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Sibling spore isolates of *Tricholoma matsutake* vary significantly in their ectomycorrhizal colonization abilities on pine hosts *in vitro* and form multiple intimate associations in single ectomycorrhizal roots

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Abstract

Tricholoma matsutake is a commercially important edible ectomycorrhizal mushroom. The ecology of this species has emerged from studies of the genetic background of mycelial colonies in nature. Single putative colonies sometimes comprise several genets. This complex mycelial structure should be subjected to analysis to determine its ecophysiological significance. We tested the ectomycorrhization ability of nine *T. matsutake* sibling spore isolates. The ectomycorrhizal colonization ratio differed significantly among isolates, and was dependent on the soil nitrogen content. Mixed inoculations of three selected isolates into soils in which single pine seedlings were grown showed that the isolates interacted in the seedling roots. Paired inoculations of isolates #52/#99 and #52/#84, and a triple inoculation of isolates #52/#84/#99 resulted in levels of ectomycorrhizal colonization that significantly exceeded the colonization levels following single isolate inoculation. We suggest that mycelial interaction between sibling isolates is a significant phenomenon that operates within individual ectomycorrhizal pine root tips.

Keywords: Agaricomycotina; Competition; Ectomycorrhizal symbiosis; Fungus-plant interaction; Genetic diversity; Mutualism; Non-timber forest resources; Shiro structure; Soil microbial ecology; Wild edible mushrooms

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1. Introduction

Tricholoma matsutake is one of the most commercially valuable wild edible ectomycorrhizal mushrooms. The Japanese colloquial name for this species and its taxonomic relatives is "matsutake" (Turdell et al., 2017; Vaario et al. 2017). *Tricholoma matsutake* is associated with *Pinus densiflora* and several other coniferous species in Far Eastern Asia, with *Pinus yunnanensis* and several other coniferous and fagaceous species at the foot of the Tibetan Plateau, and with *Pinus sylvestris* in Northern Europe (Vaario et al., 2010, 2017; Yamada et al., 2010; Endo et al., 2015). The market price of this mushroom in Japan is *ca*. 200–500 USD/kg. However, the domestic harvest has continuously declined over the past 80 y, as a result of social, economic, and ecological changes in Japan after World War II (Ogawa, 1978; Saito and Mitsumata, 2008). Most *P. densiflora* forests in lowland areas were cultivated or secondarily established, and were maintained by the local human populations for diverse uses. As this way of life declined after World War II, many of the pine forests, including those that produced matsutake, were abandoned. Pine forest management for *T. matsutake* production can be successful when previous practices are implemented over a decadal time frame (Ogawa, 1978; Furukawa et al., 2016). Although appropriate forest management practices have been in place in diverse geographic regions over several recent decades (The Matsutake Research Association, 1964; Ogawa, 1978; Iwase and Ito, 1997), the harvest of matsutake in many previously productive sites has declined to zero since the 1970s due to severe damage inflicted by pine wilt disease, which is caused by the pathogenic nematode *Bursaphelenchus xylophilus* (Suzuki, 2005). Thus, the habitat range of *T. matsutake* has declined dramatically, especially in western Japan. Domestic production in recent years has been limited to just a few provinces, e.g., Nagano and Iwate (Ministry of Agriculture, Forestry, and Fisheries, Japan). These overall trends may trigger a negative feedback leading to declines in the natural populations. Hence, the development of alternative, novel cultivation techniques for this

fungus in pine forests and the recovery of the domestic harvest to levels recorded several decades ago are highly desirable.

Tricholoma matsutake cultivation trials in sites other than managed experimental pine forests have been conducted in Japan since the 1960s. Although *in vitro* primordium formation of the fungus on nutrient rich soil (additions of glucose and dried yeast) in the absence of a host plant has been recorded once (Ogawa and Hamada, 1975), the experiment has not been repeated. Inoculations of cultured *T. matsutake* mycelia into pine forest sites have also been attempted, but most of the inocula disappeared before ectomycorrhizal associations could be established (Sugawara et al., 2012). Guerin-Laguette et al. (2004) reported successful pinpoint inoculation of cultured mycelium into pine roots that lacked natural ectomycorrhization. A different inoculation technique involves *in vitro* ectomycorrhization of pine hosts, followed by subsequent transplantation of ectomycorrhizal seedlings to the field. Although *in vitro* ectomycorrhizations have been repeatedly and reliably performed (Yamada et al., 1999, 2006; Guerin-Laguette, 2000, 2004; Vaario et al., 2002, 2010; Kobayashi et al., 2007; Murata et al., 2013, 2015b; Saito et al., 2018), field trials of these established ectomycorrhizal systems are as yet limited in scope. Successful ectomycorrhizal introductions can be confirmed when the symbiosis survives for 2 y (Kobayashi et al., 2008, 2015). We foresee the development of novel techniques that will enable the efficient establishment of *T. matsutake* ectomycorrhizas on pine seedlings in forest sites and the expansion of mycelial areas required for fruiting. Progress will depend on a better understanding of the mechanisms by which the ectomycorrhizal system in the *T. matsutake*-pine association is controlled by biotic (internal) and abiotic (external) factors (Vaario et al., 2019).

Recent investigations of the genetic structures of *T. matsutake* mycelial colony (“shiro”) in forest conditions have suggested that what superficially looks like a single genet

based on the epigeous fruiting pattern actually comprises several genets (Murata et al., 2005b; Lian et al. 2006). The shiro mycelium produces basidiomata annually for over a decade (Hamada, 1970; Ogawa, 1978; Narimatsu et al., 2015; Furukawa et al., 2016). The genetic structure of a single shiro may conceivably change over time because spore deposits from fruit bodies can theoretically result in di-mon mating or sibling di-di mating among germinated individuals within maternal shiro mycelium (Yamada et al., 2019). The observed genetic mosaic structure of a shiro requires experimental analysis to determine its ecophysiological significance. A fuller understanding of (i) the mechanism by which this mosaic structure is established and (ii) its influence on host nutrition may lead to advances in matsutake cultivation techniques. Progress will depend on the development of techniques for *in vitro* production of shiro mosaic formations. These techniques should enable manipulation of the mosaic structure to achieve sustainable harvests of the fungus under appropriate forest management practices.

We recently established a line of *T. matsutake* sibling isolates using a spore isolation technique. Mycelial growth varied among 100 sibling isolates maintained on nutrient medium. Carbon and nitrogen nutrition and commensal interactions among isolates on nutrient agars also differed among selected isolates (Yamada et al., 2019). Thus, in the current study we first aimed to determine the extent to which sibling isolates differed in ectomycorrhizal colonization ratios and host responses. Subsequently, we aimed to (i) establish a shiro mosaic structure *in vitro* and (ii) investigate the way in which this structure affected host plant growth. We used several DNA markers previously employed to estimate genetic diversity in *T. matsutake* (Guerin-Laguette, et al. 2002; Lian et al., 2003, 2006; Murata et al., 2005b, 2008; Xu et al., 2007, 2008; Amend et al., 2010) to analyze the mosaic structure. Intraspecific genetic variation in *T. matsutake* has been investigated by comparisons between specimens or isolates from different geographic regions and hosts, but equivalent

comparisons of sibling isolates are very few (Murata et al., 2015a). In this study, we investigated the significance of the mosaic structure of *T. matsutak*e shiro mycelium using data obtained from several new *in vitro* experiments and previous reports on the ecophysiology of this fungus.

2. Materials and methods

2.1. *Cultured* T. matsutake *isolates*, *host plant*, *and soil types*

Nine sibling isolates (#31，#45，#52，#79，#84，#99，#111，#121，and #126) of *T. matsutake* were used in the experiments. They were selected from a line of 100 isolates established by spore isolation from a single basidioma collected in Takagi Village, Nagano, Japan, and showed physiological differences in their carbon and nitrogen nutrition (Yamada et al., 2019). These selected sibling isolates, which had been stored as slant cultures on modified Norkrans' C (MNC) agar medium (Yamada and Katsuya, 1995), were restored to mycelial growth on MNC agar plates.

Seeds of *P. densiflora* that we used as host plants in our study of *in vitro* ectomycorrhization were collected in the experimental forest owned by the Ibaraki Prefectural Forestry Research Institute, Japan, and stored at −60°C until use. These seeds were harvested as a mixture from several selected mother trees. Although these trees were open-pollinated, and the harvested seeds should thus have genetic variations, we did not consider this aspect in the present study.

Soil samples used for ectomycorrhizal synthesis were obtained from the soil B-layers in *P. densiflora* stands located in the Tera Experimental Forest, Faculty of Agriculture, Shinshu University, Ina City, and in Koshibu, Nakagawa Village, both in Nagano Prefecture. Both are granite-based weathered soils (Saito et al., 2018). The soil from the Tera Experimental Forest was used in a single isolate inoculation experiment that compared ectomycorrhization abilities

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363 between sibling isolates. The soil from Koshibu was used in a mixed isolate inoculation
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365 experiment that determined the interactions between sibling isolates in a host root system.
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367 Soil samples were dried at 50°C, sieved through a standard mesh (5 mm mesh), and stored in
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369 the laboratory until use. Small amounts of these soil samples were used to measure pH and
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371 the contents of water-soluble inorganic phosphorus ($H_2PO_4^-$) and nitrogen (NH_4^+, NO_3^-,
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373 NO_2^-). A 100-g sample of dried soil was mixed with 100 mL of distilled water in a flask and
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375 stirred for 1 h with a magnetic stirrer, after which the sample was filtered; the pH of the
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377 filtrate was then determined. To measure water-soluble phosphate and nitrogen contents, a 50-
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379 g soil sample was mixed with 100 mL of distilled water in a flask and stirred for 1 h with a
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381 magnetic stirrer. The soil solution was centrifuged at 1,000 *g* for 10 min (TOMY LC-200
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383 centrifuge, Tokyo, Japan). The supernatant was filtered and subjected to the following tests:
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385 PACKTEST WAKPO$_4$ (D), WAK-NH$_4$, WAK-NO$_3$, and WAK-NO$_2$ (KYORITSU
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387 CHEMICAL-CHECK Laboratory, Tokyo, Japan), which measured the concentrations of
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389 $H_2PO_4^-$, NH_4^+, NO_2^-, and NO_3^-, respectively. The concentrations were measured by
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391 absorption spectroscopy (JASCO V-530 UV/VIS spectrometer, Tokyo, Japan), and standard
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393 curves were prepared using standard solutions of KH_2PO_4, NH_4Cl, KNO_3, and KNO_2
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395 (Nakalai Tesque, Kyoto, Japan). The average value from three measurements of a single
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397 sample was used in the analyses. The averages for the Tera Experimental Forest (i.e., "Tera"
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399 soil) were: pH, 5.67; $H_2PO_4^-$, 0.008 mg/kg dried soil; NH_4^+, 0.750 mg/kg dried soil; NO_3^-,
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401 0.647 mg/kg dried soil; and NO_2^-, 0.002 mg/kg dried soil. The averages for the Koshibu soil
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403 were: pH, 5.89; $H_2PO_4^-$, 0.033 mg/kg dried soil; NH_4^+, 0.569 mg/kg dried soil; NO_3^-, 0.658
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405 mg/kg dried soil; and NO_2^-, 0.004 mg/kg dried soil.
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412 *2.2*. In vitro *ectomycorrhizal synthesis through single inoculation of* T. matsutake *isolate into*
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The stored *P. densiflora* seeds were washed, surface sterilized, and germinated on MNC agar plates (Yamada et al., 2010). Seedlings between 7 and 10 d old were transplanted into soil prepared in culture vessels using the methods described below. Two mycelial segments (5 × 5 mm) of each *T. matsutake* isolate that had been previously cultured on a MNC agar plate for 2 months were inoculated into 10 mL of autoclaved MNC liquid medium in a 75-mL wide-mouth glass bottle and incubated at 20°C for 1 month to prepare a mycelial inoculum for ectomycorrhization. Previously dried and stored soil was dried again at 70°C for 24 h to a relative water content of 0%. The dried soil was saturated with distilled water to produce a relative water content of 75%. Approximately 180 mL water was required to increase the water content to 75% for 1 kg dried soil (Saito et al., 2018). In the nitrogen-added soil treatment, 1.0 g of powdered dried yeast (Ebios, Asahi Group Foods, Ltd., Tokyo, Japan) was added to 1 L dried soil. A 200-mL sample of the prepared soil was autoclaved in a 250-mL polycarbonate wide-mouth vessel (No. 2116-0250; Thermo Scientific Inc., Waltham, MA, USA) at 124°C for 60 min. This nitrogen-added condition was intended to reveal physiological variations among the nine tested isolates under symbiosis with pine hosts. The polyethylene cap of the autoclaved vessel was removed under a sterile hood; the soil was then inoculated with liquid-cultured *T. matsutake* mycelium (equivalent to approximately 30 mg dry weight). The inoculum was divided into five portions before being dispersed through the soil in the polycarbonate vessel (four portions toward the outer sides of the vessel and one at the center at middle soil depth). At the same time, an axenically germinated *P. densiflora* seedling was transplanted into each polycarbonate vessel. A second (open) autoclaved polycarbonate vessel was inverted and placed over the top of the planted vessel such that the two vessels were mouth to mouth (Kobayashi et al., 2007). The necks of the vessels were subsequently sealed with transparent polyvinyl chloride film (Riken Tape; Kyoei Plastic MGF Co. Ltd., Tokyo, Japan), after which we weighed the whole assembly. Four 6-mm diameter

