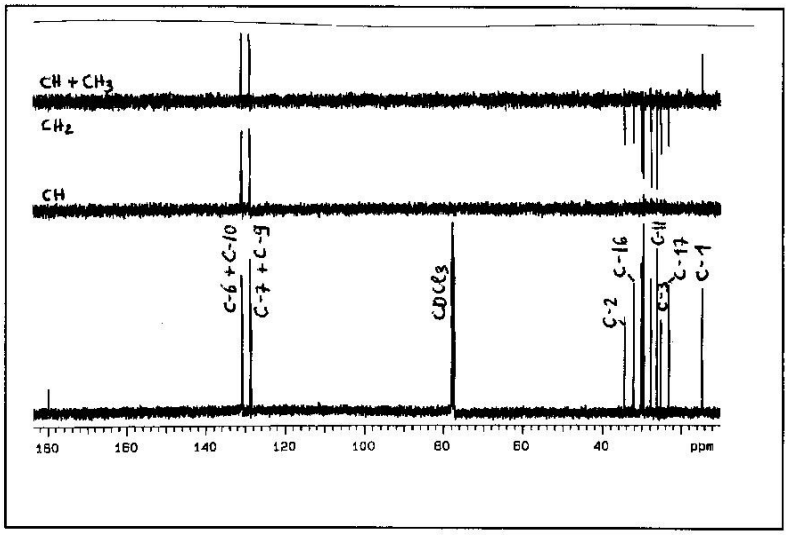

Figure 4-50: DEPT RMN spectrum of the active molecule against B. glabrata

GHSQC (figure 4.50), GHMBC (figure 4.51) and COSY (figure 4.52) have allowed elaborating the structure. Information given by the literature confirms it.

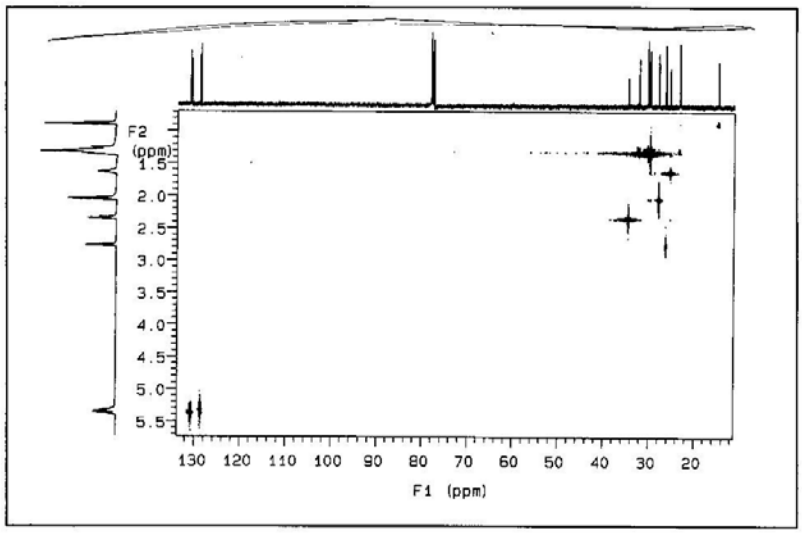

Figure 4-51: GHSQC RMN spectrum of the active molecule against B. glabrata

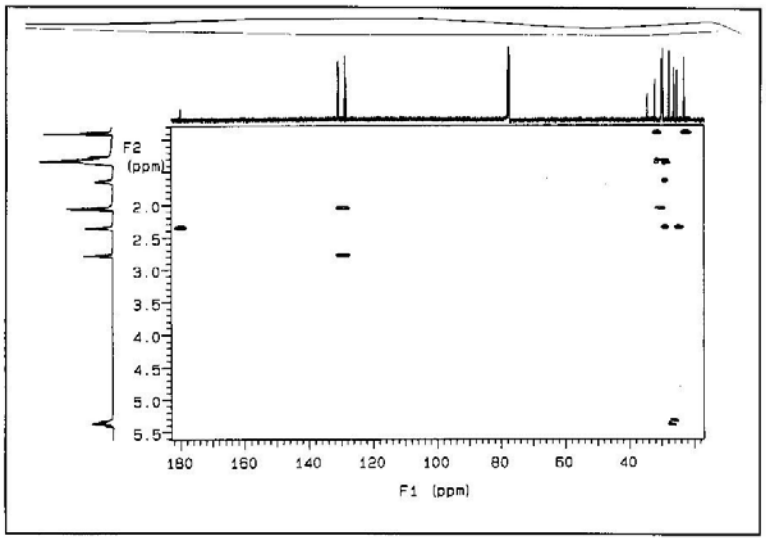

Figure 4-52: GHMBC RMN spectra of the active molecule against B. glabrata

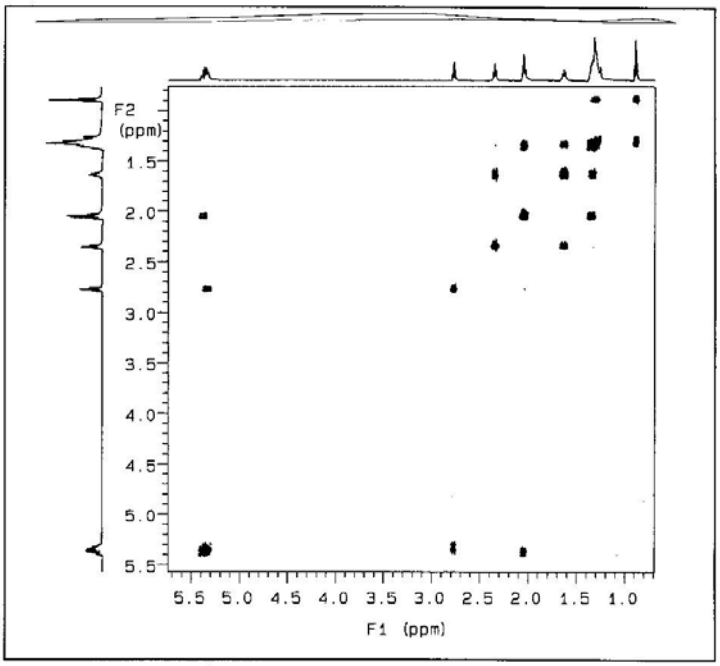

Figure 4-53: COSY RMN spectrum of the active molecule against B. glabrata

Gunstone et al. (1977), in a study on the ^{13}C NMR fatty acids spectra showed that chemical shifting of the-CH_2 group included between two double bonds is strongly influenced by cis or trans configuration of the two double bonds:

-CH=CH-CH2-CH=CH-

Cis-cis:	25.7ppm
Cis-trans	30.6ppm
Trans-trans	35.7ppm

This is not valuable when the system is affected by close substitutes; however it is not our case. The δ of methyle between the two double bonds being of 26.35 in our molecule, the deduced configuration is cis-cis. The fatty acid is therefor linoleic acid (figure 4.53) or 9-cis,12-cis-octadecadienoic acid, also called emersol 310, emersol 315, polyin no.515 and telfairic acid.

