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Saprobic potential of *Tricholoma matsutake*: growth over pine bark treated with surfactants

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Abstract Saprotrophic growth of *Tricholoma matsutake* isolates was investigated over *Pinus densiflora* bark fragments either on soil or on agar media. Preferential colonization of pine bark fragments by hyphae, in glucose-deprived environments suggested that Matsutake was able to extract some nutrients to sustain its growth. This was confirmed in glucose-free liquid nutrient medium, where bark as sole carbon source significantly stimulated (up to two-fold) growth of *T. matsutake* isolates. The addition of surfactants (Tween 80 and Tween 40) in liquid medium further stimulated mycelium growth over pine bark by up to 55%. Such growth stimulation was associated with a sharp increase in protein and β-glucosidase excretion by hyphae in culture filtrates. As *T. matsutake* has some saprotrophic ability, the initiation and extension of Matsutake Shiro in forest soil might require simultaneously nutrients derived from the host plant and from soil organic compounds. Data reported here may contribute to the formulation of new culture substrates adapted to the co-culture of *T. matsutake* and its host plant under controlled conditions.

Keywords Glucosidase · Pine bark · *Pinus densiflora* · Surfactant · Tricholoma matsutake

Introduction

Ectomycorrhizal fungi in situ primarily derive carbon from the host plant. However, to sustain their growth, some may rely on below-ground carbon sources as well as on host plant photosynthates. This may be true, for example, for fruit-body formation, a developmental step requiring the continuous supply of a large amount of carbon. The significance of ectomycorrhizal fungi in below-ground carbon cycling has been previously highlighted (Durall et al. 1994) and the ability of ectomycorrhizal fungi to hydrolyze enzymatically various components of the plant cell wall in vitro has been reported (Hutchison 1990; Cao and Crawford 1993). The use of soil organic compounds by ectomycorrhizal fungi should, therefore, reduce the carbon drain imposed on the host plant by the mycobiont (Finlay and Söderström 1992).

The trophic status of the edible fungus *Tricholoma matsutake* has been widely discussed for many years. Its capacity to colonize roots and to form typical ectomycorrhizal Hartig net structures has been reinvestigated recently (Gill et al. 1999, 2000) and unequivocally confirmed by in vitro synthesis (Yamada et al. 1999; Guerin-Laguette et al. 2000; Vaario et al. 2000). However, these observations do not exclude the possibility that *T. matsutake* also has some saprotrophic potentialities, as frequently suggested (Wang et al. 1997; Guerin-Laguette et al. 2001). Norkrans (1950) noted that several *Tricholoma* species had a range of starch utilization abilities; Ohta (1997) reported that starch-containing media supported *T. matsutake* growth very well and, according to Terashita et al. (1995), *T. matsutake* exhibits high levels of both CM-cellulase and avicelase activities in potato dextrose medium.

Considering the difficulty of promoting the growth of *T. matsutake* hyphae colonizing host plant roots in controlled conditions (Ogawa 1978), we investigated its saprotrophic potential. Indeed, the successful development of *T. matsutake* on roots and in their vicinity may rely both on its symbiotic and saprotrophic status. Preliminary observations of various mixed organic substrates inoculated with *T. matsutake* in sterile conditions indicated that *Pinus densiflora* (Japanese Red Pine) bark was preferentially colonized by hyphae. Here, we confirm that pine bark could be a carbon source for *T. matsutake* and highlight the role of surfactants in promoting bark catabolism.

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Materials and methods

Isolates and culture conditions

Tricholoma matsutake (S. Ito et Imai) Sing. strains T2, T61, T89, and T945 were isolated from fruit-bodies collected beneath *Pinus densiflora* Sieb. et Zucc. in Nagano, Niigata, Shiga, and Kyoto Prefectures, respectively. Identification was confirmed by PCR-RFLP analysis of the rDNA intergenic spacer (Guerin-Laguette et al. 1999). Stock cultures were maintained on agar medium (Ohta 1990). Mycelial cultures were incubated in the dark at 23°C.