aeration holes were drilled through the top vessel; each hole was covered with a polytetrafluoroethylene membrane seal (pore size 0.45 mm; Milliseal, Merck, Darmstadt, Germany). These vessel assemblies were incubated in a growth chamber at 20°C under continuous fluorescent illumination at a photon flux of 140 μmol/m^2/s for 150 d. Each experimental unit comprised six replicate seedlings. Control treatments (no fungal inoculation) were established in both soil conditions (with or without dried yeast fertilizer). Sterilized distilled water was supplied monthly to the soil substratum in each vessel under a sterile hood to maintain a constant vessel weight.

2.3. In vitro *ectomycorrhizal synthesis through mixed inoculation with three sibling* T. matsutake *isolates*

Based on the results of ectomycorrhizal synthesis *in vitro* through the single inoculation of nine *T. matsutake* isolates, we selected three (#52, #84, and #99) for a mixture inoculation experiment. These three isolates differed in their levels of ectomycorrhization: #84 was high, #52 was moderate, and #99 was low. In this experiment, eight inoculation units were prepared: #52, #84, #99, #52/#84, #52/#99, #84/#99, and #52/#84/#99, with two inoculation designs, *i.e.* Arrangement-A and Arrangement-B. Preparations for the ectomycorrhizal synthesis experiment were similar to those described in section 2.2, except for the steps of fungal inoculation and seedling transplantation, and without the addition of dried yeast. Prepared mycelia of two or three isolates were concurrently inoculated into three points at middle soil depth in a vessel containing a pine seedling. A total amount of approximately 30 mg (dry weight) inoculum was supplied to each vessel for all mixture inoculation experiments, with equal proportions of each isolate. Two types of inoculum design were established in the triple inoculation experiment: Arrangement-A, in which three different pairs of mycelia (#52/#84, #52/#99, and #84/#99) were inoculated into each of the three points in

the soil; and Arrangement-B, in which each of the three isolates was inoculated singly into each of the three points in the soil (Fig. 1). Arrangement-A was expected to promote more mycelial competition than Arrangement-B. The different arrangements were designed to distinguish direct and indirect competition between different mycelia in the association with the host (Kennedy and Bruns, 2005). Single isolate inoculations (#52, #84, or #99) were also set up because the soil used in this mixture inoculation experiment was different from that used in the single inoculation experiment (2.2). Each experimental unit comprised five replicate seedlings. A control treatment (no fungal inoculation) was also set up.

One pine seedling was transplanted into the soil of the vessel 30 d after fungal inoculation. Direct fungal competition was expected to have occurred during the pretransplantation period. The seedlings were then maintained in the vessels for a further 120 d.

2.4. Harvesting and measurement of seedlings grown with single isolates

After a 150-d incubation, a small volume of the soil particles in each vessel was taken and inoculated onto an MNC agar plate to check for the presence/absence of contaminating microbes and the growth of inoculated *T. matsutake* mycelium. Following this procedure, each seedling was removed from the vessel and separated into shoot and root portions. The shoot was dried at 60°C for 24 h, after which the dry weight was determined. The root system was washed in flowing tap water and cut into segments <1.0 cm in length. Root lengths were measured using the grid-line intersect method (Brundrett et al., 1996). We determined the total actual ectomycorrhizal root length. The total root length of a seedling was estimated as one-quarter of the measured root length. We examined small samples of sound ectomycorrhizal root tips microscopically to determine the presence/absence of morphological and anatomical characteristics of ectomycorrhizal development (Yamada et al.,

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2010). After the measurements were completed, the root system was dried and weighed. We calculated (i) total seedling weight, (ii) shoot/root (S/R) biomass ratios, and (iii) ectomycorrhizal root colonization ratio from the individual data points, i.e., shoot and root weights, and total and ectomycorrhiza root length, for each seedling.

2.5. Harvesting and measurement of seedlings grown with isolate mixtures

After a 120-d incubation, a small volume of the soil particles from each vessel was inoculated onto an MNC agar plate to check for the presence/absence of contaminating microbes. Following this procedure, three aluminum plates (7 × 3 cm) were inserted vertically into the culture vessels to separate the soil and the root system into three portions, each of which had been previously inoculated with different mycelium (Fig. 1). The seedling shoots were removed from the vessel, after which the separated soil portions and the roots they contained were extracted. Five ectomycorrhizal root tips were randomly sampled from each of the soil samples and stored in a refrigerator for DNA analysis, as described below (2.6). The roots and shoots were prepared following the methods described in section 2.4.

2.6. DNA analyses of ectomycorrhizal root tips in the mixed culture experiment

The three isolates we tested (#52, #84, and #99) had two different restriction fragment length polymorphism (RFLP) patterns in the intergenic spacer (IGS) 1 region of the rRNA gene cluster (Yamada et al., 2019). We, therefore, first targeted this site to determine how mixed fungal inoculations develop ectomycorrhizal symbioses in the root system of a single host. Fungal DNA was extracted from a single ectomycorrhizal root tip using the procedure described by Endo et al. (2015) with minor modifications. The primer pair CNL12/5S-Anderson was used for PCR amplification of the IGS1 region (Duchesne and Anderson, 1990; Anderson et al., 1992). PCR was performed in a thermal cycler GeneAmp PCR System

2700 (Applied Biosystems, Waltham, MA, USA). We amplified extracted template DNA by PCR using DreamTaq Polymerase (Thermo Scientific, USA) following the manufacturer's recommendations. Cycle parameters for PCR were as follows: first denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. Because the PCR amplicons of the IGS1 regions of the three *T. matsutake* isolates tested have different RFLP patterns following digestion with *Hae*III (Yamada et al., 2019), we used this restriction enzyme (Takara Bio Inc, Shiga, Japan) for the analysis, following the manufacturer's instructions. The digestion samples were electrophoresed following the procedures of Endo et al. (2015). When necessary, cloning of the IGS1 region was performed following the procedure of Ogawa et al. (2018).

We used the retrotransposon marker for *T. matsutake* typing at the genet level (Murata et al., 2005a) to distinguish the three isolates. This DNA marker distinguishes the tested isolates on MNC agar (Yamada et al., 2019), and we therefore used it on the ectomycorrhizal root samples. We subjected the extracted DNA samples to PCR using the primers pL281/pS48, pDGSL313-1/pS48, pDGSL719-2/pS48, and pS1 following the procedures described by Murata et al. (2005a, 2005b, 2008), with minor modifications. PCR was performed using the ProFlex PCR System (Applied Biosystems). Cycle parameters for PCR were as follows: first denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 90 s, and a final extension at 72°C for 10 min.

To cross compare the distinguishing abilities of DNA markers among isolates, we also tested microsatellite markers (Trma01, Trma02, Trma07, Trma08, Trma14, and Trma16) that can distinguish genets of *T. matsutake* (Lian et al., 2003, 2006) following the procedures of Lian et al. (2006).

2.7. Data analyses

One-way ANOVA was used to test for significant differences among means in each experiment; the analyses were performed with Kaleidagraph ver. 4.5 software (Synergy Software, USA). Tukey's HSD post hoc test or Dunnett's post hoc test were used for multiple pairwise comparisons ($P < 0.05$) of treatment means. When necessary, *t*-tests were used for additional comparisons between pairs of selected treatments. Two-way ANOVA was used to identify significant effects of the inoculated fungal strains, soil nitrogen condition, and their interaction on host pine growth. S/R ratios were arcsine transformed prior to ANOVA. Regression analyses were also used to determine (i) the effects of ectomycorrhizal root length on plant growth parameters, and (ii) the effects of soil nitrogen and phosphorus on ectomycorrhizal root length and plant growth parameters.

3. Results

3.1 Ectomycorrhization by the nine sibling isolates inoculated into Tera soil

Ectomycorrhizas were formed by all isolates tested, and on most seedlings, with the exception of a seedling inoculated with isolate #99 into soil with added nitrogen. We observed a thin fungal mantle on the lateral root surface and Hartig net development in the root cortex of the ectomycorrhizal root tips (Fig. 2). No ectomycorrhizal formation was observed on un-inoculated control seedlings. Mycelia of *T. matsutake* were detected from all inoculated soils as recovered mycelia on MNC agar plates. No microbial contamination was observed in any of the treatment or control soils.

Seedlings grown in soils inoculated with isolate #84 had the longest ectomycorrhizal root lengths and greater ectomycorrhization colonization regardless of the presence of additional nitrogen (Fig. 3A, B). The level of ectomycorrization produced in the symbiosis

with isolate #84 was similar between soil treatments (with/without added nitrogen), but for isolates #45, #79, and #121, the level of ectomycorrhization was significantly higher in the presence of added nitrogen ($P < 0.05$).

Inoculation with isolates #111 and #126 produced significantly higher seedling biomasses than the controls when no supplementary nitrogen was added to the soil (Fig. 3C; $P < 0.05$). Seedling biomasses were not significantly different from the controls in the absence of supplementary nitrogen when the other isolates were inoculated, although inoculation with isolate #121 showed a trend toward higher seedling biomass than the control ($P < 0.1$). When supplementary nitrogen was added, total plant biomass was not significantly different from the control in any isolate inoculations, although inoculation of isolate #84 did show a trend toward higher biomass than the control ($P < 0.1$). Total plant biomass was generally higher in the no supplementary nitrogen soil condition than in the supplementary nitrogen soil condition, and the control and inoculations of isolates #31, #99, #111, #121, and #126 showed significant differences ($P < 0.05$) in response to the soil nitrogen condition.

Shoot biomasses varied among fungal isolate inoculations (Fig. 3D). When no nitrogen was added, shoot biomasses were significantly larger than in the control after inoculations with six of the isolates ($P < 0.05$). When supplementary nitrogen was added, shoot biomass significantly exceeded that of the control only when isolate #84 was inoculated ($P < 0.05$). Shoot biomass was generally higher in the soil condition with no supplementary nitrogen than in soil with added nitrogen, and inoculations of isolates #31, #99, #111, #121, and #126 showed significant differences ($P < 0.05$) in response to the soil nitrogen condition.