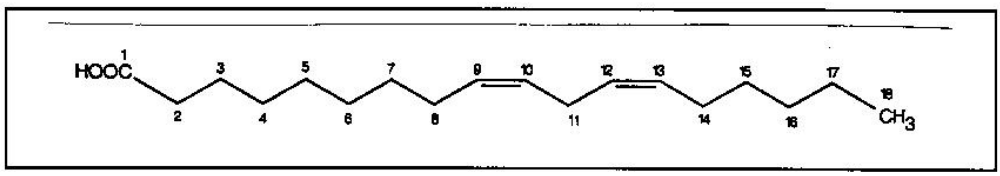

Figure 4-54: linoleic acid

Rough formula: $C_{18}H_{32}O_2$
Developed formula: $COOH(CH_2)_7(CH)_2CH_2(CH)_2(CH_2)_4CH_3$
Molecular weight: 280.45

Spectral data:

^{13}C RMN ($CDCL_3$): δ 179.94 (COOH, C-1), 130.91, 130.71 (CH, C-9, C-13), 128.76,128.59 (CH, C-10, C-12), 34.48 (CH_2, C-2), 32.22 (CH_2, C-16), 30.27, 30.04, 29.83, 29.77, 29.72 (CH_2, C-4, C-5, C-6, C-7, C-15), 27.90, 27.88 (CH_2, C-8, C-14), 26.32 (CH_2, C-11), (CH_2, C-3), 23.26 (CH_2, C-17), 14.75 (CH_3, C-18).

4.3.5 Discussion

We have here developed a *T. viride* bioassays on the basis of other bioassays performed at the Laboratory of Pharmacognosy and Phytochemistry (Geneva, Switzerland) led by Dr. K. Hostettmann. The development of this test was important as many problems arise in development process of cultivation and there are almost no existing compounds to fight this pest. A few positive results were found in basidiomes extracts: *F. hepatica*, *F. velutipes*, *G. lucidum*, *G. frondosa* Gf13 extract and *S. commune*, representing 26,3 % of the tested species. No active compounds were isolated during this work although in our laboratory Magali Kocher used this bioassay to test medium and mycelium extracts of resistant strains toward *Trichoderma* (selected by the method developed during this work chapter 4.2) and found a strong activity. She isolated a phenolic compound active against *T. viride*, validating the developed technique.

As for the other bioassays, many tested DCM extracts showed antibacterial activity against *B. subtilis*. RMN analysis of an active *G. frondosa* DCM extract identified it as a fatty acid; however we did not work further on the molecule identity as there are thousands of antibacterial fatty acids already known.

No antibacterial activity was previously reported from *A. aegerita* fruit bodies, however Stransky et al. (1992) have showed that submerged culture mycelium produces antibacterial substances with bacteriostatic and bactericide effects on Gram+ and Gram- bacteria. *G. lucidum* total extract was reported by Hsu (1990) as antibacterial against *Staphylococci, Streptococci* and *Bacillus pneumoniae*. Such an activity was not reported from *G. frondosa*. Ethylacetate extract of *L. edodes* already showed anti-bacterial (as well as antifungal) activities due to disulphide derivative (Takazawa et al., 1982, from English abstract). In a study on the efficiency of diverse natural substances in preventing tooth decay and periodontitis *L. edodes* chloroform, ethylacetate and water extracts inhibited the main bacteria species responsible of these problems: *Streptococcus* spp*., Actinomyces* spp*., Lactobacillus* spp*., Prevotella* spp*.,* and *Porphyromonas* spp. (Hirasawa et al., 1999). Recently, *L. edodes* extracts were also reported to inhibit growth of *S. aureus* and *E. coli* due in part to the formation of oxalic acid (Bender et al., 2001). No antibacterial activities were previously reported from the 3 *Pleurotus* spp. studied.

No extracts were active against *C. albicans*. However Stransky et al. (1992) have detected an antifungal activity against this fungus in submerged culture of *A. aegerita*. We could therefore conclude that this (these) active substance(s) is only produced by the mycelium and is not present in the fruit body. However we should be aware that some substances can vary in function of the strain as well as in function of trophic and environmental conditions as will be further discussed in chapter 4.4. *L. edodes* is also used against *Candida* spp however; it is active by stimulation of the immune system, so that an in vitro assay can not show this activity.

All the DCM extracts tested in the mollusquicide test were active. Linoleic acid 9-cis,12-cis-octadecadienoique revealed to be the molecule responsible for the snail *B. glabrata* death at least in *G. frondosa* extract. This widespread linoleic acid is one of the main fatty acids in mushrooms along with oleic acid, stearic acid and palmitic acid. Linoleic acid can represent up to 60% of the total fatty acid quantity. We don't know the mode of action, but as this molecule is not soluble in water, it might just block the respiration system of the snail, leading to its death. Narbe et al. (1991) have noticed that a fatty acid mix containing mainly oleic, palmitic and linoleic acid could possess an immunosuppressive activity. Stadler et al. (1994) have demonstrated a nematicidal activity against *Caenorhabditis elegans* for linoleic acid.

In the antiradicalar bioassay it resorted that *G. lucidum* was the only one to show strong antiradicalar activity. Wang et al. (1985) and Lin *et al*. (1995) already showed that *Ganoderma* have an antioxidant activity by elimination of free hydroxyls radicals. Thus the active molecules were not isolated. It is interesting to note that in these first extracts tested, all activity was in the DCM extracts, due to an apolar molecule, except these antiracalar activities in *G. lucidum*.

The molecule responsible for the activity against *C. cucumerinum* in *G. frondosa* stipe and cap extracts could not be isolated: a try resulted in the disappearance of the activity, probably by degradation. Remark that the activity in the stipe was about twice that observed in the cap. Of high interest in a biological point of view is the fact that in further extracts of the same strain but grown differently and submitted to a different treatment after collection, no activity could be detected. It is unknow whether the activity was linked to a molecule which was not synthesized in the second case or whether it was an unstable molecule which was degraded by treatment after collection (freezing then drying). This species would therefore be an interesting model organism for the analysis of modulation (see chapter 4.4) in which influence of treatment, but also of strain and substrate composition will be studied on morphological characters or secondary metabolites profiles. Indeed very little is known about the influence of different factors after harvest of the fruit bodies on metabolites degradation: boiling and storage has shown to have an influence in *G. frondosa* (Takama et al. 1981), while other Basidiomycetes species metabolite profiles were showed to be influenced by damage to the fruit body (Pang and Sterner 1991, Pang, Sterner and Anke 1992), by environmental factors like light (Adam, Campbell and McCorkindale 1967). Moreover as Nishitobe et al. (1986) have shown that high temperature and prolonged oxidation must be avoided in order to conserve intact the structure of the mushroom components. Reason why reducing the chemical modification in the extracts seemed so important to us, that it was decided to freeze the collected fruit bodies and the extracts, as well as practicing the extraction at low temperatures with the least light possible.