The mycelial slurry used as inoculum was prepared as previously described (Vaario et al. 2000). Before inoculation, the mycelium was rinsed and resuspended in a modified rinse medium (RM): $(NH_4)_2HPO_4$ 0.35 g; KH_2PO_4 0.63 g; $MgSO_4.7H_2O$ 1 g; $CaCl_2.2H_2O$ 0.05 g; KCl 0.19 g; micronutrients (except $FeCl_3$) and vitamins according to Ohta (1990); ferric citrate (1%) in 1% citric acid solution 3 ml; distilled H_2O to 1 l. The pH was adjusted to 5.1 with 1 N HCl before autoclaving at 121°C for 20 min.

Growth on bark fragments over solid substrates

Soil collected on the University of Tokyo campus was sieved (ca. 3 mm), and dried (24 h, 60°C). Dry soil was then moistened with the RM medium (humidity, 32.5% w/w dry soil) and autoclaved twice with a 2-day interval (121°C, 20 min) before being spread aseptically into Petri dishes (35 ml of soil per 9-cm diameter plate). *P. densiflora* bark fragments (5–6 pieces of 1–4 cm^2 per plate) were autoclaved in distilled water and laid on soil in triplicate Petri dishes. Each plate was aseptically inoculated with 5 ml of mycelial slurry (isolate T2) distributed all over the substrate surface (including bark fragments) as drops. The plates were incubated for 45 days. Alternatively, bark crushed in an electric coffee mill was autoclaved and spread (0.1 g/plate) over RM agar medium in triplicate Petri dishes inoculated with one 5-mm mycelium plug collected at the edge of an actively growing colony (isolate T2). The hyphal growth was observed after 21 days. All plates were incubated in the dark at 23°C.

Growth in bark powder-supplemented liquid medium

Erlenmeyer flasks (200 ml) containing 20 ml of liquid medium (RM) were inoculated with 4 ml of mycelial slurry. The nutrient medium was eventually supplemented with glucose (10 g/l) or *P. densiflora* bark, crushed as described above and sieved (400 mg per flask, granulometry 1–2 mm). When specified, the surfactants Tween 80 (polyoxyethylene sorbitan monooleate) and Tween 40 (polyoxyethylene sorbitan monopalmitate), purchased from Sigma (St. Louis, Mo., USA), were added to the nutrient medium (0.5% w/v). Ethanol was used to dilute surfactants before incorporation into the nutrient medium (final ethanol concentration in the medium before autoclaving, including Tween-free controls was 1.8%). The cultures were incubated stationary in the dark at 23°C for 4 weeks. The mycelium together with bark was collected over a nylon mesh filter (24×30 µm) and freeze-dried. Ergosterol was then extracted into absolute ethanol (6 ml) and assayed by HPLC (Martin et al. 1990). The culture filtrate was collected for estimation of glucosidase activity and protein content.

Glucosidase activity and protein content of culture filtrates

The 1,4-β-glucosidase activity was assayed in filtrates, collected as described above, by monitoring the release of *p*-nitrophenol from *p*-nitrophenol β-D-glucopyranoside (PNPG; Sigma N-7006), according to Habu et al. (1997), except that the incubation time was extended to 30 min. One unit (U) of β-glucosidase was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute. Protein content was determined by the Bradford method (Bradford 1976) with the Bio-Rad Protein Assay Kit (Calif., USA).