When no supplementary nitrogen was added, root biomass was significantly higher than the control only when isolate #126 was inoculated (Fig. 3E). When supplementary nitrogen was added, root biomasses were not significantly different from the control across all fungal isolate inoculations. Root biomass was generally higher in soil without supplementary

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843 nitrogen than in the supplementary nitrogen condition, and the control and inoculations of
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845 isolates #31, #52, #79, #111, #121, and #126 showed significant differences ($P < 0.05$) in
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847 response to the soil nitrogen condition.
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850 The S/R ratio of seedlings (Fig. 3F) showed a similar pattern to that of shoot dry weight
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852 (Fig. 3D). Inoculations of isolates #111 and #121 showed significantly higher S/R ratios in
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854 the condition without supplementary nitrogen than in the control. S/R ratio was significantly
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856 higher in the supplementary nitrogen condition than in the control only in the inoculation of
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858 isolate #111. S/R ratio was generally the same for each isolate between soil nitrogen
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860 conditions, but was significantly higher in the supplementary nitrogen condition than in the
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862 no supplementary nitrogen condition when isolates #52 and #79 were inoculated.
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865 Soil nitrogen addition influenced the measured plant parameters (Table 1). Fungal
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867 isolate identity affected host shoot weight, the S/R ratio, and ectomycorrhizal biomass.
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869 Because the level of ectomycorrhizal colonization varied markedly among fungal isolates and
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871 between two levels of nitrogen fertilization, we used regression analysis to investigate the
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873 relationship between ectomycorrhization level and host biomass. Although the
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875 ectomycorrhization level was not correlated with plant biomass when no supplementary
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877 nitrogen was added, the two variables were significantly positively correlated when
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879 supplementary nitrogen was available (Fig. 4).
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884 *3.2 Ectomycorrhization following inoculations of mixtures of sibling isolates into Koshibu*
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888 Ectomycorrhizas formed following all inoculations except when isolate #99 was
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890 inoculated alone. No ectomycorrhizas formed on the roots of uninoculated control seedlings.
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892 Mycelia of *T. matsutake* were detected from all inoculated soils as recovered mycelia on
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894 MNC agar plates. No microbial contamination occurred in any of the soils, including the
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903 controls.
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906 The longest ectomycorrhizal root length was obtained when paired mixtures of isolates
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908 #52/#99 were inoculated (Fig. 5A), and was significantly longer than that developed
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910 following single inoculation with isolate #52 ($P < 0.05$; *t*-test). Ectomycorrhizal root lengths
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912 measured following paired inoculations of isolates #52/#84 tended to be longer than those
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914 measured following single inoculation of either isolate ($P > 0.1$; *t*-test). The paired inoculation
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916 of isolates #84/#99 produced shorter ectomycorrhizal root lengths than the single inoculation
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918 of isolate #84 ($P < 0.1$; *t*-test). In the triple inoculation experiments, ectomycorrhizal root
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920 lengths that developed in Arrangement-A tended to be longer than those that developed
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922 following single inoculations of isolates #52 and #84 ($P > 0.1$; *t*-test), but were similar to the
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924 average ectomycorrhizal root lengths that developed following inoculation with three paired
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926 isolates (#52/#84, #84/#99, and #52/#99). The average ectomycorrhizal root lengths that
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928 developed in Arrangement-B were similar to those that developed following single
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930 inoculations of isolates #52 and #84, but were significantly smaller than the average
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932 ectomycorrhizal root lengths that developed following paired inoculations of these three
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934 isolates ($P < 0.01$; *t*-test).
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937 Host plant biomass was significantly greater in the control than in treatments inoculated
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939 with isolate #52, especially in shoots (Fig. 5B, C). Shoot biomasses were also significantly
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941 reduced following single inoculations of isolates #84 and #99, all three paired inoculations,
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943 and in the Arrangement-B ($P < 0.05$). The root mass that developed following the inoculation
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945 of isolate #52 showed a lower trend than that in the control, but this was not the case for other
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947 isolates. Ectomycorrhization level was not correlated with plant biomass (Fig. 6).
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952 *3.3 RFLP patterns in the IGS1 region of ectomycorrhizal root tips following mixed isolate*
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We analyzed a single ectomycorrhizal root tip from each harvested ectomycorrhizal sample. Most samples, except for one obtained following triple inoculation in Arrangement-B (sample ID: B3-2), had a single PCR product of *ca*. 460 bp. Samples from paired inoculations had a single RFLP pattern, i.e., 310 bp and 110 bp bands (Fig. 7), which was identical to the pattern for isolates #52 and #84. Most samples from Arrangement-A also had a RFLP pattern identical to that of isolates #52 and #84; sample ID: A5-2 was an exception, as it had a RFLP pattern identical to that of mixed isolate #99 (205 and 110 bp) and those of the other two isolates, i.e., 310, 205, and 110 bp bands. Most samples from Arrangement-B also had a RFLP pattern identical to that of isolates #52 and #84, but sample ID: B5-3 had a RFLP pattern identical to that of a mixture of three isolates.

3.4 Retrotransposon-based molecular marker (LTR) detection of inoculated isolates in ectomycorrhizal root tips that grew after mixed isolate soil inoculation

The LTR markers were able to detect the three individual sibling isolates. The primer pairs pL281/pS48, pDGSL719-2/pS48, and the single primer pS1 performed especially well (Supplementary Figs. S1–S5; Table 2). However, PCR amplicons obtained using the primer pair pDGSL313-1/pS48 did not clearly distinguish the individual *T. matsutake* isolates (data not shown). Two samples from the Arrangement-B triple inoculation experiment were not included the LTR analysis because the DNA samples were insufficient for PCR. Isolates #52 and #84 were regularly detected in single root tips by LTR, even though this was not possible in the IGS1 analysis because the isolate RFLP patterns were identical. The detection ratio for isolate #52 was higher than the detection ratio for #84 (Table 2). Isolate #99 was rarely detected by either LTR or RFLP analyses (Fig. 7). None of the three isolates was detected in the ectomycorrhizal root tips after triple soil inoculations.

3.5 Comparison of DNA markers that distinguished sibling isolates of T. matsutake

In our microsatellite marker analysis, primers Trma01 and Trma16 produced two band patterns in the cross comparison of the three sibling isolates #52, #84, and #99 (Fig. 8). However, Trma02, Trma07, Trma08, and Trma14 produced a single pattern. We tested Trma01 and Trma16 on the nine sibling isolates. Trma01 and Trma16 produced only two band patterns (Fig. 9). This microsatellite marker was, therefore, considered inadequate for discriminating sibling isolates. Hence, we did not use microsatellite markers for the analysis of ectomycorrhizal root tips.

Discussion

Sibling spore isolates of *T. matsutake* obtained from a single basidioma varied greatly in their *in vitro* ectomycorrhizal colonization ability when tested on *P. densiflora* seedlings. The patterns of variation differed between soil nitrogen levels (Fig. 3). Thus, the matsutake–pine associations appear to be influenced by the fungal genetic background, although the effects of the pine genetic background should not be ignored (Karst et al., 2009). We were unable to consider the genetic background of pine seedlings based on our experimental data, which is a weak point in the present study. The variability in colonization abilities among isolates was not apparent in the growth patterns of the nine isolates on MNC agar media. These nine isolates were selected from >100 based on their mycelial growth ratios. The slow-growing isolates #31 and #99 (on MNC agar) (Yamada et al., 2019) had reduced levels of ectomycorrhizal development, but the rapidly growing isolate #79 (on MNC agar) also had a reduced level of ectomycorrhizal development in the absence of nitrogen fertilization (Fig. 3). In contrast, the slow-growing isolates #84 and #111 (Yamada et al., 2019) had higher levels of ectomycorrhizal development, regardless of soil nitrogen level. Therefore, the growth of different *T. matsutake* isolates on nutrient agar is not a good predictor for selecting appropriate strains for symbiotic

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development with pine tree hosts. Nevertheless, most of the intraspecific data available for ectomycorhizal fungus performance has been obtained from cultured mycelial isolates grown on nutrient agars in the absence of host plants (e.g., Cairney, 1999; Colpaert et al., 2000; Guidot et al., 2005; Wilkinson et al., 2010), and the isolates in these studies often had different geographic origins or different host plant species. These combinations of isolates with different provenances have much more diverse genetic backgrounds than sibling isolates from a single basidioma. A very diverse genetic background hampers the determination of the genetic components of the fungus-plant interaction.

Importantly, we found that sibling isolates of *T. matsutake* can together colonize a host root system, thereby increasing ectomycorrhizal biomass. The combined presence of inoculates #52 and #99 increased the ectomycorrhizal biomass by a factor of five in comparison with biomasses resulting from single isolate inoculations (Fig. 5). Our DNA analysis of this paired inoculation (#52 and #99) demonstrated a significant dominance of #52 over #99 (Table 2). Colonization by isolate #99 was only detected in two of the triple inoculation samples (Fig. 7). Thus, competition among sibling isolates appears to promote ectomycorrhizal development by enhancing fungal colonization ability during occupation of the root system. This "competitive activation" hypothesis is also congruent with data from the triple inoculation experiment, especially in the case of Arrangement-A, in which we expected more competition among sibling mycelia than in Arrangement-B. Arrangement-A inoculation resulted in considerable ectomycorrhizal biomass. The combination of the two isolates #52 and #84 doubled the ectomycorrhizal biomass over levels produced in the single inoculations of these isolates (Fig. 5); our DNA analysis demonstrated co-dominance of these two isolates (Table 2). The boosting effect on ectomycorrhization resulting from mixed inoculations of *T. matsutake* isolates to a single host should have commercial implications for the production of ectomycorrhizal pine seedlings for cultivation and future field transplantation trials. However,

the combination of isolates #84 and #99 was a neutral association, i.e., ectomycorrhizal biomass did not exceed the average value of the single inoculations, and the DNA analysis demonstrated complete dominance of isolate #84 in the association. Hence, the competitive activation hypothesis was not applicable to the #84/#99 combination. In our paired and triple inoculation experiments, we did not observe the priority effect of ectomycorrhizal competition that has been reported for *Rhizopogon*-pine combinations. The timing of ectomycorrhizal colonization (growth rate of ectomycorrhizas) strongly affects interspecific competition between *Rhizopogon* species (Kennedy and Bruns, 2005; Kennedy et al., 2009). Our experimental data and the competitive activation hypothesis partially explain why large mycelial colonies of *T. matsutake* may be maintained over long periods (decades) (Hamada, 1970, Lian et al., 2006; Yamada et al., 2010, Narimatsu et al., 2015). If competition among sibling isolates in a shiro were to decrease annually or cease completely due to reductions in basidiospore dispersal from epigeous basidiomata or from outside the shiro, a shiro mycelium would likely decline gradually in area and biomass.