No important activities were found during this work. However it has been clearly stated that mushrooms represent a wide source for new products (Lindequist et al., 1990; Miles and Chang, 1997). To our point of view if analysing fruit bodies grown *in vitro* restrain the number of species, it has the advantage not to be dependant on the generosity of nature, but also to work on known strains in controlled trophic and environmental conditions. To counterbalance the restricted number of species, we should better multiply the diversity of biotests. However our laboratory has neither the skill nor possesses the material necessary to test mushroom extracts against various biological targets. Thus it seems essential to built up a network of partnership so that each member realizes the work in which it is specialized. For instance, our laboratory could focus on cultivating mushrooms and studying the influence of trophic, environmental and genetic factors on production of active metabolites while a

chemist or pharmacologist could focus on biological tests, isolation and characterization of active molecules

4.4 MORPHOLOGICAL, PHYSIOLOGICAL AND CHEMICAL VARIATIONS IN THE CULTIVATE EDIBLE AND MEDICINAL MUSHROOM GRIFOLA FRONDOSA IN REGARD TO STRAIN, SUBSTRATE AND TECHNICAL TREATMENTS.

4.4.1 Morphological and physiological comparison between strains

The results presented in this paragraph were obtained during the experimentation treated during this work (cultivation and resistance) but are united here to compare the different *G. frondosa* strains.

Growth rates in a 2% AM medium at different temperatures (figure 4.55) already show that if all the species grow better at 25°C, GfJ strain is the only one which was able to grow at 30°C. This might show an adaptation of the strains to higher temperature conditions.

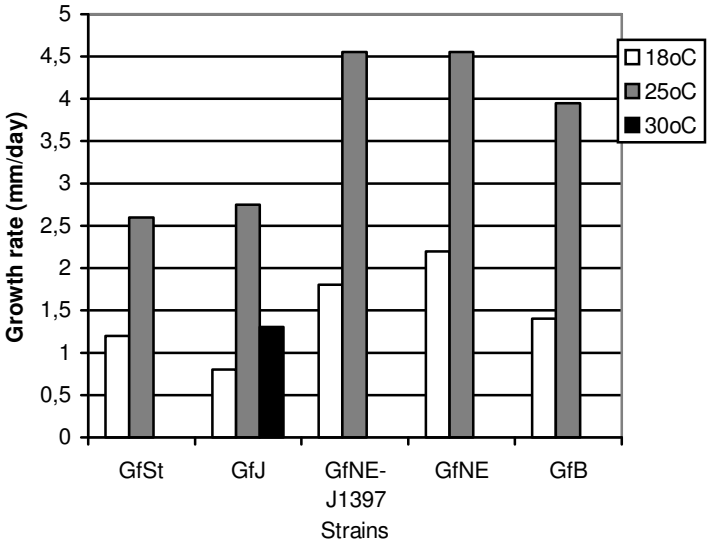

Figure 4-55: Growth rate (mm/day) on 2% AM medium of 5 G. frondosa *strains at 18°C, 25°C and 30°C.*

When considering substrate colonisation rates on the four substrates A, B, C and D at 25°C (figure 4.56) we can also observe variations in a given strain on different substrates, however all strains grew better on A substrate which has the highest nitrogen content then on C and D and finally B. It could mean that for all tested strains, the higher nitrogen content tends to enhance growth.

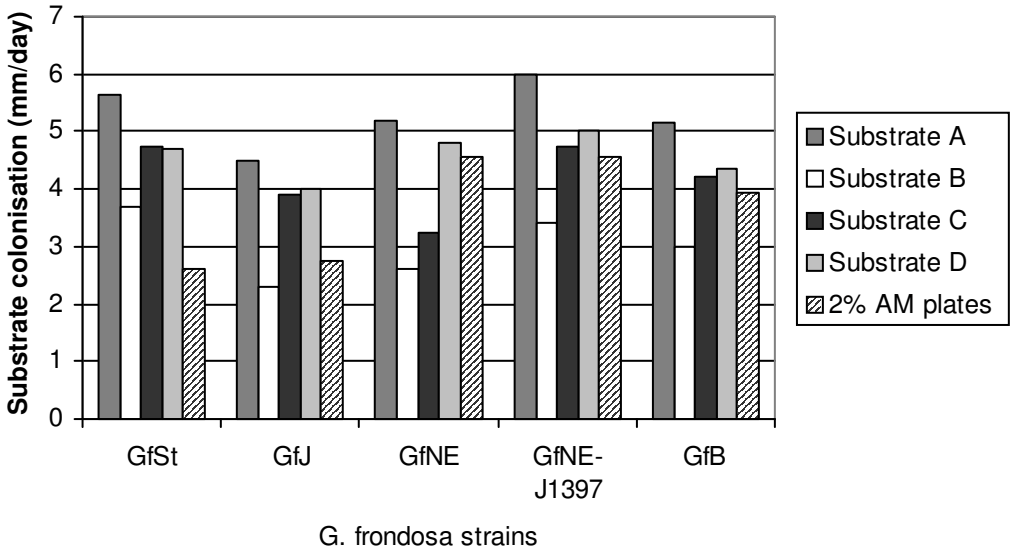

Figure 4-56 Substrate colonisation rate (mm/day) of 5 strains of G. frondosa *on 4 lignocellulositic substrates and on 2% AM medium at 25°C.*

Resistance tests on 2% AM medium showed that none of the strains could resist at all. There was not even latency before *Trichoderma viride* overgrew *G. frondosa* mycelium. On the substrates, however, strains could resist more or less (figure 4.57) by stopping *T. viride* progression after a while.

