Mycoscience (2003) 44:249–251
DOI 10.1007/s10267-003-0098-z

SHORT COMMUNICATION

Akiyoshi Yamada · Hisayasu Kobayashi · Hitoshi Murata

Tricholoma matsutake IFO6933 and IFO30604, "matsutake" isolates that have been maintained on slants and widely used in vitro for a quarter to half a century, can form ectomycorrhiza in *Pinus densiflora*

Received: August 22, 2002 / Accepted: January 29, 2003

Abstract *Tricholoma matsutake* isolates IFO6933 and IFO30604 have been maintained on slants for 46 and 26 years, respectively, and widely used for studies of matsutake in vitro without any contact with potential host plants. In the present study, we demonstrate that both isolates are able to form a typical ectomycorrhiza in association with *Pinus densiflora*. The result shows that *T. matsutake* is hardly attenuated in its symbiotic potential and that the fungal cultures maintained for years are useful in further analysis in vitro.

Key words Ectomycorrhiza · Edible mushroom fungi · In vitro mycorrhiza synthesis · *Pinus densiflora* · *Tricholoma matsutake*

Tricholoma matsutake (S. Ito et Imai) Singer is one of the well-studied edible ectomycorrhizal mushrooms in the world because of its economic importance (Hosford et al. 1997; Yamada et al. 1999a). Some *T. matsutake* isolates have been cultured on nutrient media over the past 60 years, and such isolates, without proof of their symbiotic behavior, have been widely used in elucidation of the basic biology of this fungal species (Hamada 1940, 1950, 1964; Kawai and Abe 1976; Kawai and Ogawa 1976, 1977; Kawai and Terada 1976; Matsumoto et al. 1999; Ogawa 1964; Ogawa and Hamada 1975; Ogawa and Kawai 1976; Ohta 1986, 1990; Terashita and Kono 1987; Terashita et al. 1991, 1995). Although all the studies were conducted with cultures grown in nutrient media, those data greatly contributed to our understanding of matsutake, such as physiology involved in vegetative growth, karyotypes, and chemistry of compounds produced by the fungus. Recently, methods that allow symbiotic fungi to form ectomycorrhizas in vitro were established, and such methods with newly isolated *T. matsutake* enabled us to demonstrate that the fungus is a typical ectomycorrhizal symbiont (Gill et al. 1999; Guerin-Laguette et al. 2000; Vaario et al. 2000; Yamada et al. 1999b).

To prove that *T. matsutake* isolates that have been widely used for decades retain their potential in plant–microbe interactions, we explored whether such authentic isolates can associate with *Pinus densiflora* Sieb. et Zucc. as an ectomycorrhizal symbiont. Two *T. matsutake* isolates were chosen from the collection of Institute for Fermentation, Osaka, Japan (IFO). The isolate IFO6933 (original number: M-141S) was purified as a single mycelial culture from the basidiospores collected in Nagano Prefecture, Japan, in 1952, and the isolate IFO30604 (original number: Tm 3) was purified in Shiga Prefecture, Japan, in 1973 (IFO 1996; Ogawa and Hamada 1975; Ohta 1990). Characteristics of both isolates, such as the mycelial growth rate and dikaryotic status of the mycelium, as examined on nutrient media have been described elsewhere as standard characteristics of *T. matsutake* (Ogawa and Hamada 1964; Ohta 1990; Yamada and Terasaki 1998). These isolates were also systematically analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the internal transcribed spacer (ITS) region of rDNA (Yamada et al. 2001).

Mycorrhizal association with *P. densiflora* was examined in vitro after Yamada et al. (1999b) with slight modifications. Inocula were prepared by culturing the fungal mycelia in the MNC liquid medium after regeneration of the cultures stored on slants of Hamada's agar medium (IFO 1996) by allowing them to grow on Modified Norkrans's "C" (MNC) agar plates (Yamada and Katsuya 1995;

A. Yamada (✉)
Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, 8304 Minami-minowa, Kami-ina, Nagano 399-4598, Japan
Tel. +81-265-77-1631; Fax +81-265-77-1629
e-mail: akiyosh@gipmc.shinshu-u.ac.jp

H. Kobayashi
Ibaraki Prefectural Forestry Center, Naka 311-0122, Japan

H. Murata
Department of Applied Microbiology and Mushroom Science, Forestry and Forest Products Research Institute, Kukizaki 305-8687, Japan