The genetic mosaic structure of the *T. matsutake* shiro mycelium was recently demonstrated using fine DNA markers that distinguish genets of this fungus (Murata et al., 2005b; Lina et al., 2006). The large shiro mycelium of *T. matsutake* in the soil assumes a circular shape at ground level, often forming fairy rings of basidiomata (Hamada, 1970; Ogawa, 1978; Narimatsu et al., 2016). These rings have been a focus of attention among mycologists interested in their genetic construction, i.e., single genets or consortia of genets? A mosaic structure was demonstrated by analyses of basidiomata (Murata et al, 2005b; Lina et al, 2006), shiro mycelia, and ectomycorrhizal root tips in the soil at a resolution scale of 30 cm between soil samples (Lina et al., 2006). In the present study, we found evidence that sibling isolates provided a mosaic structure even in a single ectomycorrhizal root tip, as speculated by Murata et al. (2005b), but deemed unrealistic by Lina et al. (2006): paired inoculations of isolates #52

and #84 consistently co-existed in single ectomycorrhizal root tips (Table 2). *Tricholoma matsutake* does not have a demarcation line between different dikaryotic isolates when they are paired on nutrient agar plates (Ogawa, 1978; Yamada et al., 2019), unlike other diverse saprobic mushroom fungi (Esser, 2006; Boddy et al., 2007) or several ectomycorrhizal mushroom taxa that have been tested (Fries, 1987; Dahlberg, 1995). Although ectomycorrhizal fungi, such as *Pisolithus*, have functional territoriality and distinct boundaries between different dikaryotic isolates in symbioses with host plants (Wu et al., 2012), our data indicate that *T. matsutake* has a different mycelial strategy in the vegetative growing phase. However, the mechanism by which genetically different dikaryons can grow in a single ectomycorrhizal root tip to build a general symbiotic structure is as yet unexplored. Although our data are not definitive, there may be an unknown genetic mechanism in the basidiomycetous ectomycorrhizal fungi. We strongly recommend that future studies focus on ectomycorrhizations initiated from basidiospores or monokaryotic mycelial isolate inocula. Monokaryotic isolates of *T. matsutake* growing on agar medium have recently been reported; the mycelial growth rates were slow (Murata et al., 2015a). If di-mon mating between a dikaryotic shiro mycelium and a monokaryotic mycelium were to occur, some combinations of previously existing dikaryotic shiro mycelium and newly established dikaryotic shiro mycelium should initiate competitive activation within a single shiro structure.

We confirmed the co-existence of two sibling isolates in a single ectomycorrhizal root tip using IGS1 and LTR markers (Fig. 7, Table 2). PCR-RFLP analysis of the IGS1 region has demonstrated variation in *T. matsutake* populations (Guerin-Laguette et al., 2002; Matsushita et al., 2005), but not in the ITS region that is used to distinguish species. The IGS1 analysis of isolates #52 and #84 in the present study produced the same patterns (Fig. 5). Although both LTR and microsatellite markers distinguished the sibling isolates #52, #84, and #99 (Table 2, Fig. 8), the microsatellite markers did not fully discriminate these three isolates

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when they were mixed in a single DNA sample, likely because these markers provided only three patterns among the 9 sibling isolates tested when using primers Trma01 and Trma16 (Fig. 7). LTR markers were previously reported to discriminate among the nine sibling isolates that were tested for ectomycorrhizal synthesis in the present study (Yamada et al., 2019). Therefore, the controversy over whether a single basidioma of *T. matsutake* can provide different heterozygotic genets (Murata et al., 2005b; Lian et al., 2006) may be a result of the different discriminating abilities of the DNA markers used. The results of our LTR marker analysis were largely congruent with those of Murata et al. (2005b). The shiro mycelium reported by Lain at al. (2006) should perhaps be re-analyzed with finer DNA markers. The DNA analysis of a single ectomycorrhizal root tip conducted in this study (Fig. 7, Table 2), however, does not completely preclude shortcomings. The result may reflect contamination of extramatrical hyphae of one isolate on an ectomycorrhizal root tip colonized by another isolate. Our future research should, therefore, include a study with a higher burden of proof involving DNA analysis of sectioned root fragments in an ectomycorrhizal root tip.

We used “Tera” and “Koshibu” soils in our *in vitro* experiments. With Tera soil we observed the symbiotic effect of *T. matsutake* on pine growth and a positive correlation between ectomycorrhizal root length and pine seedling biomass (Fig. 4). In the experiment using Koshibu soil we found a commensal effect of *T. matsutake* inoculation on pine growth, but no correlation between ectomycorrhizal root length and pine seedling biomass. We have consistently reported the symbiotic effect of *T. matsutake* on pine growth *in vitro* when using different soil samples (Yamada et al., 2006, Murata et al., 2013, Saito et al., 2018). The Koshibu soil experiment was the first to show a commensal effect of *T. matsutake* on pine growth. This soil had a higher phosphorus content than Tera soil, but the nitrogen contents were similar. The biomass of seedlings in Koshibu controls exceeded the biomass in Terra soil controls (P = 0.138; t-test). Therefore, we suggest that the commensal effect of *T. matsutake* on pine growth

in Koshibu soil was not related to soil nutrient deficiencies. However, as ectomycorrhizal root length and seedling biomass in Tera soil were positively correlated only when supplementary nitrogen was added, the N/P balance in Koshibu soil may have influenced the commensal effect of *T. matsutake* on pine growth. The ectomycorrhizal root lengths of #52-inoculated pine seedlings were highly similar between Tera and Koshibu soils (P = 0.432; *t*-test), but the ectomycorrhizal root lengths of #84-inoculated pine seedlings were significantly lower in Koshibu soil than in Tera soil (P = 0.018; *t*-test). These isolate-dependent responses in ectomycorrhizal development should be further studied to identify the factors that were the primary determinants of these experimental outcomes. We recently reported that some specific combinations of *T. matsutake* isolates and soil types had positive effects on both ectomycorrhizal length and pine seedling biomass *in vitro* (Saito et al., 2018). Thus, the following isolate/soil combinations will be beneficial for the production of matsutake-associated pine seedlings that may be used in future cultivation studies of this fungus: isolate #84 in Tera soil, paired isolates #52/#99 in Koshibu soil, and paired isolates #52/#84 in Koshibu soil. The granite-based, weathered B-layer mineral soils used in the present study and in our previous work (Yamada et al., 2006, 2010; Saito et al., 2018) were deficient in both nitrogen and phosphorus. This type of soil is quite common in the Japanese natural habitat of *T. matsutake* in *P. densiflora* forests (Ogawa, 1978; Vaario et al., 2017; Saito et al., 2018). Nitrogen addition to these soils at a rate of 1.0 g Ebios dried yeast/L soil relieved likely nutrient deficiencies and increased both ectomycorrhizal and host pine growth *in vitro* (Yamada et al., 2006; Kobayashi et al., 2007), but overdosing at a rate of 10 g Ebios/L soil had negative effects (unpublished data). Nitrogen addition to forest soils decreases the external mycelium of ectomycorrhizal fungi (Nilsson and Wallander, 2003) and changes their species composition (Peter et al., 2001; Parrent and Vilgalys, 2007). Therefore, optimization of soil nutrient conditions for better growth of both ectomycorrhizas and hosts in the matsutake-pine

association in this soil type is desirable for further practical cultivation studies of this mushroom.

Disclosure

The authors declare no conflict of interest. All of the experiments undertaken in this study comply with the current laws of Japan.

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Figure Legends

Fig. 1. Inoculation locations of *Tricholoma matsutake* mycelium in the triple inoculation experiment. Two configurations (A, B) were set up in this experiment. The numbers in each circle enclosed by a dashed line are the code numbers of the inoculated isolates. Dashed straight lines show where the soil was separated, when the root system was measured, and the root tips were sampled for fungal DNA content.

Fig. 2. Ectomycorrhizas synthesized *in vitro* following inoculation of single *Tricholoma matsutake* isolates. (A) External morphology of a Y-shaped ectomycorrhizal root tip grown in soil inoculated with isolate #84 without additional nitrogen. (B) External morphology of young ectomycorrhizal root tips grown in nitrogen-fertilized soil inoculated with isolate #84. (C) Transverse section of ectomycorrhizal root tip grown in soil inoculated with isolate #84 without additional nitrogen. (D) Semi-longitudinal section of young ectomycorrhizal root tip grown in nitrogen-fertilized soil inoculated with isolate #126. Arrows indicate Hartig net hyphae in the root cortex. Ep, epidermal cells, some of which have tannin cell characteristics (C); Co, cortical cell; En, endodermal cell; Nu, nucleus of a cortical cell. Bars, 0.5 mm (A, B), 20 µm (C, D).

Fig. 3. Ectomycorrhizal development and host biomasses following the inoculation of single *Tricholoma matsutake* isolates into Tera soil. (A) ectomycorrhizal root length; (B) ectomycorrhizal colonization ratio; (C) total seedling biomass; (D) shoot biomass; (E) root

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biomass; (F) shoot/root biomass ratio. Values are means + SE ($n = 6$). S, significant difference between treatment and control means ($P < 0.05$); s, significant difference between treatment and control means ($P < 0.1$); *, significant difference between means with and without supplementary nitrogen ($P < 0.05$; *t*-test); **, significant difference between means with and without supplementary nitrogen ($P < 0.1$; *t*-test); Cont, control. Different upper and lower case letters (A–C, a–d) identify significant pairwise differences between *T. matsutake* isolates in each soil treatment ($P < 0.05$).

Fig. 4. Relationships between biomass and ectomycorrhizal root length in ectomycorrhizal seedlings separately inoculated with each of nine *Tricholoma matsutake* isolates. Controls were not inoculated. Treatments were applied with or without supplementary nitrogen in the Tera soil substrate. Data were extracted from Fig. 3 and subjected to correlation analysis ($n = 60$).

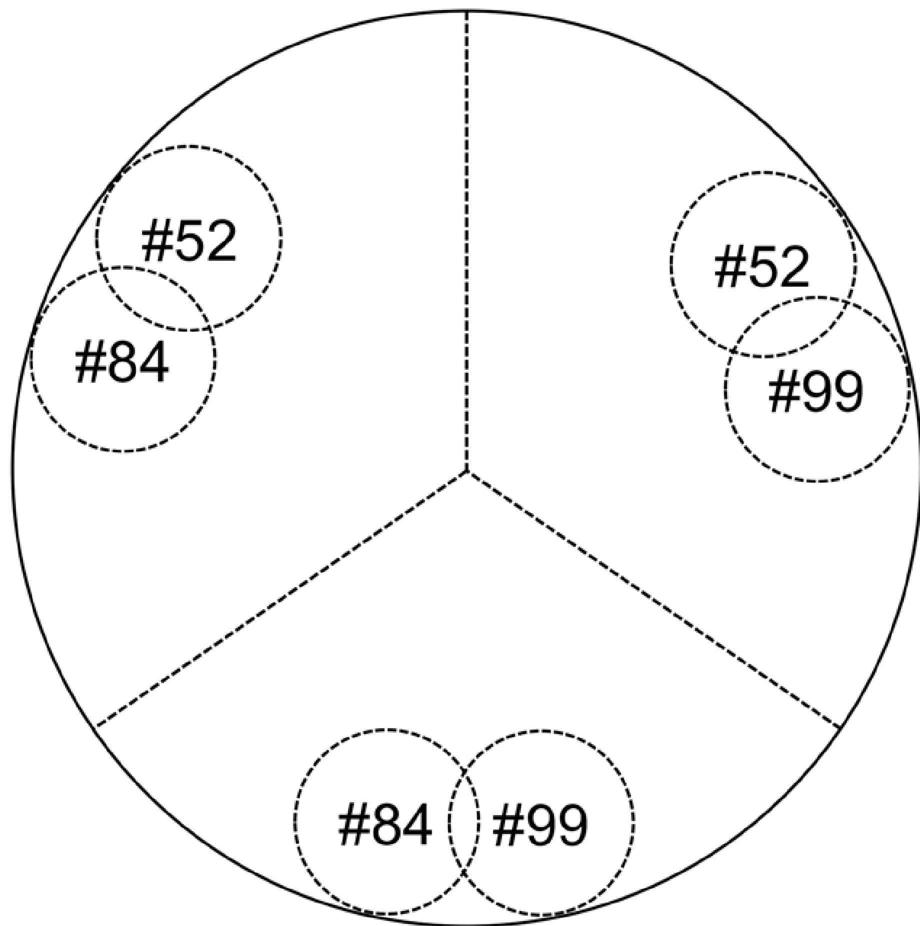
Fig. 5. Ectomycorrhizal development and host biomasses after single and paired inoculations of three *Tricholoma matsutake* isolates into Koshibu soil. (A) seedling total biomass and ectomycorrhizal root length; (B) shoot and root biomasses; (C) ectomycorrhizal colonization ratio and shoot/root biomass ratio. Values are means + SE ($n = 5$). S, significant difference between treatment and control means ($P < 0.05$); s, significant difference between treatment and control means ($P < 0.1$). Different lower case letters (a, b) identify significant pairwise differences between means ($P < 0.05$).

