Part I Shiitake

Chapter 2

Shiitake Spawn and Strain

IMPROVEMENT OF SPAWN FOR CULTIVATION IN ALTERNATIVE SUBSTRATES

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Introduction

Spawn, frequently called "inoculum," is the vegetative tissue of the fungus and consists of a medium which has been permeated by mycelium. Shiitake spawn commercially prepared on wooden pegs is known as "plugs," on supplemented sawdust called "sawdust spawn" (Przybylowics and Donoghue, 1988; Kozak and Krawczyk, 1989), and recently also produced on cereal grains (Mata *et al*., 1998). The process of introducing spawn into the substrate is called inoculation or spawning. An adequate spawn is one possessing a mycelium that is capable of rapid growth when invading a particular substrate (Leatham and Griffin, 1984). Shiitake cultivated on sterilized substrates must have a competitive advantage over other colonizers (present in the substrate or introduced without intention at spawning) that might potentially utilize the same space and nutrients.

The use of non conventional substrates for shiitake cultivation is promising for the treatment of agricultural by-products but it implies the preparation of a special spawn that assures an adequate mycelial development on the substrate (Donoghue *et al*., 1996). In sterilized substrates, there is a risk of contamination at spawning by mould spores or bacteria present in the environment. Generally, competition between shiitake and antagonistic organisms occurs during the first days after spawning. The use of efficient shiitake strains selected specifically for their ability to colonize a non-conventional substrate and the use a vigorous mycelium as spawn could be decisive for substrate colonization and the success of the cultivation. The components used for spawn making play very important roles in mycelium vigor and can reduce the incidence of antagonistic organisms affecting shiitake cultivation (Mata *et al*., 1998, 2002; Ohmasa and Cheong, 1999; Savoie *et al*., 2000).

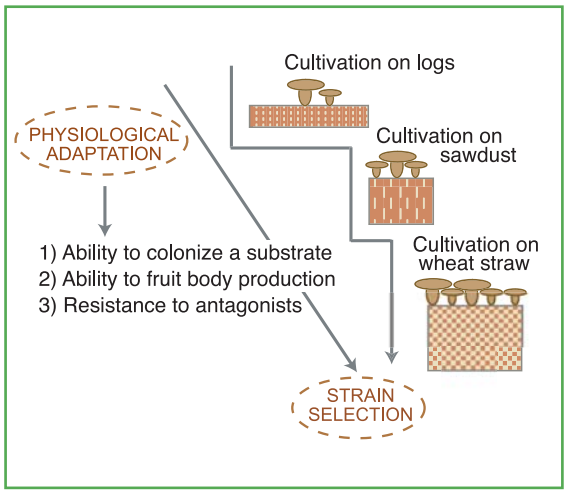

Figure 1. Factors to consider in shiitake strain selection

When alternative substrates are used for shiitake cultivation, strains must be selected carefully and their abilities have to be improved by using supplemented spawn (Fig. 1). Information is given to reach these goals through the cases of the development of shiitake cultivation in pasteurized cereal straw or coffee pulp.

Strain selection

Strain selection is one of the most important selections in mushroom cultivation. Different strains should be chosen according to other growing parameters and market demand pattern. For example, farmers grow high-temp. strain and low-temp.

strain in turn where they have several seasons. If they have specific mould problems with high density cultivation in some areas, the farmers can use specific strains that resist the pathogen. When stipe consumption increases in the market, farmers will change to a strain that produces mushrooms with long and thick stipes. And when consuming preference shifts from dark to bright, bright-color strain can be chosen.

The capacity of a mushroom to grow on a lignocellulosic substrate is related to the vigor of its mycelium, as well as to its capacity to activate physiological mechanisms necessary to adequately exploit the medium (Buswell *et al*., 1993). This capacity is defined as its competitive saprophytic ability. This capacity is dictated by the characteristic genetic make-up of each strain and is substrate specific. Competitive saprophytic ability depends on 1) the ability to grow rapidly when stimulated by soluble nutrients and to use an appropriate enzyme system for degradation of resistant constituents, and 2) the ability to produce fungistatic and bacteriostatic compounds and to tolerate fungistatic compounds (Shearer, 1995).