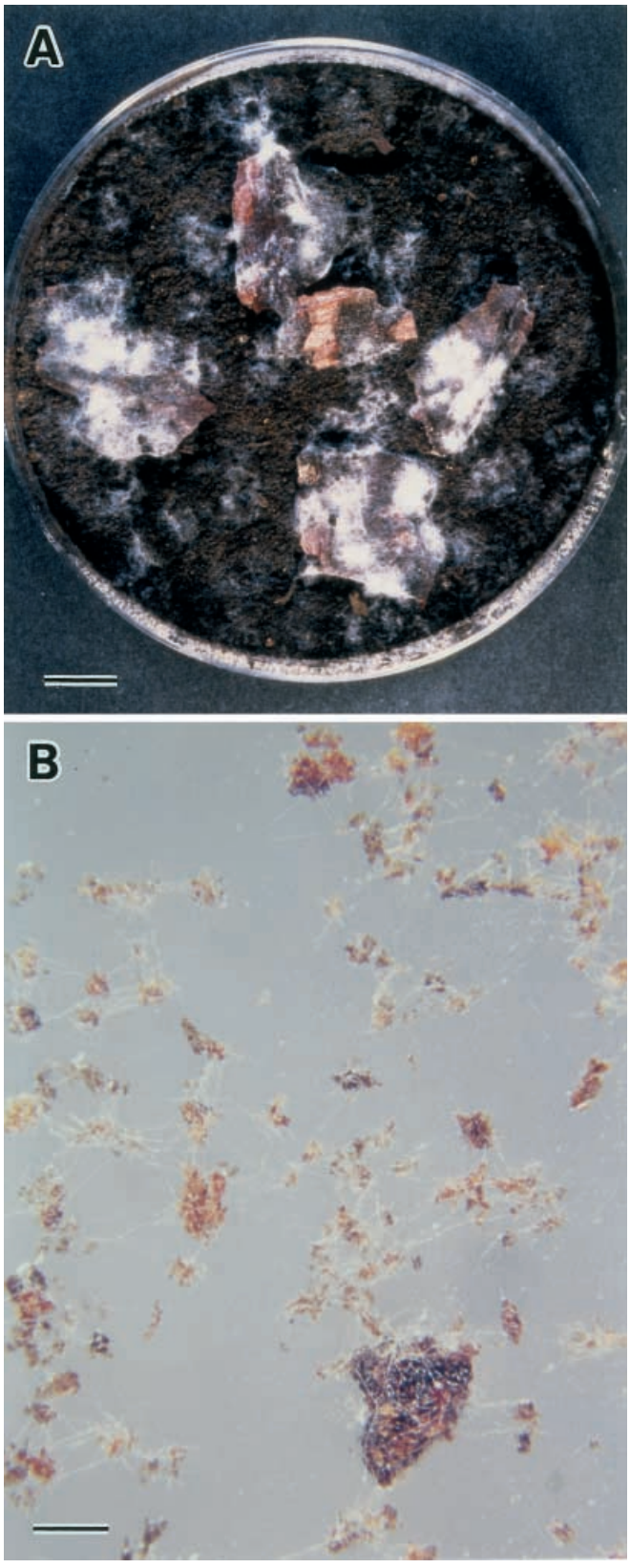

Fig. 1 Microscopic features of *Tricholoma matsutake* (isolate T2) growing on soil (**A**) and on RM agar medium (**B**) supplemented with *Pinus densiflora* bark fragments, after 45 and 21 days, respectively. Fungal hyphae appeared to colonize preferentially pine bark fragments either on soil or on agar medium; *bars* 1 cm (**A**), 1 mm (**B**)

Sugar concentration of bark-containing medium after autoclaving and prior to inoculation

Autoclaved RM medium (an aliquot of 1 ml) containing pine bark powder (20 g/l), crushed and sieved as described previously, was freeze-dried and resuspended in STOX (125 µl) and silylation grade pyridine (125 µl) solutions, according to Long and Chism (1987). The mixture was heated for 60 min at 70°C and gently swirled after 20 and 40 min. After cooling to room temperature, trifluoroacetic acid (20 µl) and hexamethyldisilazane (200 µl) were added. After 60 min incubation the mixture was filtered onto a 0.2-µm nitrocellulose filter (Whatman Inc.). The filtrate was analysed by gas chromatography (Hitachi G-3500) using a 2% OV-17 on 80–100 mesh Chromasorb, glass column (ID 3 mm×1 m), according to Long and Chism (1987). The carrier gas (N_2) flow-rate was 35 ml/min.

Statistical analyses

Pairwise comparisons of fungal ergosterol contents were made using Student's *t*-tests (P <0.05). Other data, for each isolate, were subjected to multiple comparisons (one-way ANOVA, Tukey-Kramer test) (P<0.05). Computations were carried out by using Statistica 2000 (Statsoft Inc.) for Windows.

Results

Tricholoma matsutake hyphae appeared to colonize preferentially pine bark fragments either on soil (Fig. 1A) or on agar (Fig. 1B) surfaces, suggesting that the bark provided some nutrients to sustain growth in glucose-deprived environments.