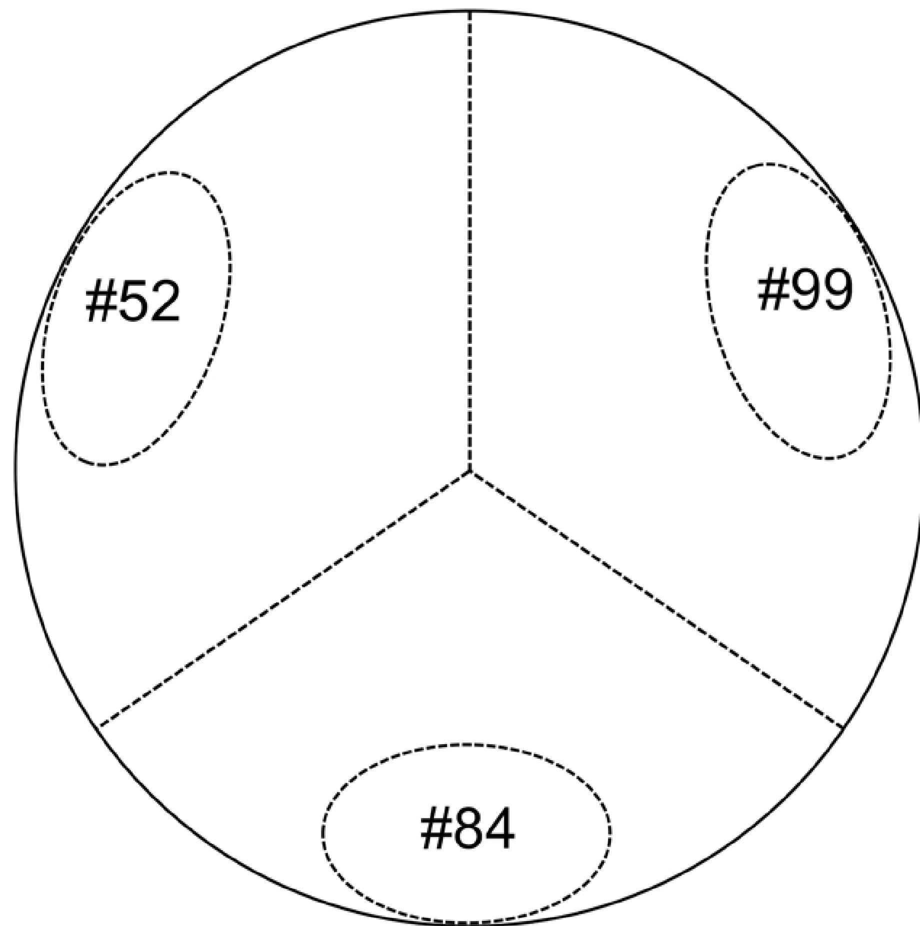
Fig. 6. Relationships between biomass and ectomycorrhizal root length in ectomycorrhizal seedlings inoculated with three selected *Tricholoma matsutake* isolates and control seedlings. All seedlings were grown in Koshibu soil. The graph was plotted from data extracted from Fig. 5; the correlation coefficients were calculated from these data ($n = 45$).

Fig. 7. Restriction fragment length polymorphism (RFLP) patterns in the intergenic spacer (IGS) 1 region of the rRNA gene cluster obtained from ectomycorrhizal samples that developed after mixed inoculations of three sibling *Tricholoma matsutake* isolates. Paired inoculations of three sibling isolates (#52/#84, #52/#99, and #84/#99) and triple inoculations of these three isolates in Arrangement-A (A1–A5) and Arrangement-B (B1–B5) (Fig. 1) were performed. M: molecular ladder marker (100 bp–3 kbp).

Fig. 8. Polymorphic patterns of six microsatellite markers in three sibling *Tricholoma matsutake* isolates. In the Trma01 electrophoresis, isolates #52 and #84 produced identical bands; isolate #99 had a slightly larger band size (two bands present). In the Trma16 electrophoresis, isolates #84 and #99 had identical bands, but isolate #52 had a slightly smaller band size. The remaining four markers had the same band pattern among the three isolates. NC, negative control; M, DNA ladder marker (100–1,000 bp).

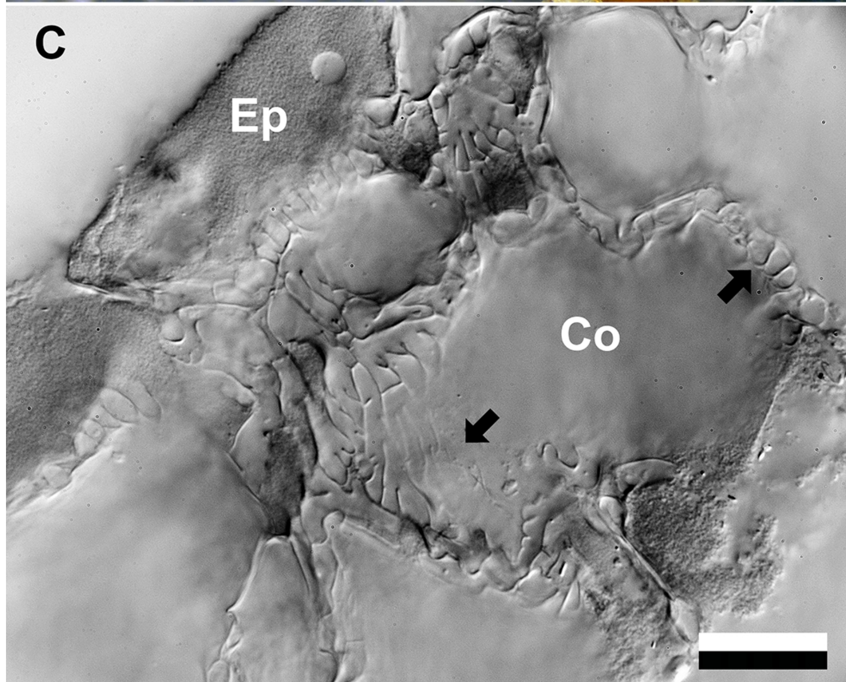
Fig. 9. Polymorphic patterns of two microsatellite markers in nine sibling *Tricholoma matsutake* isolates. In the Trma01 electrophoresis, seven sibling isolates (#31, #45, #52, #84, #111, #121, and #126) had identical single-band patterns; the remaining two (#79 and #99) had identical band patterns. Isolate AT-0740 (740 in the figure) had a unique three-band pattern. Y1 had a band pattern identical to those of #52 and #84. In the Trma16 electrophoresis, eight sibling isolates had identical two-band patterns. Isolate #52 had a single-band pattern. Isolate AT-0740 produced no bands. The band pattern of Y1 was identical to that of #52. Isolates Y1 and AT-0740 were known *T. matsutake* isolates (Yamada et al., 2019).