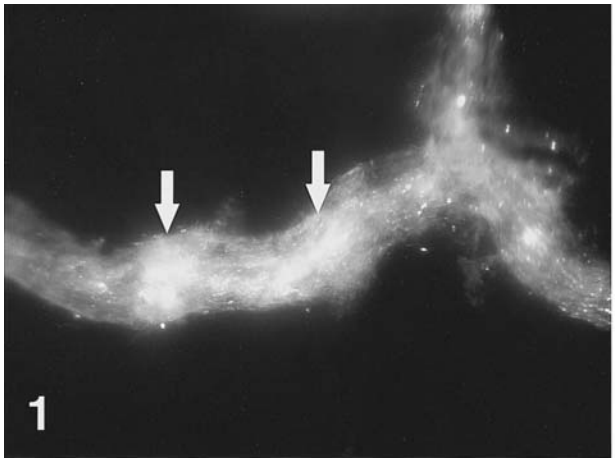

Fig. 1. Dissecting micrograph of a pine ectomycorrhiza synthesized with *Tricholoma matsutake* isolate IFO6933. *Arrows* indicate fungal sheath

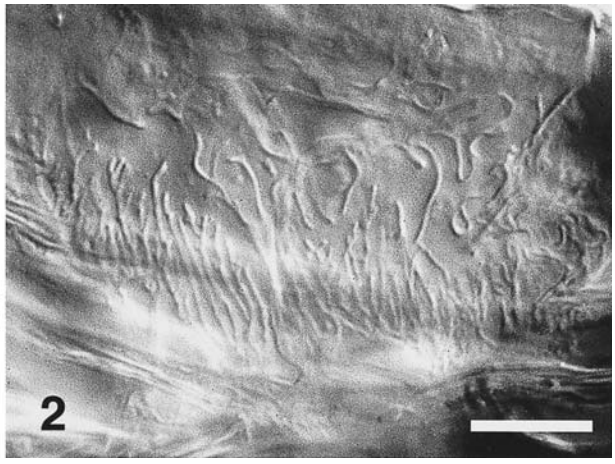

Fig. 2. Differential interference micrograph of the mycorrhiza showing labyrinthine Hartig net mycelium at the root cortex. A whole-mounted mycorrhizal tip is observed. *Bar* 20 μm

Yamada et al. 1999b). Colony morphology and growth rate of both IFO6933 and IFO30604 on the MNC agar plate were essentially the same as those of Y1 (=IFO33136), Y4, and F (=IFO33137), the *T. matsutake* isolates that were isolated during the period from 1993 to 1996, and proven to behave as ectomycorrhizal symbionts in vitro (Yamada et al. 1999b). The culture of each fungal isolate and two axenically germinated seedlings of *P. densiflora* were placed in a 450-ml culture substrate composed of vermiculite, sphagnum, and the MNC liquid medium in a 900-ml polycarbonate bottle to allow the association. The experiment was conducted with five replications. The result consistently observed throughout the replications was as follows.

Six months after inoculation, the root system of the host plants was observed microscopically. Sections of lateral roots colonized by the fungal mycelia showed typical structures of the ectomycorrhiza such as thin fungal sheath and the Hartig net (Figs. 1, 2). The mycorrhizal morphology and the section anatomy were the same as those of Y1, Y4, and F (Yamada et al. 1999b). All mycorrhizas examined thus far were formed on 1-year-old pine seedlings, and healthy plant growth was sustained throughout the incubation period, the phenomena consistent with observations in Y1, Y4, and F (Yamada et al. 1999b). However, no primordium formation was observed during the incubation period.

Plant pathogenic fungi as well as other infectious microbes are apt to attenuate in their parasitic or symbiotic behaviors during a prolonged in vitro preservations (Agrios 1997; Davis et al. 1980). Instability of such important characters in a given isolate often causes serious problems in reproduction of past experimental results as well as in generation of new data on the same standardized genetic background. Similar phenomena have also been reported in ectomycorrhizal fungi, and most of such phenomena have been ascribed to the attenuation of symbionts during a long-term vegetative growth state in vitro (Brundrett et al. 1996; Heinonen-Tanski and Holopainen 1991; Marx and Daniels 1976; Molina and Palmer 1982; Thomson et al. 1993). In the present study, however, we demonstrated that *T. matsutake* isolates subcultured on slants for so long as half a century were able to form a typical symbiotic organ in association with *P. densiflora*. We speculate that the symbiotic phenotype markedly stable in *T. matsutake* may be attributed in part to its tremendously slow growth in vitro even under optimum nutrient medium conditions, such as mycelial growth at most 2 cm/month in Hamada's or MNC agar at 20°–25°C (Hamada 1950; Yamada and Terasaki 1998). Further analysis in the process of colonization of the fungus in association with host plants and its effect on plant growth may provide us with a clue to the mechanisms involved in the genetic stability of *T. matsutake* (Thomson et al. 1993).