1) *Lentinula edodes* is a white rot fungus that is found in the wild on woods. For its cultivation on other lignocellulosic substrates, it is necessary to select strains well adapted to this alternative substrate. Shiitake generally grows slowly by producing dense mycelia and it utilizes both available nutrients and polysaccharides bounds to lignin thanks to its ability to produce extracellular enzymes useful for degrading lignocellulosic materials: phenoloxidases and hydrolases (Savoie *et al*., 2000). However different shiitake strains show different abilities to grow, fructify and resist antagonistic moulds. By comparing the metabolic activities and the production of extracellular enzyme activities of different shiitake strains cultivated on sterilized wheat straw, we observed that the strains with the earlier production and higher yield were those able to hydrolyze and utilize straw cell wall components soon after inoculation and that developed high metabolic activities (Mata and Savoie, 1998).
   The number of strains in a collection adapted to a specific substrate could be relatively low. For their selection, also for the improvement by breeding, it is then necessary to have a large collection and to develop screening methods evaluating their ability for space and nutrient capture. For example, estimates of mycelial growth on malt and yeast extracts agar supplemented with water soluble lignin derivatives were proposed as indicator of a strain's potential for mycelial growth on substrates derived from wheat straw (Mata *et al*., 2001).

2) During competition between shiitake and moulds such as the *Trichoderma* genus or bacteria in non-sterilized or contaminated substrates, several mechanisms of chemical interference are involved. Abilities to produce and to resist various antibiotics, to limit the growth of potential antagonists and to resist the attack of antagonists that can be present in a specific cultivation substrate are another set of important traits of strains that can be selected. Confrontation experiments between mycelia or between mycelia and bacteria are often used on both agar and solid media for evaluating their relative competitive ability. Several emergence patterns of non-assimilative mycelia including pigmented barrages have been identified during such confrontations and contribute to the defense against the antagonist (Rayner *et al*., 1994; Savoie *et al*., 2001). Visual observations of such reactions can easily be used to evaluate this component of the competitive saprophytic ability of shiitake strains (Figs. 2). Overgrowing and cell lyses can also be observed during interactions (Tsuneda and Thorn, 1994). Alternatively, the production of specific compounds of the defense reaction such as laccases can be assayed when laboratory facilities are available (Savoie *et al*., 1998).
   When strains with efficient competitive saprophytic abilities are selected for an alternative cultivation substrate, it is interesting to give them the better cultivation conditions allowing the expression of their potentialities. That can be reached by improving the spawn.

Figure 2. Confrontations of shiitake mycelium with Trichoderma moulds. Shiitake mycelium is blocking mould progression on wheat straw.

Improvement of the resistance to moulds by spawn adaptation

It was shown that the competitive ability of shiitake strains in wheat straw could be improved during the first days after spawning by modifying the composition of components used for spawn production, and consequently the incidence of moulds was reduced by 75% (Mata *et al*., 1998; Savoie *et al*., 2000). In these experiments, the preparation of a grain spawn supplemented with components rich in lignin and phenols has allowed a considerable reduction of contamination during the first growth stages by comparison to non supplemented grain spawn. By pre-adaptation of mycelium to the degradation of the lignocellulosic substrate in which the cultivation of shiitake was performed, the supplements acted as inducers of the defense systems. Actually most of shiitake strains are able to produce emergent hyphae and a dark brown line in the contact zone with moulds in order to block moulds progression (Figs. 2), but due to the delay for developing this defense, large area of the substrate is colonized by the antagonists before being stopped in their growth by this barrage.

The barrage is the result of an over production of phenoloxidases (laccases) and this over expression is also induced by lignin derivatives and various phenolic compounds. Consequently due to the presence of such compounds in the spawn, the reaction time is decreased when the mycelia face moulds in the cultivation substrate. In addition its overall competitive ability is improved because it is also adapted for the degradation of lignified components of the cultivation substrate.

Some strains of shiitake are able to grow on artificial media added with crude extracts of cultures of *Trichoderma harzianum*. Some commercial preparations are partially purified extracts of *Trichoderma*, usually used for their properties of plant and fungal cell wall lyses (for instance, Lysing Enzyme from Sigma®). This ability of shiitake mycelium pre-adapts the mushroom to *Trichoderma* moulds antagonism. During this pre-adaptation the growth rate is decreased and laccases are induced as above, but rapidly the mycelium reaches again its initial growth rate. When such mycelium is confronted by *Trichoderma* mycelium the lag time for the production of the barrage was decreased by one third and the production of spores was reduced by comparison with non pre-adapted mycelium (Mata *et al*., 2001; Savoie and Mata, 2003). Improvement in the resistance to antagonists by introduction of some of their metabolites to the culture medium is a very efficient method by which to select strains with a high level of resistance.

Production and Use of Supplemented Grain Spawn

The use of different spawn formulas has demonstrated that the strains have different adaptive possibilities to grow on the components of supplemented spawn. These differences could be limitative to select strains for supplemented spawn production.

However, strains having these abilities are very important genetic resources that open the possibility to use wheat straw and other agricultural by-products to shiitake cultivation. Based on the knowledge of the competitive abilities of shiitake strains, it is possible to propose means for giving an advantage to the mushroom, during spawn preparation, and decreasing the impact of *Trichoderma* spp. when shiitake is cultivated on wheat straw: 1) addition of raw material rich in phenolic compounds with high adsorption capacity, 2) increase of the number of inoculum points per volume of substrate by using spawn supports with a low granulometry, 3) increase of the vigor of mycelium by strain selection and pre-adapting the mycelium to the degradation of lignocellulosics.

