Interactions of Heterologous Mycelia Colonized in the Substrate Govern Fruit Body Production in the Cultivated Homobasidiomycete Pholiota nameko

Katsuhiko BABASAKI,¹ Kazuhiko MASUNO,² and Hitoshi MURATA¹,†

¹Department of Applied Microbiology and Mushroom Sciences, Forestry & Forest Products Research Institute, Tsukuba, Ibaraki 305-8687, Japan
²Department of Mushroom Science, Nagano Forest Research Center, Shiojiri Kataoka, Nagano 399-0711, Japan

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The spawn of cultivated mushrooms are generally produced, propagated, and distributed to growers as a mycelial culture without genetic purification, in which phenotypic variants frequently occur. We investigated how heterologous mycelia present in a spawn influence fruit body production in the cultivated basidiomycete Pholiota nameko. The ‘di-mon’ dual cultivation of protoplast clones produced mosaic fruit bodies, which could result from the ‘di-mon’ mating. In the ‘di-di’ dual cultivation of heterologous strains with different fruiting times, authentic fruit bodies of each dikaryon and chimera showing a feature combining characteristics of the two dikaryons emerged simultaneously. Mycelia isolated from the chimera produced all three types of fruit bodies, indicating unlikeliness of the occurrence of anastomosis. These results suggest that mycelia colonized in the substrate interact with each other and coordinately promote fruit body production in P. nameko. This phenomenon masks a clonal variability that may be surfaced through multiplication and distribution of the spawn, occasionally bringing about abnormal fruiting.

Key words: basidiomycetes; cell-to-cell communication; fruit body production; Pholiota nameko

Pholiota nameko is a wood-decaying homobasidiomycete that produces an economically important edible mushroom, ‘nameko’¹,³) The fungus is heterothallic and bipolar; its sexual compatibility is controlled by a pair of alleles, Aa on a chromosomal locus.¹) Unlike Agaricus bisporus and Lentinula edodes, which produce fruit bodies exclusively from dikaryons, P. nameko produces fruit bodies not only from dikaryotic mycelia but also monokaryotic ones that are frequently generated through conidia in the secondary asexual life cycle.¹)

The production of ‘nameko’ has been much improved in Japan by introducing a cultivation system with a substrate composed of hardwood sawdust and nutrient supplements such as wheat bran in environment-controlled facilities.¹,³) In such a cultivation system (= sawdust cultivation), the sexual growth stage is induced at 15°C and humidity of 95% after the 60-day vegetative growth stage at 20°C and humidity of 70%, in which mature fruit bodies develop about 20 days after the induction, requiring a total production cycle of some 80 days.¹,³) Therefore, the sawdust cultivation permits growers to harvest an expected amount of mushrooms daily as planned throughout a year, which is more efficient than with a conventional cultivation system with bed logs of hard woods in the field (= log cultivation) that allows growers to harvest a variable amount of crops only during the late autumn.¹,³)

Recently, however, ‘nameko’-growers are facing serious problems of poor cropping and of the occurrence of abnormal fruit bodies in the sawdust cultivation. Most poorly cropping spawns were, however, found to be capable of producing fruit bodies after a prolonged sexual growth stage of variable length, suggesting that the poor cropping phenomenon may be due to some physiological events in the colonized substrate rather than infectious diseases. Our preliminary analysis showed that the cytoplasmically homogeneous spawn are in fact composed of genetically heterologous mycelia.

It has long been questioned whether fruit bodies are produced by mycelia originated from a single fungal cell or by those of multicellular origins in homobasidiomycetes.⁴–¹¹) Recent RFLP-based analysis in Armillaria gallica and L. edodes provided some evidence that fruit body formation could occur involving genetically heterologous mycelia.¹²,¹³) Despite these progresses, it still remains uncertain how heterologous mycelia in the colonized substrate affect fruit body production in cultivated mushrooms.