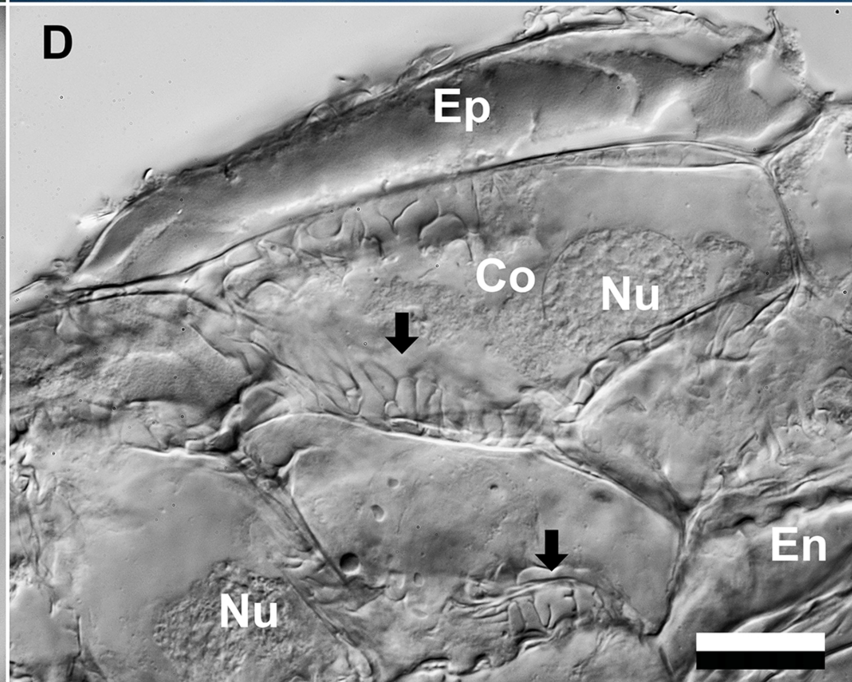

#52
#84
#52
#99
#84
#99
Arrangement-A

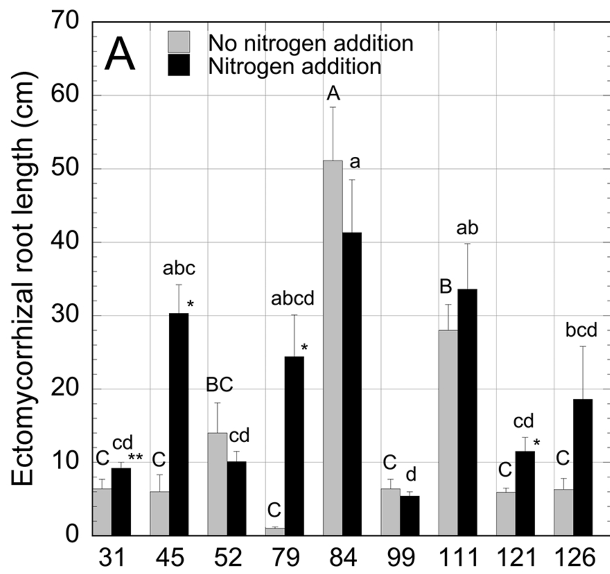

#52
#99
#84
Arrangement-B

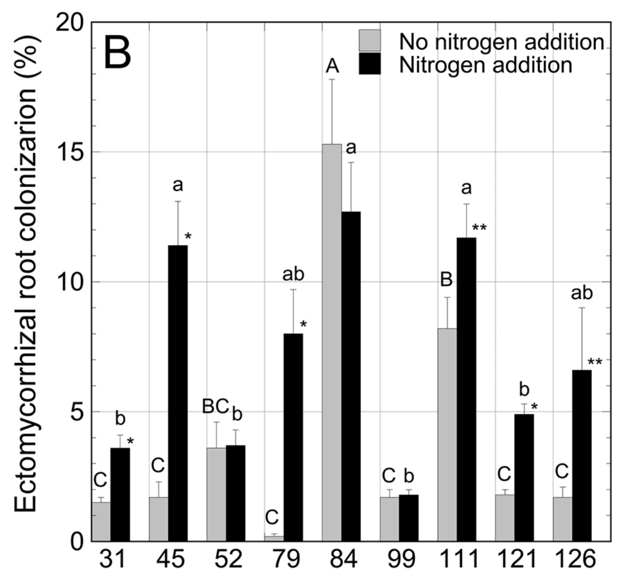
A

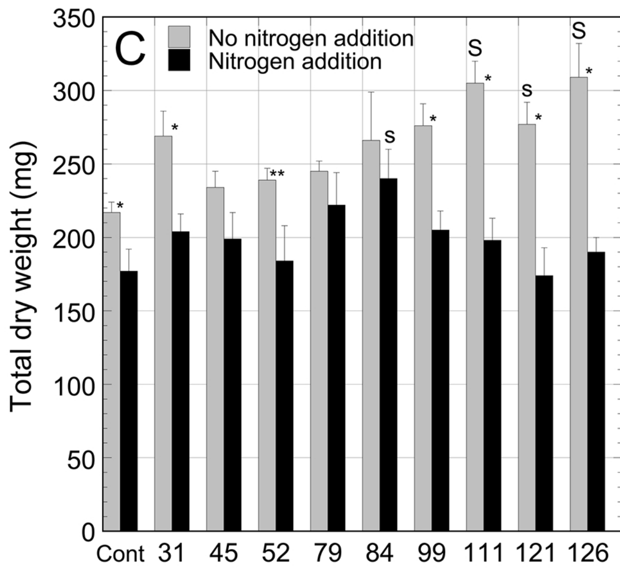
B

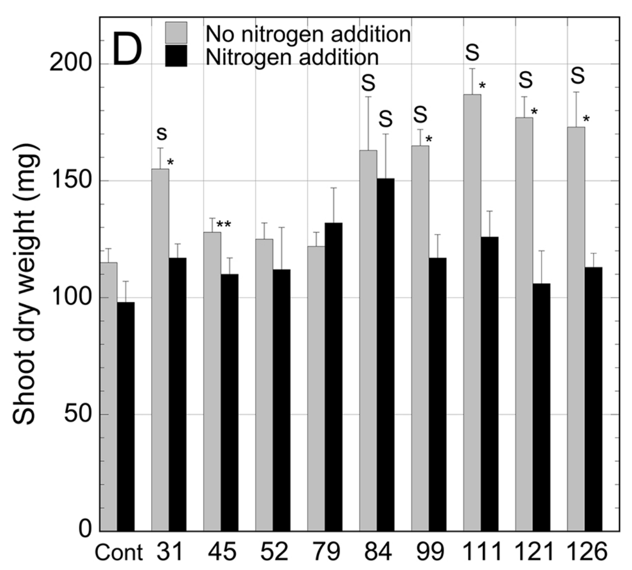

C
Ep
Co

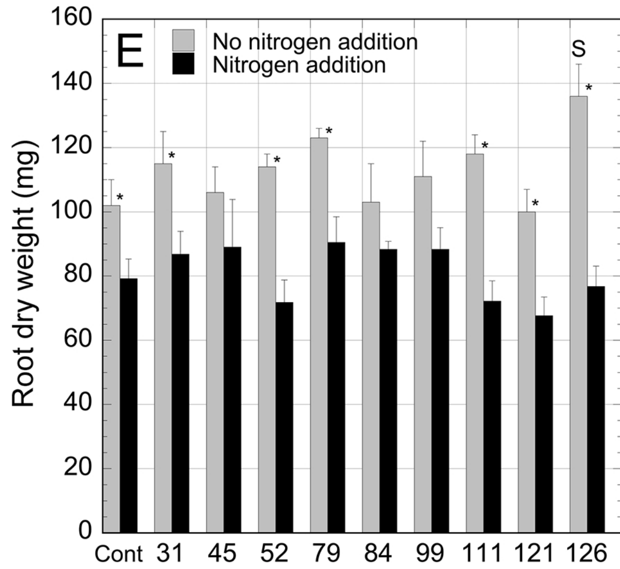

D
Ep
Co
Nu
En
Nu

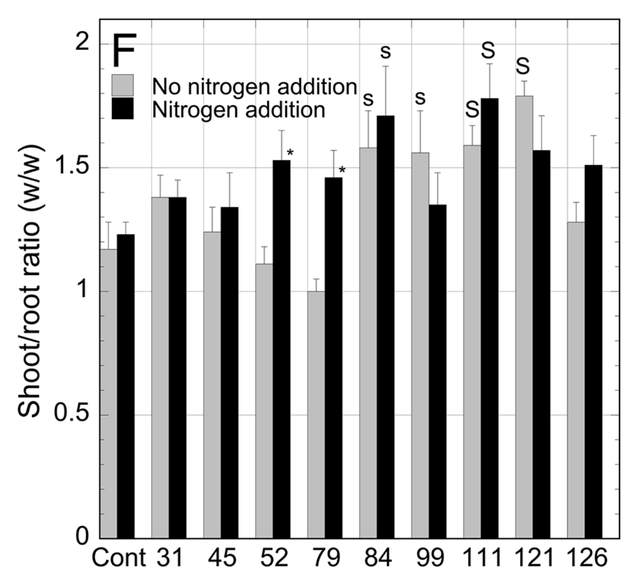

A
No nitrogen addition
Nitrogen addition
Ectomycorrhizal root length (cm)

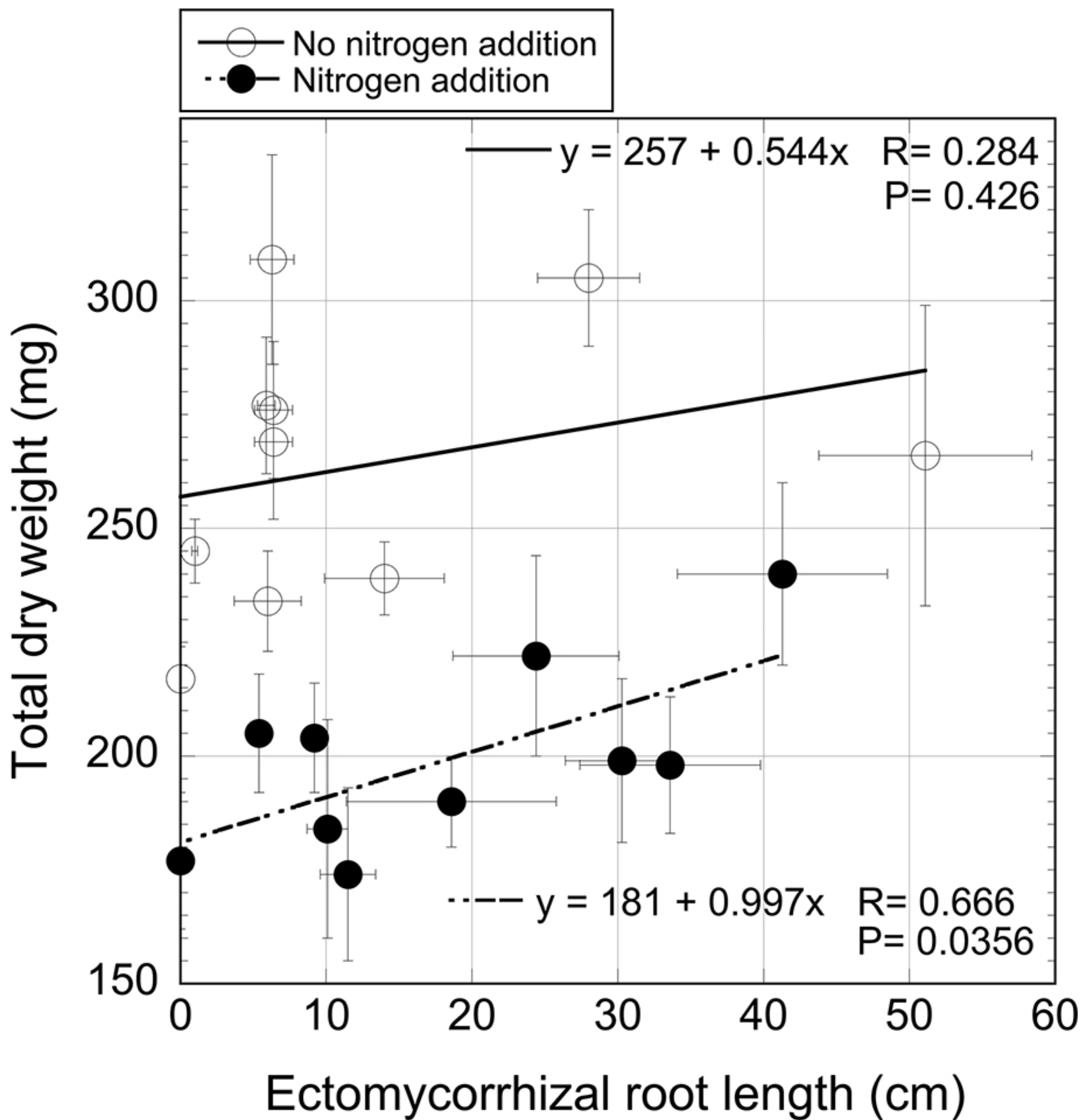

B
No nitrogen addition
Nitrogen addition
Ectomycorrhizal root colonizarion (%)

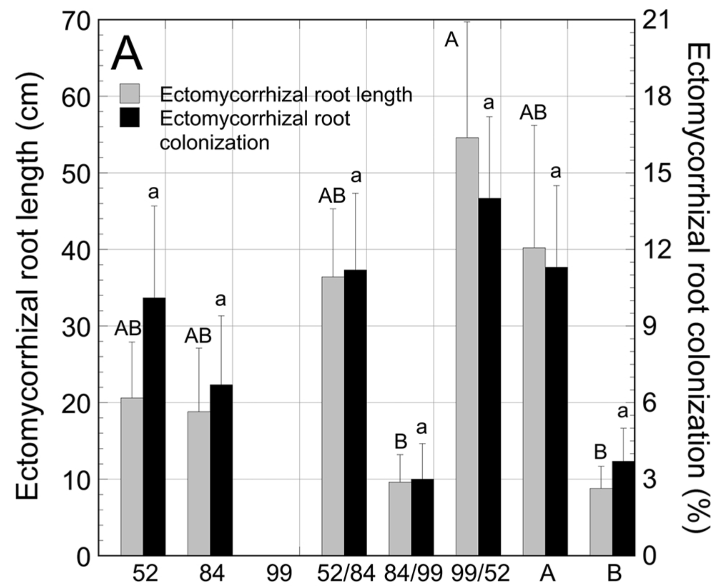

C
No nitrogen addition
Nitrogen addition
Total dry weight (mg)

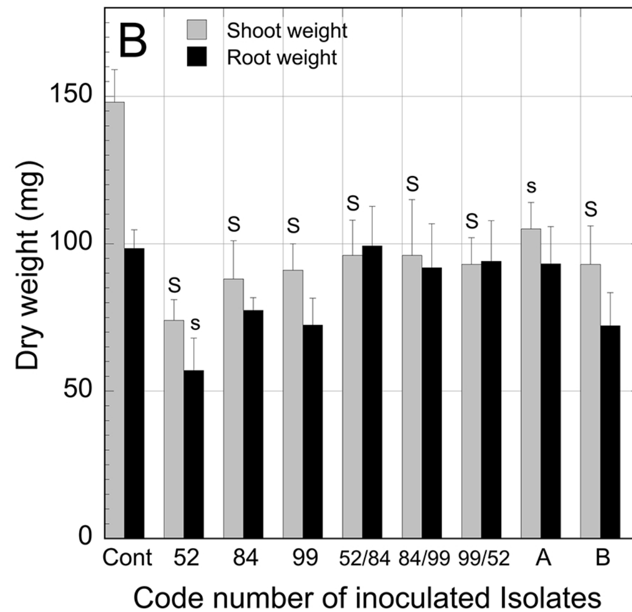

D
No nitrogen addition
Nitrogen addition
Shoot dry weight (mg)

