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NOTE

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Estimation of viability of inner bark tissue of *Quercus serrata*, a substrate for log cultivation of *Lentinula edodes*, using the TTC assay method

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Abstract In the log cultivation of Shiitake (*Lentinula edodes*), early colonization of this fungus is extremely retarded in living wood tissues, in particular in inner bark tissues. To estimate the viability of inner bark tissues of *Quercus serrata*, a substrate for log cultivation of Shiitake, we employed a colorimetric assay utilizing a tetrazolium salt (2,3,5-triphenyltetrazolium chloride, TTC) and investigated the relationships between degree of decrease in viability and increase in growth of *L. edodes* in the tissues. When the mixtures of different proportions of living and dead tissues were assayed, formazan production was proportional to the percentage of living tissues. When logs dried for various time periods were inoculated with *L. edodes*, the fungus grew more extensively in tissues with reduced formazan production. These results indicate that the TTC assay is a useful method for estimation of viability and thus can be used to decide the proper timing for inoculation of *L. edodes*.

Key words *Lentinula edodes* · Log cultivation · Mycelial growth · *Quercus serrata* · TTC assay · Wood tissue viability

Introduction

Shiitake, *Lentinula edodes* (Berk.) Pegler, is an edible mushroom cultivated widely throughout Japan on logs of *Quercus serrata* Thunb. or other fagaceous wood as substrate. For the development of log cultivation techniques of Shiitake, it is important to accumulate knowledge on the colonization mechanisms of *L. edodes* in wood substrates. Komatsu et al. (1980) and Kishimoto et al. (1984) reported that mycelial growth of *L. edoes* was extremely retarded in living wood tissues, in particular in the inner bark tissues, of *Q. serrata*, and indicated that speed of colonization of the fungus in the substrates was closely related to their viability.

The ability of viable cells to reduce various tetrazolium salts has been reported for a variety of organisms. Colorimetric assays with tetrazolium salts such as 2,3,5-triphenyltetrazolium chloride (TTC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) have been shown to be effective for estimation of cell viability (Steponkus and Lanphear 1967; Towill and Mazur 1975; Monsann 1983). A previous work reported that the viability of the inner bark tissues of *Q. serrata* could be estimated by the coloration of the tissues caused by TTC (Komatsu et al. 1980). However, clear relationships between degree of decrease in viability of living tissues and increase in growth of *L. edodes* have not been investigated. In this study, we employed a simple and rapid colorimetric assay of TTC for measuring the degrees of viability of inner bark tissues of *Q. serrata* and confirmed that reduction of the viability of the tissues markedly increased early colonization of this fungus in the logs.

Materials and methods

Fungal strain and culture conditions

A dikaryotic strain of *L. edodes* (TMIC-800; Tottori Mycological Institute, Tottori, Japan) was grown on sawdust medium prepared with 400 g air-dried hardwood sawdust and 80 g rice bran wetted with 1 l tap water (Murakami et al. 1987; Hasebe et al. 1991) at 23°C for 40 days. This fungal culture was used as sawdust spawn.

TTC assay

A 23-year-old *Q. serrata* was felled in December, and logs 100 cm in length and 10–12 cm in diameter were taken from

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the trunk. Inner bark tissues, which were carefully removed and torn into small fragments about 1 mm in width and 20 mm in length, were used for the TTC assay. TTC (Wako) was dissolved in pH 8.5 potassium phosphate buffer (50 mM) to give a concentration of 2%. Then, 3 ml of TTC solution was added to the tissues (0.3 g) in assay tubes (11 × 90 mm) and incubated without shaking for 18–20 h at 30°C. After incubation, the tissues were rinsed briefly with distilled water and transferred to another set of tubes (35 × 60 mm). To extract the red formazan, 30 ml 95% ethanol was added to each of the tubes, which were incubated for 2 h at 60°C. After filtration, absorbance of the extract was measured at 485 nm with a Hitachi U-1100 spectrophotometer.

Colonization test

Living logs of *Q. serrata* about 30 cm in length and 6–8 cm in diameter were air-dried for various periods at 30°C with 40% relative humidity. After drying, the logs were weighed and viability of their inner bark tissues was measured by TTC assay. After the measurements, the logs were soaked in water for 16 h. Then, five holes, each 12 mm in diameter and 25 mm in depth, were diagonally drilled in each of the logs. The sawdust spawn of *L. edodes* was inoculated into each hole, and the holes were then covered with styrofoam lids. Each of the inoculated logs was put into a polyethylene bag and incubated for 14 or 28 days at 20°C in the dark. After incubation, inner bark tissues were carefully removed from the logs, and linear growth of the fungal colony was assessed by measuring the distance from the inoculated hole to an advancing edge of the mycelia with a light microscope.