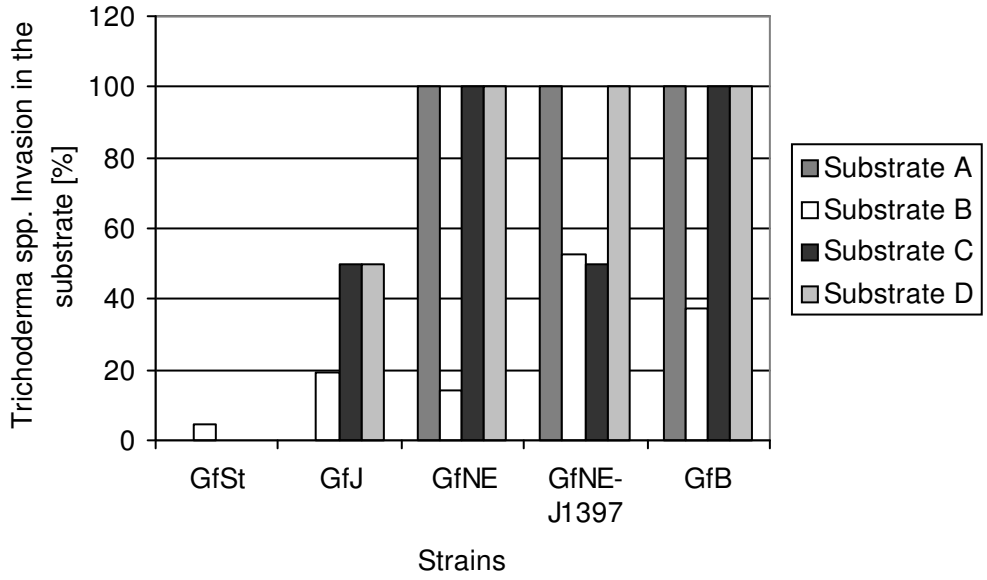

Figure 4-57: *Trichoderma viride* penetration (in percent) in different substrates colonised by *G. frondosa* strains.

Fruiting capacity also varied between strains. Yields obtained for the four tested strains on the various substrates are presented in figure 4.58. B substrate was specially designed for commercial GfNE strain fruiting (Job, unpublished), explaining this high yield obtained. GfB strain also formed primordia on substrate A.

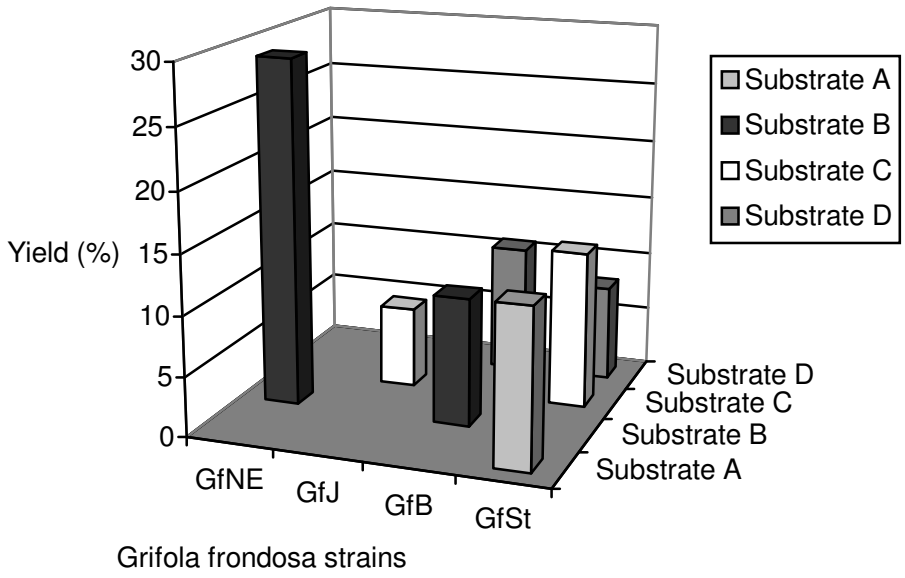

Figure 4-58 Fruit body yield (in percent) of four strains on various substrates

If there were no obvious differences in the vegetative phase, features of the fruit body obtained in the same environmental conditions did show some. For instance GfSt showed great differences in color and shape between substrates: on A the fruit body was much darker than on the other substrates and had a tiny stipe (figure 4.59), while on D it had a big stipe with the leaves turning up (figure 4.60), GfB growing on C also had a tiny stipe and a dark color, GfSt strain had a strong forest smell while GfB on B had a very strong hazelnut smell, much stronger than the GfNE strain.

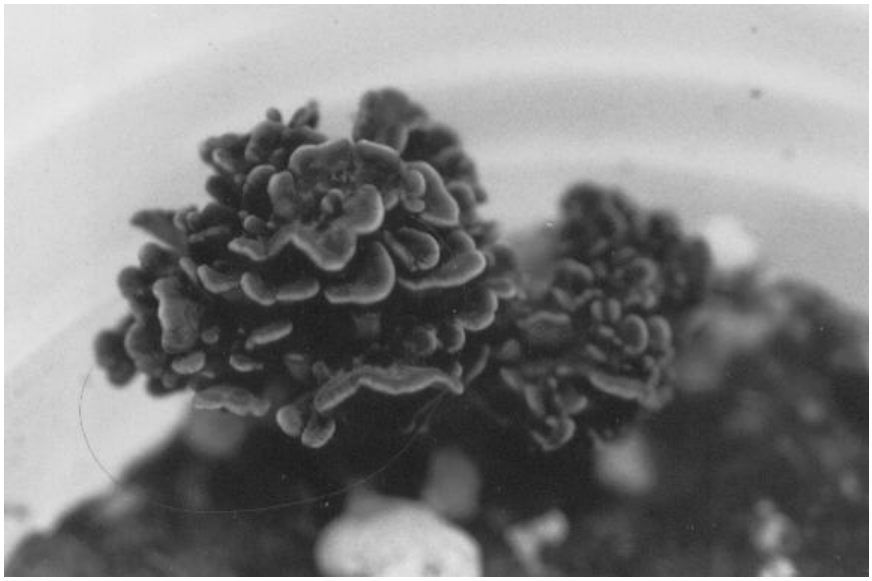

Figure 4-59:G. frondosa GfSt strain growing on A substrate

Figure 4-60: G. frondosa GfSt strain growing on D substrate.

4.4.2 Comparisons of the extracts by TLC and HPLC

The chemical profile of each extract was investigated to help identify modifications linked to the strains specificity, culture conditions or treatment. All the spots detected in DCM and MeOH extracts are listed in annex 1 and 2.

Comparisons of the DCM extract by TLC showed differences in the metabolites detected. The identity of the substances is unknown and could sometimes be different with the same RF value, but there are interesting results.

Strain specific compounds were for instance demonstrated by a visible fluorescence spot at 366 nm in any of the GfNE, Gf1397 and the hybrid GfNE-J1397 fruit body extracts but not in either of the two GfSt extracts (Gf14 and Gf15) and GfB extracts at a Rf value of 0.85 with the DCM/MeOH eluting system. It was visible independent of the treatment, thus indicating a stable compound. Another example is that all fruit body extracts except those of strain Gf J1397 (Gf9) had a spot revealed by H_2SO_4 at the base line with DCM/MeOH eluting system. It could indicate that GfJ1397 extract contains free amino-acids in much lesser quantity than in the others. Revealed with Dragendorff reagent, there was a non specific yellow spot, pink when revealed with H_2SO_4 at the base line with the same eluting system in all GfNE and GfNE-J1397 extracts but not in the GfSt or GfJ1397 extract.