In this study, we demonstrate that the interactions of heterologous mycelia colonized in the substrate strongly affect fruit body production. The observations raise an intriguing hypothesis that intermycelial
Communications, like the cell-to-cell communications observable in prokaryotes, may be involved in the physiology of fruit body differentiation in the homobasidiomycete *Pholiota nameko*.14,15)

**Materials and Methods**

*P. nameko strains.* See Table 1 for the description of *P. nameko* strains used in this study. All these fungal strains were incompatible in asexual interaction in that any combinations of strains grown side-by-side on potato dextrose agar plate (PDA, Difco, Detroit, MI) produce a borderline where both mycelia meet, limiting the further expansion of the fungal colonies. Pn2 produces white fruit bodies with no detectable spores, a phenotype that is apparently conferred by a recessive gene on the locus where the dominant allele confers brown fruit bodies with spores (M. Taniguchi, Forestry & Forest Products Research Institute, personal communication). All the *P. nameko* strains commercially available for the sawdust cultivation used in this study are siblings of the wild-type strain Pn1 as evidenced by their identical mtDNA-RFLP profiles.16) Like poor cropping-dikaryons, all the log cultivation strains and monokaryons of the sawdust cultivation strains used in this study require an unusually long and variable sexual period of > 65 days for the development of mature fruit bodies, and are inconsistent in the yield of the crop.

**Cultivation of** *P. nameko.* A substrate weighing 600 g was prepared for cultivation of *P. nameko* by the mixture of beech wood sawdust and wheat bran at the ratio of 5:1 (v/v) with water content of 65%, and packed into 800-ml polypropylene bottles (Chikuma Kasei Inc., Nagano, Japan). The substrate was then sterilized by being autoclaved at 121°C for 1 h, cooled to room temperature, and inoculated with the fungal mycelia (5 mm²/piece) cultured on PDA.1,3) *P. nameko* was grown in the substrate at 20°C under humidity of 70% for 60 days to allow colonization.1,3) After 60 days of the vegetative growth stage, the colonized substrates were placed in an environment laboratory unit (Sanyo Electric Inc., Osaka, Japan) at 15°C and humidity of 95% to induce the fruit body production, i.e., the sexual growth stage.1,3) At least three substrates were used for each test plot and the means are given.

**Dual cultivation.** ‘Di-mon’ or ‘di-di’ dual cultivation was done by co-inoculation of the substrate with a monokaryon and a dikaryon, or two heterologous dikaryons, respectively, that had been separately grown on PDA, at an appropriate ratio (Fig. 1). For example, a spawn composed of a monokaryon and a dikaryon at the ratio of 1:1 was prepared by inoculation of one half of the substrate with a monokaryon and the other half with a dikaryon (Fig. 1). Unless stated otherwise, the characteristics of *P. nameko*