Results and discussion

Determination of protocols for TTC assay

The absorption spectrum of the red formazan shows a maximum at 485 nm. Therefore, all absorbances reported here were measured at this wavelength. Figure 1 shows the effects of the pH, TTC concentration, temperature, and incubation time in the reaction buffer on the amount of formazan produced by inner bark tissues. In tissues incubated at 20°C, activity of formazan production was sensitive to H ion concentrations below pH 8.0 where formazan production decreased as pH decreased (Fig. 1A). However, in tissues incubated at 30°C a significant difference in formazan production was not detected. The production of formazan at 20°C and at 30°C increased with increasing concentration of TTC in the range from 0% to 2% and became maximum around 2% at both temperatures (Fig. 1B). Decrease in color production was observed with increasing concentration of TTC above the optimum. Figure 1C demonstrates that optimum temperature for the production of formazan is around 30°C. The time course of formazan production is shown in Fig. 1D. Because the plateau began at 12–16 h incubation at 30°C, incubation period was determined for 18–20 h. Standard incubation procedures selected as optimum are incubation without shaking at 30°C for 18–20 h in reaction buffer containing 2% TTC at pH 8.5.

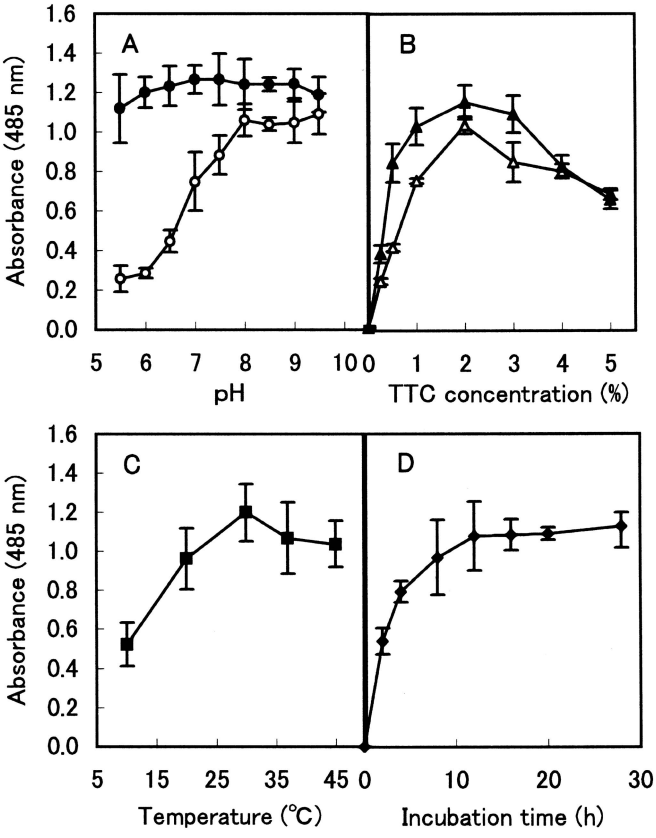

Fig. 1. Effects of pH (**A**), 2,3,5-triphenyltetrazolium chloride (TTC) concentration (**B**), temperature (**C**), and incubation time (**D**) in reaction buffer on amount of formazan produced by inner bark tissues of *Quercus serrata*. **A** Inner bark tissues were incubated in reaction buffer at various pH containing 2% TTC at 20°C (○) or 30°C (●) for 20 h. **B** Inner bark tissues were incubated in pH 8.5 reaction buffer containing various concentrations of TTC at 20°C (△) or 30°C (▲) for 20 h. **C** Inner bark tissues were incubated in pH 8.5 reaction buffer containing 2% TTC at various temperatures for 20 h (■). **D** Inner bark tissues were incubated in pH 8.5 reaction buffer containing 2% TTC at 30°C for indicated time (◆). Data show means and standard deviations of three replications

Figure 2 shows formazan production resulting from mixing different proportions of living and dead tissues. Absorbance was completely dependent on the percentage of living tissues. This result indicates that formazan production is proportional to the amount of viable tissue.