No evidence of metabolite variations was found between the two extracts of GfSt grown on different substrates, however differential technical treatments like drying, lyophilization and freezing or not, were shown to influence the metabolite profile. The only three fruit body extracts realised out of dry material without previous freezing (Gf3,Gf4 and Gf5) were revealed by a blue spot with H_2SO_4 at a Rf of 0.26 with the

eluting system DCM/MeOH. The extract 1 was the only one which showed a yellow spot at Rf 0.5 with the same eluting system. This could mean that there is a degradation product of the hybrid strain only when dried after freezing. In the same manner all the dry fruit bodies (Gf1 to Gf5) showed fluorescence at 366 nm at Rf 0.96 with the eluting system Lig/EtOAc. These can be degradation products due to drying, which are not present in lyophilized fruit body extracts. The only extract done out of a white fruit body was not revealed with Godin reagent at a Rf of 0.43 with Lig/EtOAc eluting system. Thus this spot might be correlated with the normal gray brown color of the fruit body.

Less results were obtained with MeOH extracts, however we could observe visible fluorescence spots at 366nm (Rf 0.65 and Rf 0.7 with the eluting system $ChCl_3$: MeOH:H_2O) only for the fruit body frozen before being separated from the substrate (Gf6) demonstrating possible rapidly degraded products after collection.

Strain specific compounds seem to be revealed by ninhydrine/H_2SO_4 by a yellow spot at Rf 0.42 in all fruit body extracts except the two GfSt ones (Gf14 and Gf15) with the eluting system EtOAc/ Hexane.

As for the DCM, extracts technique seems to be responsible for most compound variations as the two extracts frozen before being dried (Gf1 and Gf2) showed unique spots at the base line revealed by fluorescence extinction (254nm), at Rf 0.29 by a black spot revealed by Godin reagent and at Rf 0.68 by visible fluorescence at 365nm (eluting system $ChCl_3$/MeOH/ H_2O). Moreover spots at the base line were revealed orange by ninhydrine and H_2SO_4 for these two extracts, while it was purple in all other extracts (eluting system EtOAc/Hexane), and revealed brown by Dragendorff reagent while it was yellow in all other extracts (eluting system DCM/MeOH)

Profiling chemical extracts by HPLC revealed differences in the number of compounds extracted and in the affinity to the solvent of the compounds. Major compounds extracted with DCM have a characteristic UV absorption at 220 nm and 230 nm with a group of compounds showing absorption at 320 nm for extracts Gf8 to Gf14, whereas compounds extracted with methanol have UV absorbency mainly at 280 nm indicating differences in the chemical families. Analysis of DCM and MeOH extracts revealed 26 compounds: 22 in the DCM extracts, 14 in the MeOH extracts and 10 in both extracts types.

The total chemical compound profiles contained 26 binary variables, corresponding to the presence or the absence of the compound in each of the 15 extracts as detected by the different methods. Following the cluster analysis (Figure 4.61) using total chemical compounds revealed in both extracts, two main distinct groups could be identified.

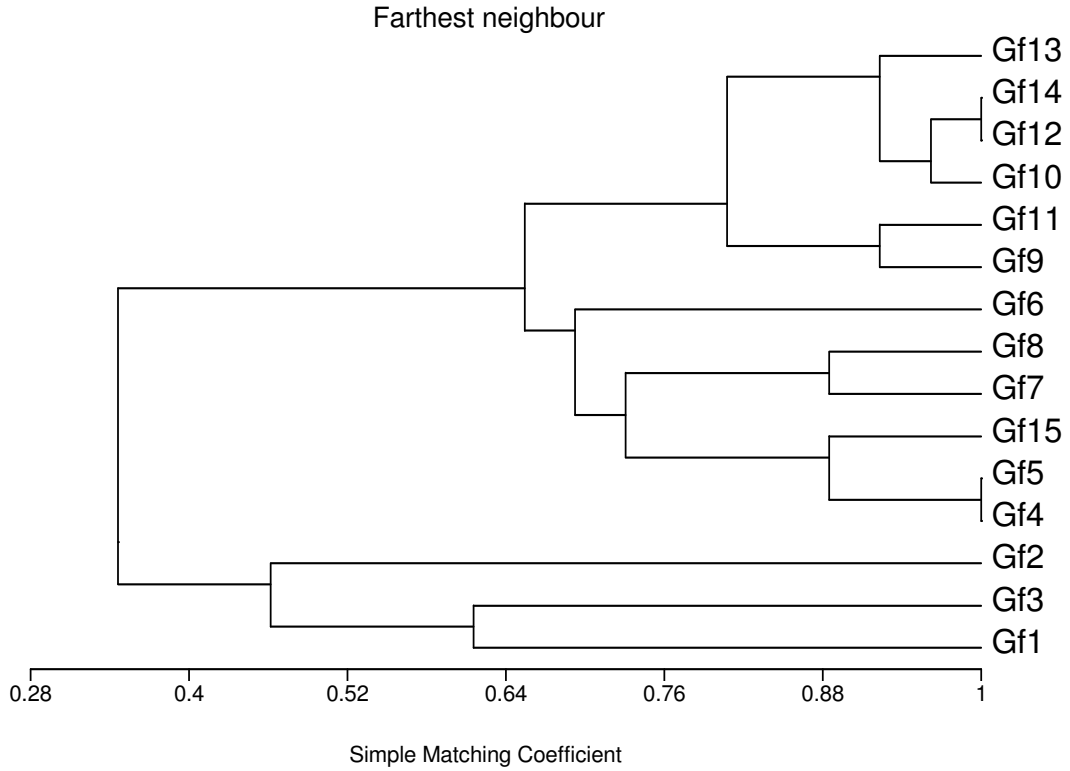

Figure 4-61 Dendrogram showing hierarchical relationships between 15 extracts grown in different physiological conditions and extracted by DCM and MeOH, either after drying or freeze-drying, using total chemical compound profiles (26 binary variables). The simple matching coefficient of Sokal and Michener was used as a similarity measure between samples and cluster analysis was computed using complete-linkage method.

Extracts Gf4 and Gf5 showed no differences, which indicates that modifying the drying conditions from room temperature to 60°C does not affect the chemical composition of this strain. Gf7 and Gf8 clustered with small differences, indicating that the major composition is similar when the fruit body is white or deformed by lack of oxygen. The same strain grown in standard condition but frozen and lyophilised still attached to the substrate where it grew (Gf6) is relatively far from Gf7 and Gf8 showing chemical differences. However we cannot explain why Gf15, resulting from another strain GfSt, grown on medium C and freeze dried, was clustered with Gf4 and Gf5.