The addition of pine bark as sole carbon source significantly stimulated the growth of all isolates (26–100% increase), expressed as ergosterol content, compared with glucose-free liquid medium (Fig. 2). However, the

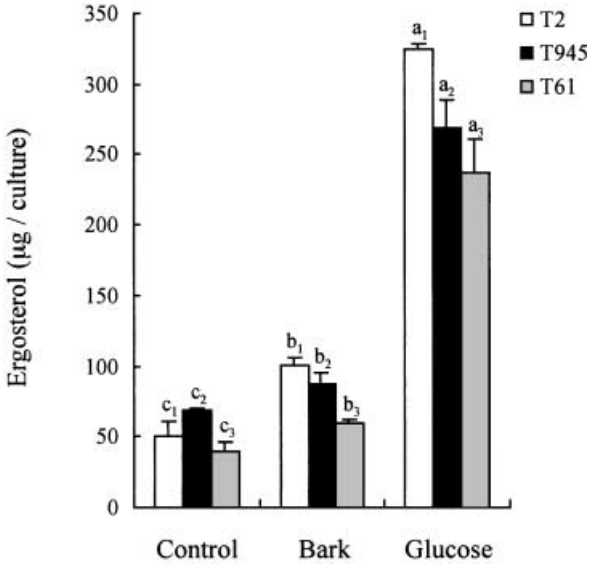

Fig. 2 Growth, estimated by ergosterol production, of *T. matsutake* (isolates T2, T945, T61) on RM liquid nutrient medium supplemented or not with *Pinus densiflora* bark fragments (20 g/l) or glucose (10 g/l), after 4 weeks. Values are the means of three replicates ± SD. For each isolate, common letters indicate non-significant differences (Student's *t* test, P<0.05)