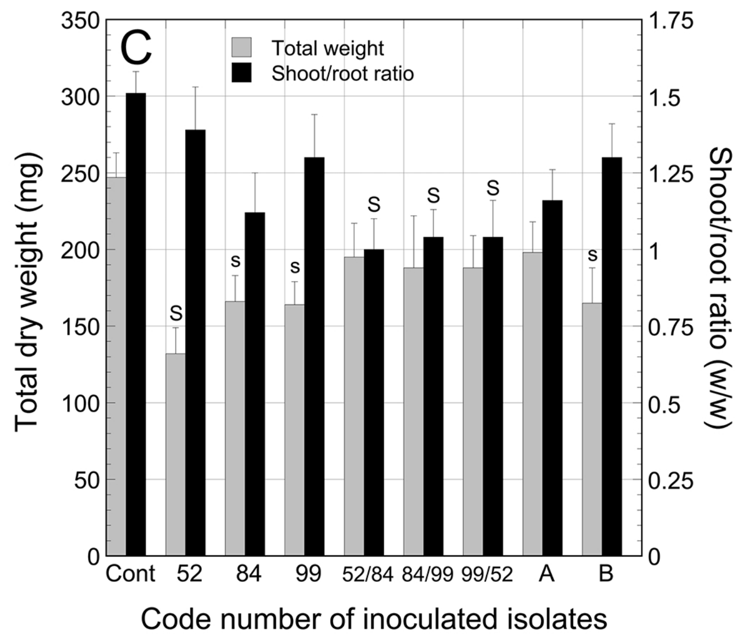

E
No nitrogen addition
Nitrogen addition
Root dry weight (mg)
Code number of inoculated isolates

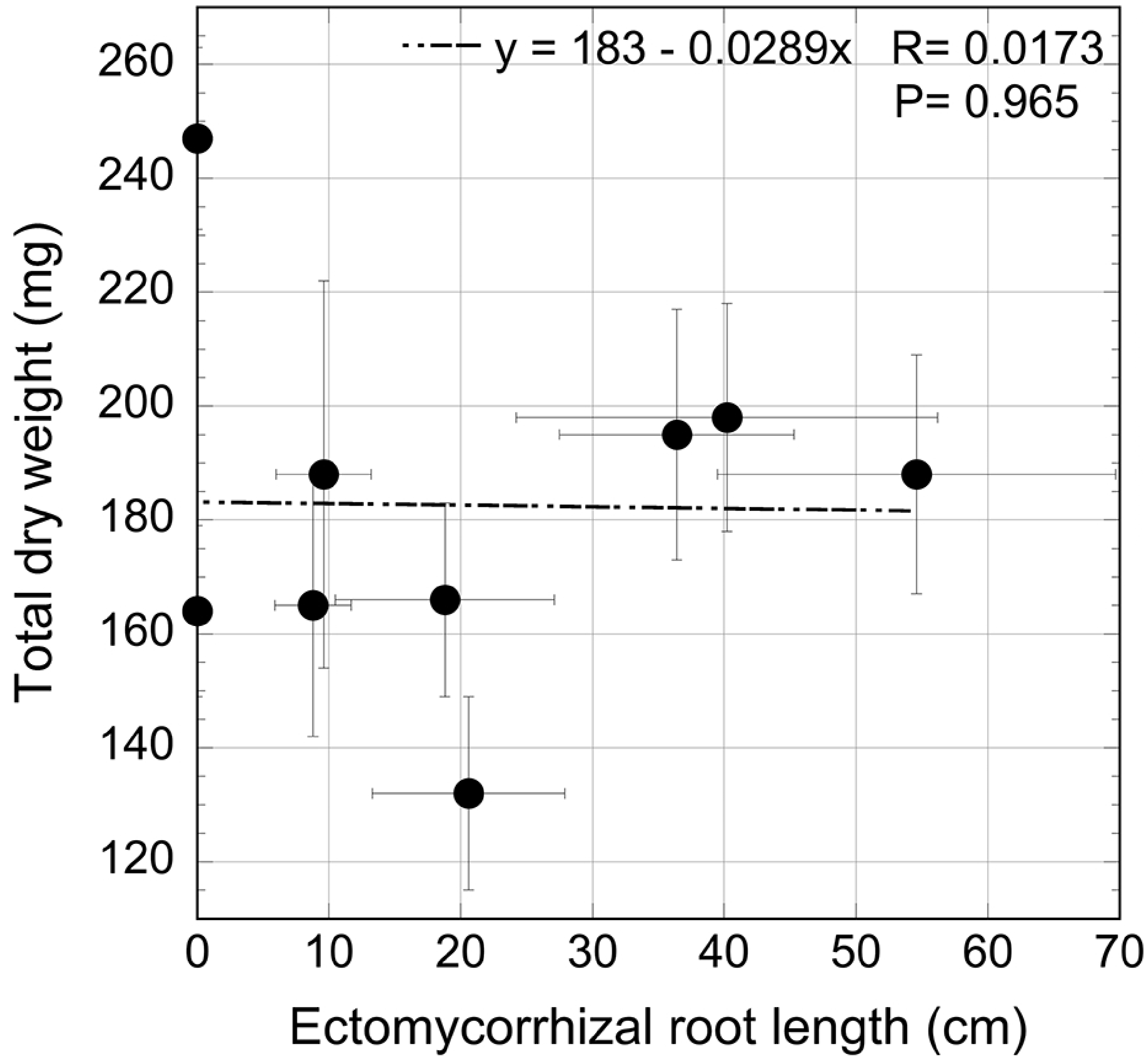

F
No nitrogen addition
Nitrogen addition
Shoot/root ratio (w/w)
Code number of inoculated isolates

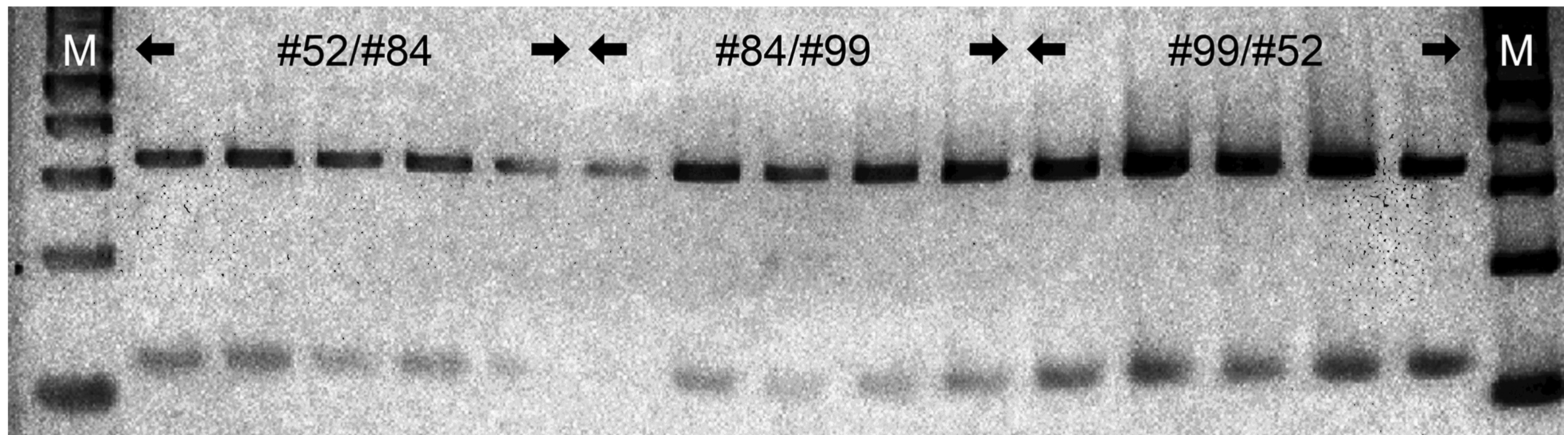

No nitrogen addition
Nitrogen addition
y = 257 + 0.544x R= 0.284
P= 0.426
y = 181 + 0.997x R= 0.666
P= 0.0356
Total dry weight (mg)
150
200
250
300
0
10
20
30
40
50
60
Ectomycorrhizal root length (cm)

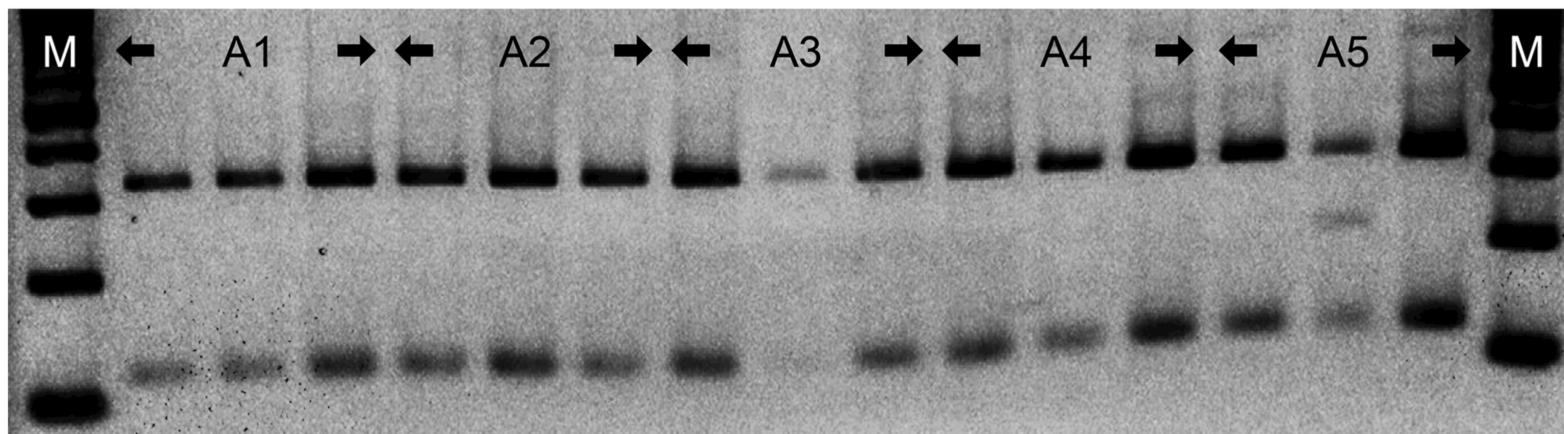

A
Ectomycorrhizal root length
Ectomycorrhizal root colonization
Ectomycorrhizal root length (cm)
Ectomycorrhizal root colonization (%)
52
84
99
52/84
84/99
99/52
A
B

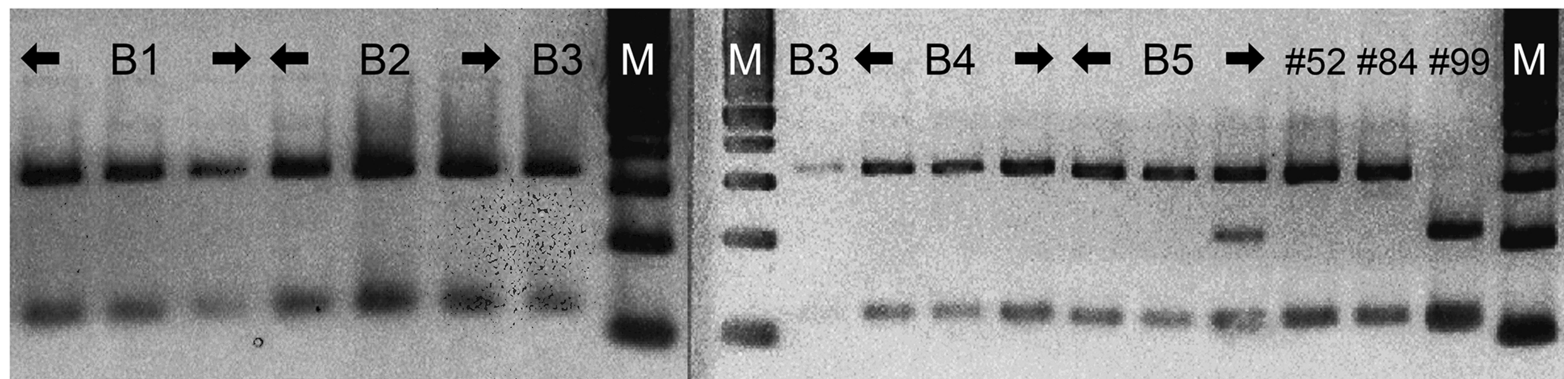

B
Shoot weight
Root weight
Dry weight (mg)
Cont
52
84
99
52/84
84/99
99/52
A
B
Code number of inoculated Isolates