Extracts Gf9, Gf10, Gf11, Gf12, Gf13 and Gf14 formed a rather homogeneous cluster. Surprisingly, Gf12 and Gf14 resulting from two different strains GfNE and GfSf, grown on two different mediums, B and D respectively, showed identical chemical profiles. Gf11 grouped with Gf12 and Gf14 although on a different branch but with less than 5 on the scale of differences. It is worthy to note that Gf11(GfNE) and Gf6, both grown on medium B and frozen when still attached to the substrate, but the first one having been damaged with a razor blade 15 minutes previous to freezing, showed a little similarity, indicating that injuries induced chemical modifications. Extracts Gf13 and Gf10 show small differences indicating that fruit body of GfNE grown on medium B

but with a stipe deformed by high CO2 level is very similar to the hybrid strain GfNE-J1397(Gf10) grown on the same medium. The wild strain GfJ1397 extract (Gf9) was very close to Gf11. It is worthy to note that the same GfNE strain grown on the same medium but one with a white fruit body (Gf8) and the other with the normal grey-brown coloured fruit body (Gf12) produced different chemical compounds. When analysing clusters obtained with DCM or MeOH extracts separately (not shown), Gf8 MeOH extract did not contain any of the 26 compounds analysed.

The cluster containing Gf1, Gf2 and Gf3 is clearly separated from the other extracts. Gf3 and Gf1 resulted from the same strain GfNE-J1397 but were submitted to a different treatments after their collection, either dried for the first or frozen before being dried for the second; their relatively slight similarity indicates that changing the harvesting technique may have a big impact on chemical composition. It is further confirmed by the fact that they are clustered at a low fusion level to the same strain grown in the same conditions but lyophilised straight after collection (Gf10). Finally, Gf2 (GfNE) was the furthest away from all the others, indicating that when the mushroom has blackened, the chemical composition changed drastically.

We would have expected the GfSt strain extracts to be grouped in the same cluster. Indeed almost no differences between the two extracts were observed by TLC analysis. Moreover, it was expected that all the GfNE strains would be in the same cluster, which is not the case.

4.4.3 Antibacterial and antifungal biological tests

Bioautographic tests were realised against the fungus *Trichoderma viride* and against the bacteria *Bacillus subtilis* and *Escherichia coli*. Only Gf13 DCM extract showed a slight inhibition spot at the point of application against *T. viride*. The same extracts were active against *B. subtilis* at a Retention factor (Rf) of 0.26 with the eluting system $ChCl_3$/MeOH/H_2O corresponding to a black spot with Godin's reagent. The only bioactivity found against *E. coli* was the one of Gf4 DCM extract at a Rf of 0.54.

4.4.4 Discussion

Growth rate measures on AM medium at different temperatures seem to show a probable adaptation of the strains to different climates, however with great dissimilarity between strains at a given temperature as already observed in previous studies (Job and Schiff Giovannini, 2004). We have found no correlation between growth rate on AM medium, invasion rate on different substrates and fruiting capacity of the strains on these substrates like those observed for *Fomitopsis pinicola* (Schiff Giovannini et al., 2004).

Invasion rates were not correlated either to fruiting as GfSt fructified on 3 of the 4 substrates, while GfJ fructified only on C were it grew the slowest in the invasion rate test, and GfB strain completed its life cycle on B and D substrates where it respectively grew the slowest and the fastest. GfNE also fructified the best on B substrate where it grew the slowest. These results seem to indicate that there is an adaptation of strains to various substrates. This is further demonstrated by published data where various material were used in the substrate composition: oak in the US and Japan (Lee, 1994), beech (Kirchhoff, 1996) and larch in Japan (Stamets, 2000), cottonseed hulls in China

(Zhao et al., 1983) with various nutrient supplements such as wheat (Mayuzumi and Mizuno, 1997) or rice bran (Takama et al., 1981), corn meal (Kirchhoff, 1996) or soybean cake (Mizuno and Zhuang, 1995). Generally no C/N values were given, however, it might be the C/N coefficient which is important and not the lignocellulositic material used (Job and Schiff Giovannini, 2004). Our results indicate that not only is resistance to *Trichoderma viride* is linked to strains, but also to substrate composition as previously demonstrated for *Lentinula edodes* or *Agaricus bisporus* (Savoie et al., 2000; Goltapeh and Danesh, 2000; Mata et al., 1998; Badham, 1991; Tokimoto et al., 1994).

Yield was variable between strains and substrates; however these data on fructifying capacities and yield were collected on few replicates, thus giving an indication, but being insignificant. Yield obtained in our tests are reasonably good if compared to Kirchhoff (1996) who obtained fruiting with a yield of about 17.5% and 7.5% for the two tested strains on one of the three tested substrates. Shen and Royse (2001) have also previously demonstrated that type and quality of nutrient supplement influence yield and basidiome quality.

TLC and HPLC analysis of the crude extracts have allowed us to confirm chemical variations according to the strain and to the substrate on which the strain grew, but mainly according to the technical treatment. The main chemical differences are observed when the mushrooms are frozen before drying, or drying alone compared to lyophilization. Variations were also observed when the fruit body was injured voluntarily as previously observed, for instance, for *Cantharellus tubeaformis* (Pang et al., 1992), *Cantharellus cibarius* (Pang and Sterner, 1991) and *Larcarius vellereus* (Sterner et al., 1985).

In the biological tests, the intensity of the activity found was very low, thus is not interesting for further pharmacological research, however we have shown that an activity can be linked to a technical treatment. Indeed all the activities of fruit body extracts were only present in two extracts realised out of fruit bodies frozen then dried at room temperature. According to the reaction with Godin's reagent, it might signify fatty acids, probably issued from the degradation of other molecules. This seems to confirm the importance of the treatment of the fruit bodies for certain biological activities linked to secondary metabolites.

The aim of our work was mainly to study specific characteristics of different strains of *Grifola frondosa*, as well as to determine the influence of substrate composition and technical treatments on the physiological and chemical traits of these strains. This work shows us that strains from different parts of the world do not have a similar behavior concerning fruiting capacities and resistance toward the world-wide pest *T. viride* Moreover it shows us the importance of constancy in experiments as strains, substrates, and treatment can have an influence on the metabolite profiles and bioactivity of fruit bodies extracts. Parallel to this work, genetic variations between the strains were also analysed by PCR analysis by Audrey Cordier in the course of her diploma work bringing complementary results.

5 CONCLUSIONS AND PERSPECTIVES

Our laboratory is mainly skilled in the domain of edible mushroom culture development. Mushrooms draw ever more interest from the scientific world for their metabolitic capacities or chemical compounds. We thus thought to extend our competences to species which could be the source of new products like pharmaceuticals, flavours, cosmetic components or nutraceuticals. Working on cultivated mushrooms indeed allows independence from the seasons, exterior trophic and environmental conditions.