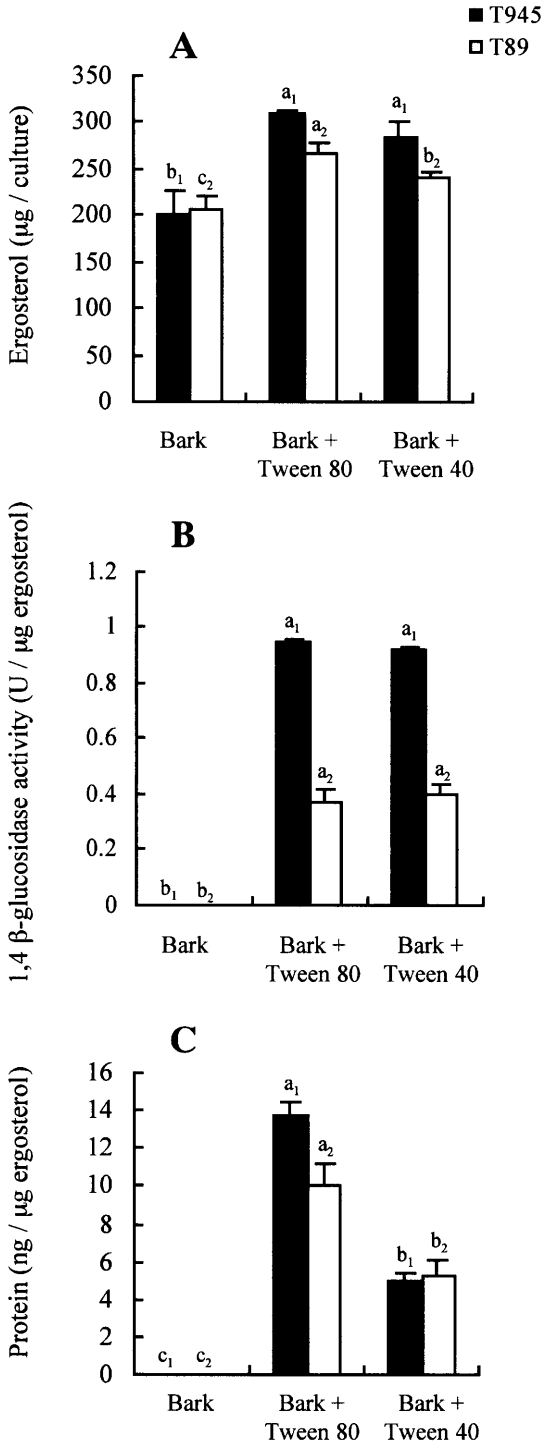

Fig. 3 Effect of surfactants (Tween 80 and Tween 40, 0.5%) after 4 weeks on growth, estimated by ergosterol content (**A**), on β-glucosidase excretion (**B**) and on protein excretion (**C**) by *T. matsutake* (isolates T945, T89) on RM liquid nutrient medium supplemented with *P. densiflora* bark fragments (20 g/l). Values are the means of three replicates ± SD. For each isolate, common letters indicate non-significant differences (one-way ANOVA, Tukey-Kramer test, P<0.05)

range of growth stimulation was still much lower than that recorded in response to glucose supplementation (Fig. 2). This growth stimulation could not be attributed to hexose release from the bark during autoclaving as no free sugar was detected in the nutrient medium (detection limit for glucose was 5 mg/l).

The addition of the surfactants Tween 40 and Tween 80 to the liquid medium significantly stimulated the growth of both isolates tested on pine bark by up to 55% (Fig. 3A). This stimulation was associated with a sharp increase in β-glucosidase activity in the culture filtrate at the end of the experiment (Fig. 3B). Cell wall-degrading enzyme activity could not be detected in the culture filtrate in the absence of surfactant. Together with β-glucosidase activity, the total amount of protein excreted by hyphae was strongly dependent on surfactant addition to the medium (Fig. 3C).

The activities of the two surfactants on hyphal growth (Fig. 3A) and β-glucosidase release (Fig. 3B) were not significantly different, but total protein excretion was stimulated twice as much by Tween 80 as by Tween 40 (Fig. 3C).

Discussion

The present results suggest that *Tricholoma matsutake* can use Japanese Red Pine bark, and maybe other plant materials, as a carbon source in soil, even though carbon may still be a growth-limiting factor on such substrates. Similarly, saprotrophic growth of *T. matsutake* in soil has been reported recently (Guerin-Laguette et al. 2001). Such saprotrophic behaviour is not incompatible with a symbiotic status whose physiological implications remain to be clarified. As for many other ectomycorrhizal species, quantification of carbon flow from the host plant to the mycobiont *T. matsutake* is still lacking. However, since matsutake mycelium does not propagate extensively following root colonization (Guerin-Laguette et al. 2000; Vaario et al. 2000), carbon transfer via the mycorrhizas may be rather low, at least in our experimental conditions.

The addition of the surfactants Tween 40 and Tween 80 seemed to greatly improve the saprotrophic potentialities of *T. matsutake* on bark. This was associated with a strong stimulation of fungal protein excretion. Only β-glucosidase activity was quantified among the excreted proteins, and the activity was also strongly up-regulated. Therefore, it seems reasonable to suggest that other lytic enzyme activities are stimulated by surfactant addition. Surfactants may promote the release of cell-bound enzymes but are also likely to increase membrane permeability. The activity of various surfactants (Tween 80, Tween 20, Triton X-100) on the yield of cell-wall degrading enzymes (β-glucosidase, endoglucanase, exoglucanase, manganese peroxidase) by various fungi has been described frequently (Gomes et al. 1994; Pushalkar et al. 1995; Rodriguez et al. 1998; Pardo and Forchiassin 1999). On the other hand, though we cannot exclude that Tween compounds may be used in part as carbon sources themselves (Caldwell et al. 1991), Tween 80 did not appear to sustain much *T. matsutake* growth in a carbon-deficient agar medium (Guerin-Laguette et al. 2001).

The present information suggests that the saprotrophic abilities of *T. matsutake* should be further investigated by testing various organic compounds, such as forest debris, in the presence of surfactants. This would contribute to the formulation of new culture substrates adapted to the co-culture of *T. matsutake* and its host plant under controlled conditions. Indeed, both symbiotic and saprotrophic carbon sources may be required for the development of Matsutake Shiro, the dense extramatrical hyphae network associated with pine roots and soil particles acknowledged to support fruiting in the forest (Ogawa 1975; Hosford et al. 1997).

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