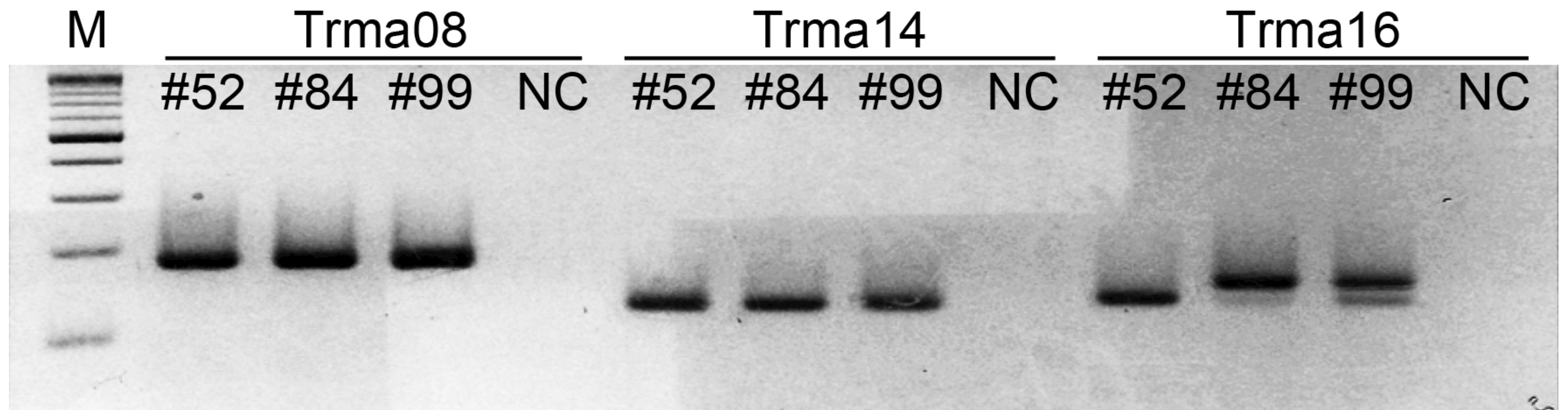

C
Total weight
Shoot/root ratio
Total dry weight (mg)
Shoot/root ratio (w/w)
Cont
52
84
99
52/84
84/99
99/52
A
B
Code number of inoculated isolates


y = 183 - 0.0289x R= 0.0173
P= 0.965
Total dry weight (mg)
260
240
220
200
180
160
140
120
0
10
20
30
40
50
60
70
Ectomycorrhizal root length (cm)


M
#52/#84
#84/#99
#99/#52
M

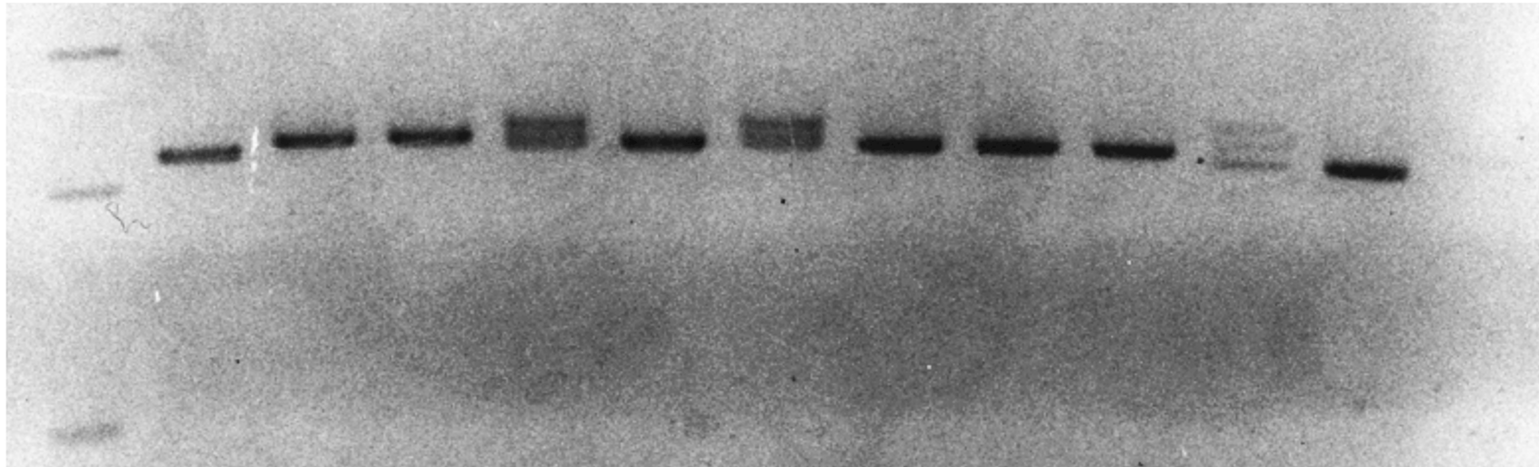

M
A1
A2
A3
A4
A5
M


B1
B2
B3
M
M
B3
B4
B5
#52
#84
#99
M


M
Trma01
#52 #84 #99 NC
Trma02
#52 #84 #99 NC
Trma07
#52 #84 #99 NC
M
Trma08
#52 #84 #99 NC
Trma14
#52 #84 #99 NC
Trma16
#52 #84 #99 NC

Trma01

M #31 #45 #52 #79 #84 #99 #111 #121 #126 740 Y1 NC



Trma16

M #31 #45 #52 #79 #84 #99 #111 #121 #126 740 Y1 NC

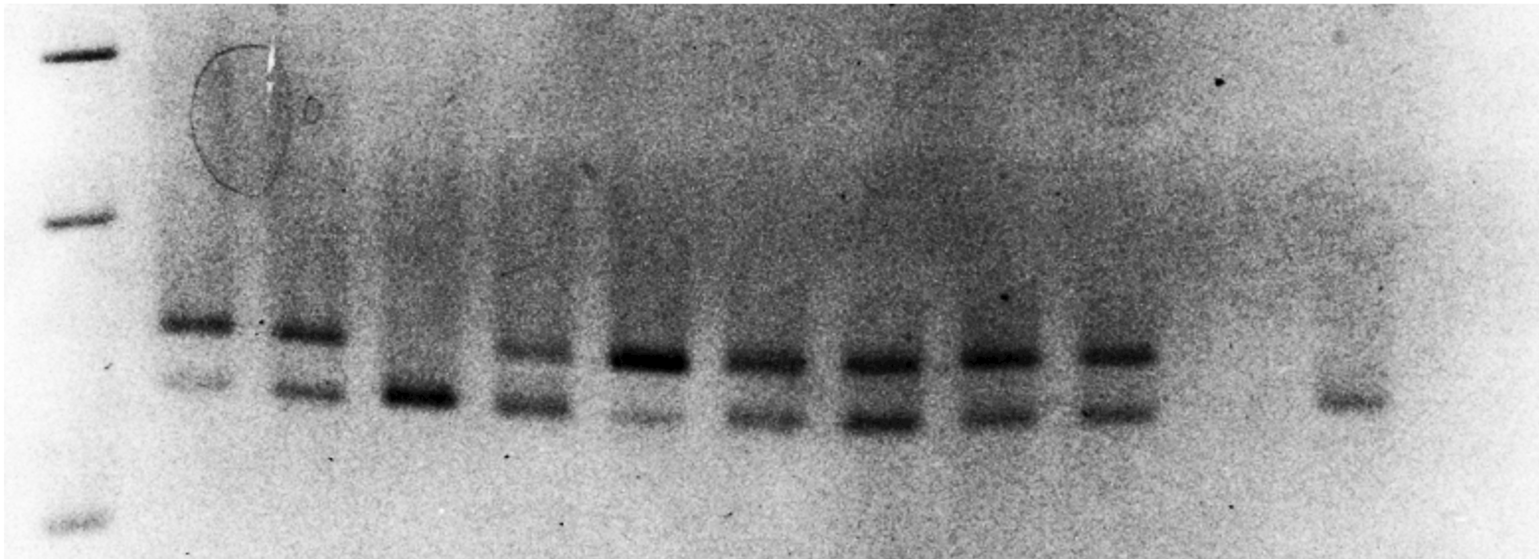


Table 1. Two-way ANOVA summary table identifying significant effects of fungal isolate identity (nine levels), nitrogen fertilizer addition (two levels), and their interaction on host plant parameters.

Parameter	Fungal isolate		Nitrogen level		Interaction	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Seedling dry weight	1.5	0.16589	63.8	<0 .0001	2.1	0.0468
Shoot dry weight	2.9	0.00608	37.4	< 0.0001	2.5	0.01883
Root dry weight	1.8	0.08767	74.4	<0 .0001	1.7	0.10468
Shoot/Root biomass ratio	4.3	0.0002	4.8	0.03156	2	0.05172
Total root length	1.0	0.47196	24.8	<0 .0001	0.4	0.90739
Ectomycorrhizal root length	21.5	<0.0001	12.2	0.00076	4.2	0.00025
Ectomycorrhizal colonization ratio	21.3	<0.0001	30.7	<0.0001	5.0	<0 .0001

Table 2. Detection of three *Tricholoma matsutake* isolates in ectomycorrhizal samples taken from soils inoculated with isolate mixtures. The retrotransposon-based molecular marker (LTR) analysis was performed with primer pair pS48/pL281.

Isolate inoculation combination	Number of ectomycorrhizal root tip samples tested	Isolate detection in ectomycorrhizal root samples		
		#52	#84	#99
#52/#84	5 (A–E)	ABCDE	A - CD -	(not subjected)
#99/#52	5 (A–E)	ABCDE	(not subjected)	- - - - -
#84/#99	5 (A–E)	(not subjected)	ABCDE	- - - - -
Arrangement-A 1	3 (A–C)	ABC	ABC	- - -
Arrangement-A 2	3 (A–C)	ABC	A - C	- - -
Arrangement-A 3	3 (A–C)	ABC	A - C	- - -
Arrangement-A 4	3 (A–C)	ABC	ABC	- - -
Arrangement-A 5	3 (A–C)	AB -	A - C	- B -
Arrangement-B 1	3 (A–C)	ABC	ABC	- - -
Arrangement-B 2	3 (A–C)	ABC	AB -	- - -
Arrangement-B 3	2 (A, B)*	AB	AB	- -
Arrangement-B 4	3 (A–C)	ABC	ABC	- - -
Arrangement-B 5	2 (A, B)*	AB	A -	- -

This table summarizes data extracted from the electrophoresed band patterns in Supplementary Figs. 1–5. See Fig. 1 for an explanation of Arrangement-A and Arrangement-B. Shared upper case letters in columns 3 and 4 indicate the presence of two isolates in a single ectomycorrhizal root tip.

*, sample data missing; -, isolate not detected