Relationship between viability of inner bark tissues and fungal colonization

Mycelial growth of *L. edodes* was extremely retarded in tissues of logs that had not been dried, and brownish pigmented lines occurred at the periphery of the limited fungal colonies (Fig. 3). Figure 4 shows the relationships between

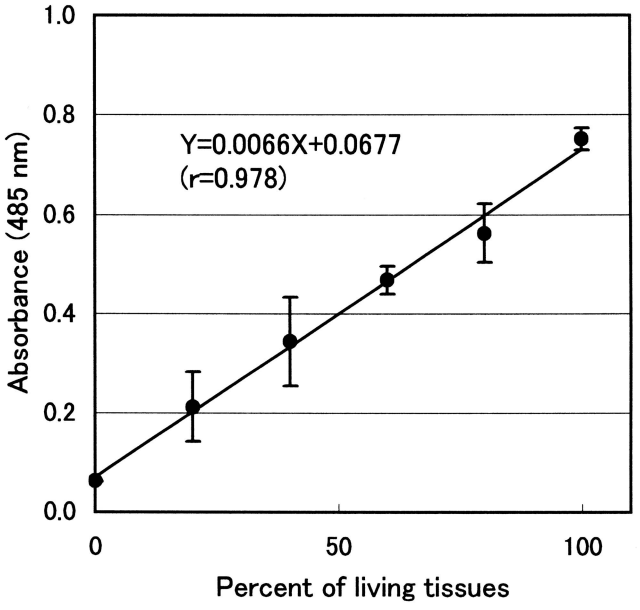

Fig. 2. Formazan produced by mixtures of living and dead tissues of inner bark of *Quercus serrata*. Different proportions of living and dead tissues were obtained by mixing tissues soaked in water at 20°C and those at 100°C for 15 min. Mixtures of living and dead tissues were incubated in pH 8.5 reaction buffer containing 2% TTC at 30°C for 20 h. Data show means and standard deviations of three replications

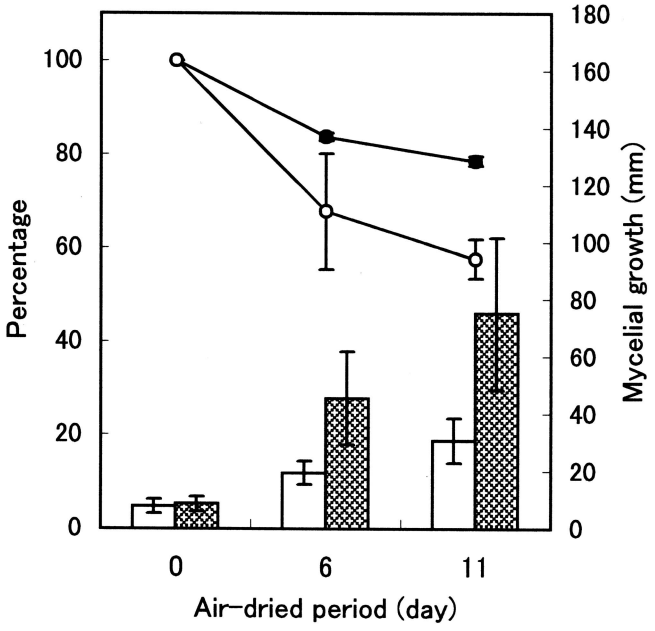

Fig. 4. Changes in formazan production by inner bark tissue of logs of *Quercus serrata* and mycelial growth rate of *Lentinula edodes* in the tissue following decrease in log weight by drying. Logs were air-dried for indicated days at 30°C with 40% relative humidity. After being air-dried, the logs were weighed and the viability of their inner bark tissue was measured by TTC assay. Relative weight of the logs (●) and viability of the inner bark tissue (○) were computed in comparison with the initial values. The logs were inoculated with sawdust spawn of *L. edodes* after being soaked in water for 16 h. Each of the inoculated logs was incubated at 20°C in the dark for 14 (*open columns*) and 28 (*shaded columns*) days; linear growth of the fungus was then measured under a light microscope. Data show means and standard deviations of three replications