Figure 3. Some different grains commonly used in shiitake spawn preparation **A**: Millet **B**: Oat **C**: Wheat **D**: Sorghum

Grain spawn can be prepared using sorghum (*Sorghum vulgare* Pers.) or millet (*Panicum milliaceum* L.) seeds. Other grains could be also used but it is very important to consider seeds size (Figs. 3).

Supplements with high capacity of water retention and containing phenolic compounds as peat moss and coffee pulp can be used. Some formulations of shiitake spawn have been tested with very good results (Del Pino *et al*., 2002; Mata *et al*., 2002) (Table 1).

Table 1. Some formulations showed very good mycelial growth (%)

	Sorghum seeds	Millet seeds	Peat moss	Gypsum	Coffee pulp powder	Wheat bran	Wheat straw powder
Formula 1	88.5		1.3	1.3	8.8		
Formula 2		88.5	1.3	1.3	8.8		
Formula 3	88.5		1.3	1.3		8.8	
Formula 4		88.5	1.3	1.3		8.8	
Formula 5	88.5		1.3	1.3			8.8
Formula 6		88.5	1.3	1.3			8.8

Seeds must be hydrated separately by soaking them at least 12 hours. After that they must be drained off excess moisture with a domestic centrifuge and then the remaining ingredients added. The mixture must be adjusted to a moisture content of 75 % (Figs. 4).

Figure 4. Supplemented shiitake spawn preparation **A**: Washing seeds **B**: Centrifugation of seeds **C**: Filling the plastic bags for sterilization

Prepared formulas can be placed in plastic bags and sterilized in autoclave for 90 minutes at 121℃. After cooling the bags containing the mixture of the formula are inoculated with mycelium cultivated for 15 days on artificial culture medium like malt extract agar (Figs. 5).

The incubation must be carried out at 25℃ in darkness. In order to obtain a good spawn the bags must be shaken carefully after two weeks of incubation in order to distribute the growing hyphae throughout the mixture and encourages a rapid growth. A good stage of maturation of shiitake spawn is obtained after 3-5 weeks of incubation (Figs. 6).

Figure 5. Supplemented shiitake spawn preparation **A**: Plastic bags with the prepared formula ready for sterilization **B**: Transferring a square of mycelium from the artificial medium into sterilized formula of supplemented spawn **C**: Mycelial growth during spawn incubation **D**: Mycelial growth on millet supplemented spawn.

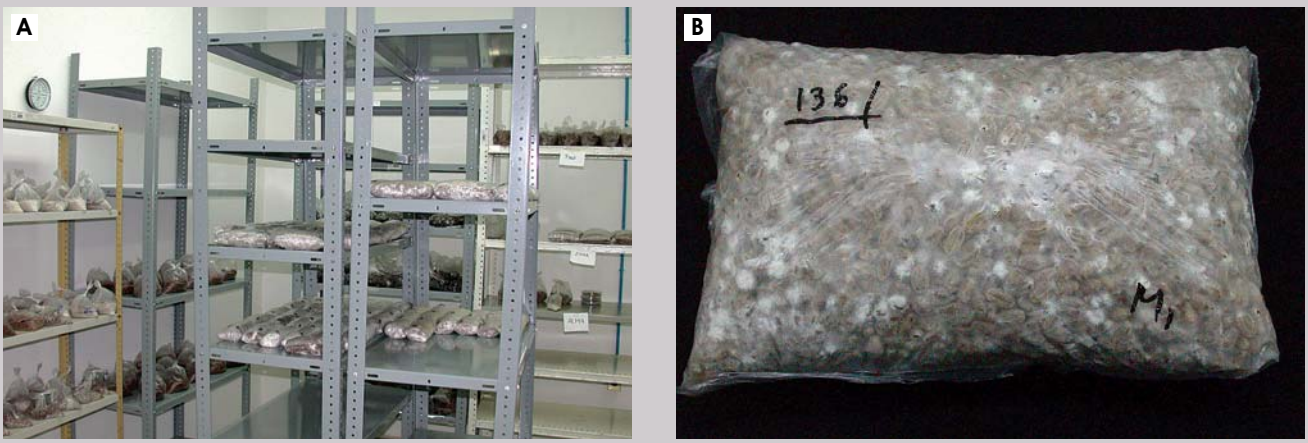

Figure 6. Supplemented shiitake spawn **A**: Incubation room with a shelf system **B**: Supplemented spawn after 3 weeks of incubation

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