### Table 1. *Pholiota nameko* Strains Used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description (Reference)</th>
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<tr>
<td>Pn1</td>
<td>Wild-type strain FMC272 carrying mtDNA type A, isolated in Fukushima, Japan (This study)</td>
</tr>
<tr>
<td>Pn2</td>
<td>Dikaryotic protoplast clone of the albino strain FMC279 carrying mtDNA type A. A recessive gene for brown fruit bodies seem to control albino fruit bodies in <em>P. nameko</em> (This study)</td>
</tr>
<tr>
<td>Pn3</td>
<td>Commercial strain KN248 carrying mtDNA type A, for sawdust cultivation (Kawamura Edible Mushroom Institute, Yamagata, Japan)</td>
</tr>
<tr>
<td>Pn4</td>
<td>Commercial strain KN231 carrying mtDNA type A, for sawdust cultivation (Kawamura Edible Mushroom Institute)</td>
</tr>
<tr>
<td>Pn5</td>
<td>Dikaryotic protoplast clone of Pn3, which produces mature fruit bodies (This study)</td>
</tr>
<tr>
<td>Pn6</td>
<td>Monokaryotic protoplast clone of Pn3, which produces abnormal dwarf fruit bodies with closed sporophores only after a long sexual stage (This study)</td>
</tr>
<tr>
<td>Pn7</td>
<td>Dikaryotic protoplast clone of Pn4, which produces mature fruit bodies (This study)</td>
</tr>
<tr>
<td>Pn8</td>
<td>Dikaryotic protoplast clone of Pn4, which produces abnormal dwarf fruit bodies with closed sporophores only after a long sexual stage (This study)</td>
</tr>
<tr>
<td>Pn9</td>
<td>Commercial strain KN232 carrying mtDNA type A, for sawdust cultivation (Kawamura Edible Mushroom Institute)</td>
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<tr>
<td>Pn10</td>
<td>Commercial strain N128 carrying mtDNA type A, for sawdust cultivation (KinoKkus, Miyagi, Japan)</td>
</tr>
<tr>
<td>Pn11</td>
<td>Commercial strain N160 carrying mtDNA type A, for sawdust cultivation (Hokken, Tochigi, Japan)</td>
</tr>
<tr>
<td>Pn12</td>
<td>Dikaryotic protoplast clone of Pn12, which produces mature fruit bodies (This study)</td>
</tr>
<tr>
<td>Pn13</td>
<td>Commercial strain KN325 carrying mtDNA type B for sawdust cultivation (Kawamura Edible Mushroom Institute, Yamagata, Japan)</td>
</tr>
<tr>
<td>Pn14</td>
<td>Dikaryotic protoplast clone of Pn12, which produces mature fruit bodies only after a long sexual stage (This study)</td>
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<tr>
<td>Pn15</td>
<td>Commercial strain KN17 carrying mtDNA type A (Hokken)</td>
</tr>
<tr>
<td>Pn16</td>
<td>Commercial strain KM1 carrying mtDNA type A, for sawdust cultivation (Kagawa Shiitake, Miyagi, Japan)</td>
</tr>
<tr>
<td>Pn17</td>
<td>Commercial strain KM2 carrying mtDNA type A for sawdust cultivation. Produces sherical stipes (Kagawa Shiitake)</td>
</tr>
<tr>
<td>Pn18</td>
<td>Commercial strain carrying mtDNA type A, for sawdust cultivation (Hokuto, Nagano, Japan)</td>
</tr>
<tr>
<td>Pn19</td>
<td>Laboratory strain carrying mtDNA type A, for sawdust cultivation (This study)</td>
</tr>
<tr>
<td>Pn20</td>
<td>Laboratory strain carrying mtDNA type A, for sawdust cultivation (This study)</td>
</tr>
<tr>
<td>Pn21</td>
<td>Commercial strain Kinko Bansei carrying mtDNA type I, for log cultivation. This strain produces few fruit bodies in sawdust cultivation (Kino Shiitake, Tottori, Japan)</td>
</tr>
<tr>
<td>Pn22</td>
<td>Commercial strain N325 carrying mtDNA type B, for log cultivation. This strain produces few fruit bodies in sawdust cultivation (Hokken)</td>
</tr>
<tr>
<td>Pn23</td>
<td>Commercial strain N572 carrying mtDNA type B, for log cultivation. This strain produces few fruit bodies in sawdust cultivation (Hokken)</td>
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were recorded in terms of the timing of maturation of fruit bodies (>2 cm tall) that filled the open area of the substrate, and by the shape and the color of the fruit bodies.

**Results and Discussion**

**Effects of monokaryotic mycelia on the fruit body production**

In our preliminary experiments, all the monokaryotic protoplast clones of *P. nameko* strains Pn3 and Pn4 showed the poor cropping phenomenon, in which abnormal dwarf fruit bodies with closed sporophores as well as normal ones with open sporophores were produced only after the prolonged sexual period. When crossed with a tester monokaryon isolated from spores, such monokaryons conferred dikaryons with large variations in the timing of maturation of fruit bodies and the yield of the crop (= fresh weight). Therefore, we questioned how such monokaryons, which are apparently a degenerative factor, would affect fruit body production in dikaryons.

To answer this question, ‘di-mon’ dual cultivation was done between Pn9 and Pn8, the former that is a monokaryotic protoplast clone and the latter a dikaryotic protoplast clone, respectively, of Pn4.\(^{5,7}\) The substrate was inoculated with mycelia of Pn9 and Pn8 at the ratio of 5:0, 3:2, 2:3, and 0:5, and the resulting substrates were incubated for fruit body production (Fig. 2(A)). No sign of basidiome formation was noted in the substrate colonized solely by mycelia of Pn9, but within the 19-day sexual stage, mature fruit bodies uniformly developed in the specimens of Pn9:Pn8 [3:2] and of Pn9:Pn8 [2:3] as in the sample exclusively with mycelia of Pn8 (Fig. 2(A)). In addition, the yield of the crop (= fresh weight) in the ‘di-mon’ dual cultivation was nearly equal (>85%) to that in the single cultivation of Pn8. The same results were scored in the experiment with protoplast clones of Pn3, *i.e.*, Pn7 and Pn5.