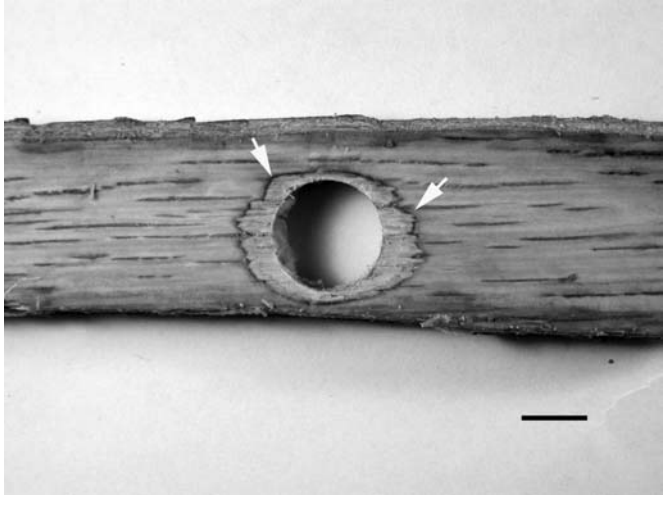

Fig. 3. Retarded colonization of *Lentinula edodes* in inner bark tissues of a living log of *Quercus serrata*. The log, which was taken from the *Q. serrata* trunk soon after it was felled, was inoculated with sawdust spawn of *L. edodes*, and then incubated for 28 days at 20°C in the dark. *Arrows* indicate brownish pigmented lines at the periphery of the limited fungal colony. *Bar* 10 mm

mycelial growth in the inner bark tissues of the logs and the amount of formazan produced by the tissues after drying the logs for various times. When the relative weight of the logs decreased to 84% and 79% by drying at 30°C for 6 and 11 days, respectively, the production of formazan by the tissues of these logs was reduced to 68% and 58% of the initial value, respectively; this result indicates that viability of the tissues decreases at these proportions. As incubation was prolonged, the fungus continued to grow without showing any visible signs of abnormal responses of interactions between the fungus and the tissues. Moreover, the fungus grew more rapidly in the tissues in which greater decrease in formazan production was recorded. These results suggest that the living wood tissues possess some defense mechanism against the fungal invasion, and that the mechanism may become defective after losing less than half the viability of the tissues.

Close correlation between tissue viability of *Q. serrata* and hyphal behavior of *L. edoes* has been reported. Previous studies demonstrated that deactivation of inner bark tissues caused by cutting down trees before the leaves had fallen (Komatsu et al. 1980) and by air-drying the logs before inoculation (Kishimoto et al. 1984) allowed vigorous colonization by the fungus. In contrast, ultrastructural study revealed that hyphal behavior of the fungus and patterns of wall degradation of the wood were strongly influenced by whether cambial and parenchyma cells were dead (Tsuneda 1988). Results of the present study indicate that considerable decrease in viability of the logs is one of the most important factors that determine how soon the fungus can establish itself in the wood.

For successful cultivation of Shiitake using living logs, it has been necessary to use various techniques that effectively inactivate the defense reaction of the living tissues against *L. edodes* invasion and promote rapid colonization of the fungus in the wood. The TTC assay is useful not only to develop these practical techniques but also to study hyphal behavior in the wood with relation to the viability of the wood tissue.

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References

Hasebe K, Murakami S, Tsuneda A (1991) Cytology and genetics of a sporeless mutant of *Lentinus edodes*. Mycologia 83:354–359

Kishimoto J, Honda K, Furukawa I, Sakuno T (1984) Relationship between mycelial growth of Shiitake (*Lentinus edodes*) and moisture condition of bed-logs (in Japanese with English summary). Bull Fac Agric Tottori Univ 36:43–53

Komatsu M, Nozaki Y, Inoue A, Miyauchi M (1980) Correlation between temporal changes in moisture contents of the wood after felling and mycelial growth of *Lentinus edodes* (Berk.) Sing (in Japanese with English summary). Rep Tottori Mycol Inst 18:169–187

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Murakami S, Hasebe K, Tsuneda A (1987) A mutant of *Lentinus edodes* forming aberrant clamp connections. Rep Tottori Mycol Inst 25:49–55

Steponkus PL, Lanphear FO (1967) Refinement of the triphenyl tetrazolium chloride method of determining cold injury. Plant Physiol 42:1423–1426

Towill LE, Mazur P (1975) Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. Can J Bot 53:1097–1102

Tsuneda A (1988) Tyloses in the vessels of *Quercus serrata* and incipient decay of the bedlogs by *Lentinus edodes*. Rep Tottori Mycol Inst 26:29–36