It is interesting to note that the isolate IFO6933 was reported not to form an ectomycorrhizal association with pine seedlings under any synthetic conditions (Ogawa 1978). Previously, we suggested that the mycorrhiza formation in vitro using *T. matsutake* isolates Y1, Y4, and F might be an isolate-specific event, and that genetic variations among the *T. matsutake* isolates used by Yamada et al. (1999b) and Ogawa (1978) might have conferred the contradictory results. However, the demonstration of mycorrhiza formation by IFO6933 in the present study clarified that the contradiction between Yamada et al. (1999b) and Ogawa (1978) should be attributed principally to different experimental conditions and not to genetic variation among the fungal isolates. According to Ogawa (1978), IFO6933 was the most efficient in all the tested isolates in primordium formation on nutrient media without a host plant (Kawai and Ogawa 1976; Ogawa and Hamada 1964, 1975). Although such morphogenesis was not observed in the present study, it is the only isolate that has been shown to achieve both mycorrhization and the development of a distinct, but immature, fruiting structure. This character set should be the key selection markers in exploring fungal isolates for the artificial cultivation of the matsutake mushroom.

References

Agrios GN (1997) Genetics of plant disease. In: Agrios GN (ed) Plant pathology, 4th edn. Academic Press, San Diego, pp 115–142

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with mycorrhizas in forestry and agriculture. Australian Centre for International Agricultural Research, Canberra

Davis BD, Dulbecco R, Eisen H, Ginsberg HS (1980) Microbiology, 3rd edn. Harper and Row, Philadelphia

Gill WM, Lapeyrie F, Gomi T, Suzuki K (1999) *Tricholoma matsutake* – an assessment of in situ and in vitro infection by observing cleared and stained whole roots. Mycorrhiza 9:227–231

Guerin-Laguette A, Vaario LM, Gill WM, Lapeyrie F, Matsushita N, Suzuki K (2000) Rapid in vitro ectomycorrhizal infection on *Pinus densiflora* roots by *Tricholoma matsutake*. Mycoscience 41:389–393

Hamada M (1940) Physiologisch-morphologische Studien über *Armillaria mellea* (Vahl) Quél., mit besonderer Rücksicht auf die Oxalsaurebildung. Ein Nachtrag zur Mykorrhiza von *Galeola septentrionalis* Reichb. f. (in German). Jpn J Bot 10:387–463

Hamada M (1950) Physiology and ecology of *Armillaria matsutake* (in Japanese). Bot Mag Tokyo 63:741–742

Hamada M (1964) Method of pure culture of the mycelia of *Armillaria matsutake* and of its allies. In: The Matsutake Research Association (ed) Matsutake (*Tricholoma matsutake*): its fundamental studies and economic production of the fruit-body (in Japanese). Matsutake Research Association, Kyoto, pp 97–100

Heinonen-Tanski H, Holopainen T (1991) Maintenance of ectomycorrhizal fungi. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology, vol 23. Techniques for the study of mycorrhiza. Academic Press, London, pp 413–422

Hosford D, Pilz D, Molina R, Amaranthus M (1997) Ecology and management of the commercially harvested American matsutake mushrooms. PNW-GTR-412. USDA Forest service, Portland, OR

IFO (1996) List of cultures, 10th edn. Institute for Fermentation, Osaka (IFO), Osaka

Kawai M, Abe S (1976) Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. I. Effects of carbon and nitrogen sources in media on the vegetative growth of *T. matsutake* (in Japanese). Trans Mycol Soc Jpn 17:159–167

Kawai M, Ogawa M (1976) Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. IV. Studies on a seed culture and a trial for the cultivation on solid media (in Japanese). Trans Mycol Soc Jpn 17:499–505

Kawai M, Ogawa M (1977) Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. V. Effects of some chemicals on the growth of mycorrhizal and non-mycorrhizal soil fungi (in Japanese). Trans Mycol Soc Jpn 18:391–398