To confirm that the effects of dual cultivation bringing about uniform fruit body production resulted from the ‘di-mon’ mating per se and was not due to the suppression of growth of a monokaryon by an overgrown dikaryon, the substrate was co-inoculated with Pn2, which is a dikaryotic albino strain, and Pn9 at the ratio of 1:1, and cultivated. The dual cultivation resulted in the production of the mosaic fruit bodies (Fig. 2(B)). We presumed that these fruit bodies involved mycelia of Pn2 and Pn9, and those resulting from the ‘di-mon’ mating.\(^{7}\) The same results were also scored in the experiment with Pn2 and Pn7.

To explore the possible positive effects of the monokaryon on fruit body production, the ‘di-mon’ dual cultivation was conducted between the monokaryon Pn9, or Pn7, and the dikaryon Pn21, Pn22, or Pn23. The dikaryotic partners were desirable for the log cultivation and did not form any basidiomes in the sawdust cultivation under the assay condition we used. When the substrate was inoculated with a
monokaryon and a dikaryon at the ratio of 1:1 and cultivated, mature fruit bodies were uniformly formed within the 38-day sexual period (Fig. 2(C)). This result, which was consistently scored with any combinations of fungal strains tested, suggests that the presence of monokaryotic mycelia in the dikaryotic ones is not necessarily harmful to the fruiting. In fact, the ‘di-mon’ dual cultivation with either a monokaryon or a dikaryon carrying the potential for expressing better qualities may be useful in improving the fruit body production in *P. nameko*.

**Interactions among dikaryotic mycelia for fruit body production**

The effect of genetically heterologous dikaryons colonized in the same substrate on the fruit body production in *P. nameko* was examined. We did the dual cultivation by co-cultivating in the same substrate at the inoculum ratio of 1:1 two dikaryotic strains carrying the potential for expressing two distinct features in fruit body production. When Pn2 and Pn5 were co-cultivated, the former that produces white fruit bodies after the 36-day sexual stage and the latter brown ones after the 15-day period, two remarkable phenomena were recognized in the morphology of fruit bodies and in the timing of maturation of fruit bodies (Fig. 3). The dual cultivation allowed chimera fruit bodies carrying both white and brown mycelia to develop at the borderline of two fungal colonies, but the authentic fruit body of each strain formed within its own colony (Fig. 4(A)). The morphology of chimera fruit bodies was of the characteristics of two strains combined. In addition, all the fruit bodies including chimera and surrounding non-chimera developed at the same time as if they were from a single fungal strain (Fig. 4(A)). The timing of the uniform formation of mature fruit bodies in the dual cultivation was different from that of in-
Fig. 5. Fruit Body Production by Mycelia Isolated from Brown Portions of a Chimera Fruit Body.

The picture shows nine mycelial samples isolated from a representative chimera fruit body resulting from the dual cultivation of the albino strain Pn2 and the brown strain Pn5 after the 30-day sexual period.

dividual strains in the single cultivation (Fig. 3).

To test whether the phenomenon observed in the dual cultivation of Pn2 and Pn5 holds true widely with P. nameko strains, various combinations of dikaryotic P. nameko strains were used in the similar experiment (Fig. 3). Of those, fruit body production in Pn2 was much stimulated in the presence of various sawdust cultivation strains that produce brown fruit bodies, such as Pn11, Pn15 and Pn18 (Fig. 3). Similarly, wild-type and log cultivation dikaryons, such as Pn1 and Pn23, efficiently developed mature fruit bodies in the substrate by the dual cultivation with Pn5 (Fig. 3, Fig. 4(B)). In contrast, the dual cultivation with the combination of Pn5 and Pn10, Pn10 and Pn11, Pn16 and Pn17, or Pn19 and Pn20, adversely affected fruit body production, in which the timing of mature fruit body formation was delayed as compared with samples independently cultivated (Fig. 3). In all cases, a borderline that separated two heterologous fungal colonies appeared in the substrate, and three types of fruit bodies, i.e., two authentic ones and one chimera, occurred simultaneously.

It is interesting to note that Pn14, a poor cropping dikaryotic protoplast clone from the sawdust cultivation strain Pn12, produced mature fruit bodies normally upon co-cultivation with Pn13, another dikaryotic protoplast clone of Pn12 (Fig. 3, Fig. 4(C)). The phenomenon may underlie the mechanisms of poor cropping occasionally encountered in 'nameko' production. A degenerative clone may behave normal in the presence of a clone that produces normal fruit bodies. Occasionally, however, the degenerative clones may be accumulat-ed through generations of selections in the spawn-to-substrate transfer and eventually express its own degenerative features on fruit body production. In other words, the potential for the occurrence of poor cropping due to the presence of degenerative clones may be overlooked by the coordinated behavior of heterologous mycelia colonized in the substrate.