Developing cultivation processes for new Basidiomycetes species helped to enhance our understanding of fruiting phenomenons. In this work 39 strains from 27 species were tested for their *in vitro* capacities, most of them on four substrates varying in their C/N coefficient. Other substrates were designed for some species, like *A. blazei* which is the only secondary saprophyte we worked on during this work. Results were encouraging as 2/3 of the species could be fructified in this *in vitro* fruiting screening. For the 2/3 of the species there was a gradient of fruiting success from substrate A with the higher nitrogen content to substrate D with the lower nitrogen content, the first one being the best.

Thus for this screening program this variety of substrates revealed to be adequate. A certain amount of observation were collected for the species and strains, increasing slightly our understanding of the physiology and fruiting process, which can however vary greatly from one species to the other. Influence of the substrate composition and genetic variations was clearly demonstrated on fruiting induction, crop time cycle, yield, odours or phenotypic characters like colour or general form.

In the future we could however focus on certain hard to please species, like the rare *L. officinalis,* which culture has never succeeded even if it was widely used in popular medicines for over 2000 years. *Poria cocos* and *Inonotus obliquus* cultivation are also examples of promising species which would be very interesting to cultivate. Moreover if a species present a interesting activity found in our laboratory or in an other the cultivation techniques should be ameliorated to disminish crop time cycle and enhance yield.

In the culture development -in the course of this work or in other projects- the worst problem encountered was substrates contamination by the mycoparasite *Trichoderma viride* leading to great waste of time and material as experiments are often long. To our point of view it was therefor important to select strains with a good aptitude to resist this pest, beside the usually performed fruiting capacity screening. Indeed no rapid test were available so that it was decided to develop one with two consequences: first it would allow less loss during culture experiments, then it might enhance greatly the chance to find an antimycotic substance against *Trichoderma* spp produced by resistant strains. Indeed there are almost no available product on the market to fight this disease in mushroom culture farms.

Transparent Teflon® tubes were used to visualise interactions between the tested strain and the pathogen on the substrates used in the fruiting capacity screening. From these experiment done by testing 5 strains of *G. frondosa* and 5 of *F. pinicola* we could stated that Teflon® tubes seemed adequate to test resistance of strains on various substrates. We could eventually criticise the fact that we worked on to few replicates. This technique was however taken over by the diploma student Magali Kocher to test tenth of mushroom species, allowing to confirm its validity. Magali Kocher even isolated a phenolic compound active against *T. viride* from the medium of a selected resistant and aggressive strain.

Our expectations were fulfilled for this part of the work. In the future we could expect to analyses systematically several strains of a new species which cultivation technique has to be developped. It could allow determining characteristics of the studies strains (substrate colonisation rate, competition ability, adaptation to a given substrate) in the early stage of cultivation process development. We could also use this technic to analyse influence of environmental conditions on spawn run, which has not been done during the present work. We could also imagine creating recombinant strains between strains with wanted characteristics like competition ability and good fruiting ability for instance.

Our laboratory had no skill in the field of active molecule research prior to that work. To learn the bases and techniques, 8 species (which culture were developed by Dr. Job) extracts were analysed in the Institute of Pharmacognosy and Phytochemistry of the University of Lausanne (now transferred to Geneva) led by Dr. K. Hostettmann. It allowed going through all the process of extraction, bioassays, molecule isolation and structure elucidation. In our laboratory we used these acquired competence to develop a bioassay against *Trichoderma viride* on the model of currently performed bioautographic assays. Testing our mushroom fruit bodies or mycelium extracts against this mycoparasite was a crucial point to try to confim our hypothesis that resistant strain produce antimycotic agents and also to develop biotesting in a way which is not in concurrence with well specialised laboratories. If no activity were found in fruit bodies extract during the present work, resistant mycelium revealed to produce active compounds in the medium (Kocher, 2004).

In the future we should keep on testing various extracts (mycelium, fruit bodies, medium) against *T. viride* however we should establish partnership with institutes specialised in the biological tests or pharmacological research to realise a battery of different test with our extracts. This should allow a great enhancement of our chance to find an interesting new molecule. Isolation and characterisation of molecules should also be realised by this partners and we should mainly focuse on cultivation process and selection of resistant strains.

Working on mushroom physiology allows observing that they are organisms with certain plasticity. The term plasticity which is used in this work refers to the morphological and physiological or chemical modifications that a strain can undergo under different external conditions, that's to say trophic or environmental. Plasticity in mushrooms was almost not studied previously. We have chosen *Grifola frondosa* as model organism. Different factors potentially influencing variations of phenotypic or chemical traits were studied: influence of the environment, of the substrate composition, of the treatment applied to fruit bodies before and after collection, of the extraction technique. Parallel a student (Audrey Cordier) had the objective to define the genetic differences between strains which could influence their behaviour in certain conditions or in other words to link genetic markers to certain phenotypic or physiological characters during the course of her diploma work.

We noticed influence of environmental factors on phenotypic characters. But the most important difference laid in the metabolites profiles depending of the treatment followed by the collected fruit body before extraction. Some variations could be observed between strains but not for a strain on different substrate, contrary to what was expected.

In the future it might be interesting, not only to obtain a better smell, colour, or higher conservation time in edible fungi like already performed, but also to induce or enhance production of potentially interesting substances, like active molecules. Parapharmaceutical

industries for instance could be interested for producing mushroom extracts with higher active compounds content, like anti-radicalar substances for instance, or oligoelement rich extracts (this subject is actually studied in our laboratory by the PhD student Gilles Farron).

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Annex 1

Comparison of the different *G. frondosa* DCM extracts on TLC

Extract	Eluting system	Fluorescence extinction under UV light at 254nm	Fluorescence under UV light at 365	Ninhydrine/H_2SO_4 reagent	Dragendorff reagent	Godin reagent
1	DCM/MeOH 9:1	Rf≈ 0.8	Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow Rf≈ 0.5: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.47 Rf≈ 0.73	Rf≈ 0.6 Rf≈ 0.96			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: orange
2	DCM/MeOH 9:1	Rf≈ 0.8 BL	Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.47 Rf≈ 0.73	Rf≈ 0.6 Rf≈ 0.96			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: purple black
3	DCM/MeOH 9:1	Rf≈ 0.8	Rf≈ 0.6 Rf≈ 0.85	BL: brown Rf≈0.26: blue	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.47 Rf≈ 0.73	Rf≈ 0.6 Rf≈ 0.96			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: purple black
4	DCM/MeOH 9:1	Rf≈ 0.8	Rf≈ 0.6 Rf≈ 0.85	BL: brown Rf≈0.26: blue	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.47 Rf≈ 0.73	Rf≈ 0.6 Rf≈ 0.96			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: purple black
5	DCM/MeOH 9:1	Rf≈ 0.8	Rf≈ 0.6 Rf≈ 0.85	BL: brown Rf≈0.26: blue	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.47 Rf≈ 0.73	Rf≈ 0.6 Rf≈ 0.96			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: purple black
6	DCM/MeOH 9:1	BL	Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96:

						purple black
7	DCM/MeOH 9:1	BL	Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: redish
8	DCM/MeOH 9:1	BL	Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.6 Rf≈ 0.96: orange
9	DCM/MeOH 9:1		Rf≈ 0.6 Rf≈ 0.85			
	Lig:EtOAc 1:1	Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: yellow
10	DCM/MeOH 9:1	BL	Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.52 Rf≈ 0.6 Rf≈ 0.96: orange
11	DCM/MeOH 9:1		Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.52 Rf≈ 0.6 Rf≈ 0.96: orange
12	DCM/MeOH 9:1		Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.52 Rf≈ 0.6 Rf≈ 0.96: orange
13	DCM/MeOH 9:1		Rf≈ 0.6 Rf≈ 0.85	BL: brown	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96:

						orange
14	DCM/MeOH 9:1		Rf≈ 0.6	BL: brown		
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: orange
15	DCM/MeOH 9:1		Rf≈ 0.6	BL: brown		
	Lig:EtOAc 1:1	BL Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.6 Rf≈ 0.96: orange
16	DCM/MeOH 9:1	Rf≈ 0.8 BL	Rf≈ 0.6 Rf≈ 0.85	BL: brown Rf≈0.26: blue	BL: yellow	
	Lig:EtOAc 1:1	BL Rf≈ 0.47 Rf≈ 0.73	Rf≈ 0.6			BL: black Rf≈ 0.43 Rf≈ 0.6
17	DCM/MeOH 9:1	Rf≈ 0.8				
	Lig:EtOAc 1:1	Rf≈ 0.47 Rf≈ 0.73 Rf≈ 0.71	Rf≈ 0.8			BL: black Rf≈ 0.43
18	DCM/MeOH 9:1	Rf≈ 0.8			BL: yellow	
	Lig:EtOAc 1:1	Rf≈ 0.73	Rf≈ 0.8			BL: black

Annex 2

Comparison of the different *G. frondosa* MeOH extracts on TLC

Extract	Eluting system	Fluorescence extinction under UV light at 254nm	Fluorescence under UV light at 365nm	Ninhydrine/H_2SO_4 reagent	Dragendorff reagent	Gibbs reagent	Godin reagent
1	DCM/MeOH 9:1		Rf ≈ 0.06	BL: brown Rf ≈ 0.54: yellow	BL: brown	BL: yellow	
	EtOAc/Hexane 7:3	BL		BL: orange Rf ≈ 0.42: yellow Rf ≈ 0.49: red Rf ≈ 0.82: yellow			
	$CHCl_3$:MeOH:H_2O 65:35:5	BL	Rf ≈0.68				Rf ≈0.29: black
2	DCM/MeOH 9:1		Rf ≈ 0.06	BL: brown Rf ≈ 0.54: yellow	BL: brown	BL: yellow	
	EtOAc/Hexane 7:3	BL		BL: orange Rf ≈ 0.42: yellow Rf ≈ 0.49: red Rf ≈ 0.82: yellow			
	$CHCl_3$:MeOH:H_2O 65:35:5	BL	Rf ≈0.68				Rf ≈0.29: black
3	DCM/MeOH 9:1			BL: brown Rf ≈ 0.54: yellow (light) Rf ≈ 0.91: yellow	BL: yellow	BL: yellow	
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow Rf ≈ 0.49: (light) Rf ≈ 0.82: yellow			
	$CHCl_3$:MeOH:H_2O 65:35:5						
4	DCM/MeOH 9:1			BL: brown Rf ≈ 0.54: yellow (light) Rf ≈ 0.91: yellow	BL: yellow	BL: yellow	
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow Rf ≈ 0.82: yellow			
	$CHCl_3$:MeOH:H_2O 65:35:5						
5	DCM/MeOH 9:1		Rf ≈ 0.06 (light)	BL: brown	BL: yellow	BL: yellow	
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow Rf ≈ 0.82: yellow			
	$CHCl_3$:MeOH:H_2O 65:35:5						
6	DCM/MeOH 9:1		Rf ≈ 0.06	BL: brown	BL: yellow	BL:	

			(light)			yellow
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow Rf ≈ 0.82: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5		Rf ≈0.65 Rf ≈0.7			
7	DCM/MeOH 9:1			BL: brown	BL: yellow	BL: yellow
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5					
8	DCM/MeOH 9:1			BL: brown	BL: yellow	BL: yellow
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow Rf ≈ 0.82: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5					
9	DCM/MeOH 9:1			BL: brown Rf ≈ 0.54: yellow (light) Rf ≈ 0.91: yellow	BL: yellow	BL: yellow
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow Rf ≈ 0.82: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5					
10	DCM/MeOH 9:1		Rf ≈ 0.06 (light)	BL: brown	BL: yellow	BL: yellow Base line: yellow
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5	Rf ≈0.2				
11	DCM/MeOH 9:1			BL: brown	BL: yellow	BL: yellow
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5	Rf ≈0.2				
12	DCM/MeOH 9:1			BL: brown	BL: yellow	BL: yellow
	EtOAc/Hexane 7:3	BL		BL: purple Rf ≈ 0.42: yellow Rf ≈ 0.82: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5	Rf ≈0.2				
13	DCM/MeOH 9:1			BL: brown	BL: yellow	BL: yellow

	EtOAc/Hexane 7:3	BL	BL: purple Rf ≈ 0.42: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5	Rf ≈0.2			
14	DCM/MeOH 9:1		BL: brown Rf ≈ 0.91: yellow	BL: yellow	BL: yellow
	EtOAc/Hexane 7:3	BL	BL: purple		
	$CHCl_3$:MeOH:H_2O 65:35:5	Rf ≈0.2			
15	DCM/MeOH 9:1		BL: brown Rf ≈ 0.54: yellow (light) Rf ≈ 0.91: yellow	BL: yellow	BL: yellow
	EtOAc/Hexane 7:3	BL	BL: purple Rf ≈ 0.49: (light) Rf ≈ 0.82: yellow		
	$CHCl_3$:MeOH:H_2O 65:35:5				
16	DCM/MeOH 9:1		Not done		
	EtOAc/Hexane 7:3	BL			
	$CHCl_3$:MeOH:H_2O 65:35:5				
17	DCM/MeOH 9:1		Not done		
	EtOAc/Hexane 7:3	BL			
	$CHCl_3$:MeOH:H_2O 65:35:5				
18	DCM/MeOH 9:1		Not done		
	EtOAc/Hexane 7:3	BL			