Kawai M, Terada O (1976) Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. II. Effect of vitamins, nucleic acid relating substances, phytohormones and metal ions in media on the vegetative growth of *T. matsutake* (in Japanese). Trans Mycol Soc Jpn 17:168–174

Marx DH, Daniels WJ (1976) Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. Can J Microbiol 22:338–341

Matsumoto M, Eguchi F, Iijima T, Higaki M (1999) Preparation and regeneration of protoplasts from mycelia of *Tricholoma matsutake*. Mokuzai Gakkaishi 45:413–418

Molina R, Palmer JG (1982) Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: Schenck NC (ed) Methods and principles of mycorrhizal research. APS Press, St. Paul, MN, pp 115–129

Ogawa M (1964) Preliminary reports on the nutrition of *Tricholoma matsutake* (S. Ito et Imai) Singer and the mycorrhizal relationship between the fungus and *Pinus densiflora*. In: The Matsutake Research Association (ed) Matsutake (Tricholoma *matsutake*): its fundamental studies and economic production of the fruit-body (in Japanese). Matsutake Research Association, Kyoto, pp 101–114

Ogawa M (1978) The biology of matsutake (in Japanese). Tsukiji-shokan, Tokyo

Ogawa M, Hamada M (1964) Method for growing the fruit-body primordium of matsutake on the artificial medium (article for patent application). In: The Matsutake Research Association (ed) Matsutake (*Tricholoma matsutake*): its fundamental studies and economic production of the fruit-body (in Japanese). Matsutake Research Association, Kyoto, pp 115–117

Ogawa M, Hamada M (1975) Primordia formation of *Tricholoma matsutake* (Ito et Imai) Sing. in pure culture (in Japanese). Trans Mycol Soc Jpn 16:406–415

Ogawa M, Kawai M (1976) Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. III. Effects of growth promotion of natural products on the vegetative growth of *T. matsutake* (in Japanese). Trans Mycol Soc Jpn 17:492–498

Ohta A (1986) Basidiospore germination of *Tricholoma matsutake* (I). Effects of organic acids on seedling and germination of the basidiospores. Trans Mycol Soc Jpn 27:167–173

Ohta A (1990) A new medium for mycelial growth of mycorrhizal fungi. Trans Mycol Soc Jpn 31:323–334

Terashita T, Kono M (1987) Purification and some properties of carboxyl proteinases from *Tricholoma matsutake*. Trans Mycol Soc Jpn 28:245–256

Terashita T, Takeuchi T, Wakayama S, Sekino Y, Terai S, Yoshikawa K, Shishiyama J (1991) Effect of low-temperature treatment on the production of 1-octen-3-ol by the vegetative mycelia of *Tricholoma matsutake*. Trans Mycol Soc Jpn 32:477–484

Terashita T, Kono M, Yoshikawa K, Shishiyama J (1995) Productivity of hydrolytic enzymes by mycorrhizal mushrooms. Mycoscience 36:221–225

Thomson BD, Malajczuk N, Grove TS, Hardy St J GE (1993) Improving the colonization of ectomycorrhizal fungal cultures by association with a host plant and re-isolation. Mycol Res 97:839–844

Vaario LM, Guerin-Laguette A, Gill WM, Lapeyrie F, Suzuki K (2000) Only two weeks are required for *Tricholoma matsutake* to differentiate ectomycorrhizal Hartig net structures in roots of *Pinus densiflora* seedlings cultivated on artificial substrate. J For Res 5:293–297

Yamada A, Katsuya K (1995) Mycorrhizal association of isolates from sporocarps and ectomycorrhizas with *Pinus densiflora* seedlings. Mycoscience 36:315–323

Yamada A, Terasaki M (1998) Characterization of *Tricholoma matsutake* isolates taken from Ibaraki Prefecture (in Japanese). Trans Jpn For Sci 109:521–522

Yamada A, Kanekawa S, Ohmasa M (1999a) Ectomycorrhiza formation of *Tricholoma matsutake* on *Pinus densiflora*. Mycoscience 40:193–198

Yamada A, Maeda K, Ohmasa M (1999b) Ectomycorrhiza formation of *Tricholoma matsutake* isolates on seedlings of *Pinus densiflora* in vitro. Mycoscience 40:455–463

Yamada A, Ogura T, Degawa Y, Ohmasa M (2001) Isolation of *Tricholoma matsutake* and *T. bakamatsutake* cultures from field-collected ectomycorrhizas. Mycoscience 42:43–50