**Fruit body production by mycelia of chimera fruit bodies**

To test how mycelia of chimera fruit bodies behave as inocula for the subsequent sawdust cultivation, ten mycelial samples were isolated from apparently brown portions of chimera fruit bodies, and each sample was individually cultured on PDA. The samples included fruit bodies resulting from the dual cultivation with the combination of Pn2 and either Pn5, Pn11, Pn15 or Pn18 (Fig. 3). Individual mycelial samples from the agar plates were inoculated separately in 800-ml bottles containing the substrate and cultivated, from which all three kinds of fruit bodies, brown, white, and chimera, were developed (Fig. 5). The timing of mature fruit body formation after the induction of the sexual growth stage, the profile of color and shape of fruit bodies, as well as the yield (= fresh weight) and the ratio of the three kinds of fruit bodies varied among mycelial samples of the same sporophores (Fig. 5). The observation suggests that the chimera fruit bodies may be produced by the aggregation, rather than the anastomosis, of heterologous mycelia, and the clonal composition of inocula randomly selected reflects variation and inconsistency in fruit body production in the P. nameko spawns.

**Conclusions**

In searching for the cause of abnormal fruit body formation occasionally encountered in the commercial production of 'nameko' mushrooms, we discovered that the heterologous mycelia present in the spawn may not only segregate into phenotypic variants but also affect the fruiting behaviors through mycelial interactions. Interestingly, the dual cultivation of any combinations of two phenotypically distinct dikaryotic strains attempted in this study conferred simultaneous production of fruit bodies of respective strains and their intermediate. The phenomena to be regarded as "intermycelial communications" not necessarily accompanying the sexual interaction affect the development of mature fruit bodies profoundly.

Several secondary metabolites have been reported to affect the fungal behaviors through intermycelial interactions. Some peptides and lectins have been reported as factors involved in aggregation of mycelia in some cultivated mushroom species.17–20 Involvement of hydrophobins has been emphasized as the
extracellular protein factor for fruit body production in homobasidiomycetes. However, our observation in *P. nameko* may not be fully explained by aggregation factors or hydrophobins.

Recently, farnesol was identified as an extracellular quorum sensing molecule from the dimorphic ascomycete *Candida albicans*. This compound prevents the yeast-to-mycelium conversion and facilitates the yeast-phase growth in *C. albicans*. Quorum sensing in *P. nameko* has been documented better than in eukaryotes. Quorum sensing is the regulation of gene expression through extracellular signal molecules called autoinducers produced by the microorganisms. The chemical properties of such autoinducers depend on the organism, such as homoserine lactons in gram-negative bacteria, secreted peptides in gram-positive bacteria and alcohol in *C. albicans*. Its concentration in the growth environment is critically important for the function of autoinducers, so activation of this regulatory system requires high-population-density of the microbial cells. In fact, quorum sensing molecules have been regarded as regulators that govern the expression of an array of physiological events leading individual cells to express cooperatively the same biological functions. Such biological functions include pathogenicity, stress response to survive in a detrimental environment, and the production of antibiotics, depolymerizing enzymes, or other secondary metabolites. Of those, the quorum-sensing system of *Myxococcus xanthus* looks relevant to our present observation, for it is involved in fruit body formation.

Our observation may be suggestive of the presence of a quorum-sensing system in basidiomycetes, since fruit body production occurs coordinately in the substrate colonized by heterologous dikaryotic mycelia. The occurrence of chimera fruit bodies may also support this notion. It may be plausible that the quantity and quality of signal molecules secreted from the mycelia in the colonized substrate affect fruit body formation. Biochemical nature of the signal molecules and molecular genetics involved need to be clarified to prove the quorum sensing system in homobasidiomycetes. This line of analysis will provide us with insights into molecular mechanisms involved in the mushroom production in basidiomycetes, and help us understand the cause of poor cropping and abnormal fruit bodies in cultivated mushrooms. It may also give us a clue to open the way to cultural production of so far uncultivable ectomycorrhizal mushrooms.

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**References**


