In vitro ectomycorrhization of *Tricholoma matsutake* strains is differentially affected by soil type

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Abstract

The Japanese delicacy *Tricholoma matsutake* has been conducted in vitro ectomycorrhizal syntheses for more than 20 y. The development of its ectomycorrhizal structures varies among experimental systems. Here, we examined the effects of soil-fungus interactions on the early stage of in vitro *T. matsutake* ectomycorrhization. Axenic *Pinus densiflora* seedlings were transplanted into autoclaved natural inorganic soil, inoculated with the cultured mycelium of *T. matsutake*, and incubated for 90 d in vitro. Both soil type and fungal strain significantly affected host plant growth; host plant growth and mycorrhization levels significantly differed among soil type/fungal strain combinations. Therefore, the selection of *T. matsutake* strains for optimal mycorrhization must take into account such fungal and soil properties.

Keywords: Ectomycorrhizal symbiosis, Fungal strain selection, Non-timber forest resources, Soil-fungus interaction, Wild edible mushroom

1. Introduction

The Japanese mushroom delicacy *matsutake* [*Tricholoma matsutake* (S. Ito & S. Imai) Singer] is a valuable food commodity in the Japanese market; the estimated annual domestic production is worth several billion yen (Ministry of Agriculture, Forestry and Fisheries of Japan 2017). However, annual production has declined sharply since reaching a peak of 12,000 t in 1941. The decline was a result of pine wilt disease and environmental changes in a wide range of productive forest areas (Ogawa 1978; Kishi 1995; Suzuki 2004, 2005). Annual production in recent years has generally amounted to < 100 t (Ministry of Agriculture, Forestry and Fisheries of Japan 2017), but consumer demand has required a supply of 1200–2900 t annually. Thus, 94.0–98.9% of domestic consumption has been supplied by imports from China, USA, Canada, Turkey, Morocco, Korea, Mexico, and several other countries (Murata et al. 2008, 2013; Ministry of Agriculture, Forestry and Fisheries of Japan 2017). Recovery of Japanese production in pine forests (based on the transplantation of mycorrhizal seedlings) is highly desirable. This transplantation procedure has been very successful for the production of black truffle (Wang and Hall 2004) and saffron milk cap (Guerin-Laguette et al. 2014).

In vitro procedures for ectomycorrhization of pine hosts with *T. matsutake* have been available for 20 y (Yamada et al. 2014; Yamanaka et al. 2014; Endo et al. 2015). Ectomycorrhizas of *T. matsutake* can be established in vitro on *Pinus densiflora* Siebold & Zucc. seedlings that are more than 3 mo old (Yamada et al. 1999b); only 2–4 wk are required for mycorrhizal formation after the mycelium makes contact with pine plants that have differentiated lateral root systems (Guerin-Laguette et al. 2000; Vaario et al. 2000). The symbiotic characteristics of the mycorrhizal association have been clarified by studies of the ultrastructural features of the Hartig net hyphae, the cortical cells of pine roots and their cell walls (Gill et al. 2000), and by investigation of pine growth promotion following *T. matsutake* inoculation and

ectomycorrhization (Guerin-Laguette et al. 2004; Yamada et al. 2006; Murata et al. 2013). Granite-based mineral soil substrata are able to provide *shiro* in vitro; *shiro* is a visible whitish soil-mycelium aggregate that covers many ectomycorrhizal root tips. The aggregates produced in vitro are closely similar to those that have established naturally in pine forests (Yamada et al. 2006; Kobayashi et al. 2007). *Shiro*-like mycelial structures have also been produced in vitro using organic soil substrate with adjuvants, i.e., non-ionic surfactants or vegetable oil (Guerin-Laguette et al. 2003).

Tricholoma matsutake strains have preferred host ranges among conifers (Yamada et al. 2010, 2014; Yamanaka et al. 2011, 2014) and can be host-specific (Vaario et al. 2010). Therefore, an appropriate selection of *T. matsutake* strains that form abundant ectomycorrhizas on a given host species will facilitate the mycorrhization process in vitro. In vitro mycorrhization of *T. matsutake* with host *P. densiflora* plants is affected by the nutrient composition of the soil (Yamada et al. 1999b, 2006). Soil physiochemical properties also affect the mycorrhizal relationship: mineral soils, such as a granite-based substratum, promote the formation of a densely packed soil-mycelium complex aggregated with ectomycorrhizal root systems, as indicated above. Therefore, host-fungus-soil relationships require full exploration to improve progress in *T. matsutake* strain selection procedures in vitro.

The largest dimensions of a *T. matsutake-P. densiflora* association obtained in vitro in a 1 L soil volume by Kobayashi et al. (2007) were approximately 4 g fresh shoot weight, 60 m total root length, and 10 m ectomycorrhizal root length. The mycorrhizal seedlings were established after incubation for 1 y at 20 °C under continual illumination of 100 µmol photons/m^2/s. This system produced a *shiro* structure approximately 5 cm diam on the mycorrhizal seedlings, but the procedure was very protracted; the minimum times for experimental *T. matsutake*

ectomycorrhization of juvenile pine seedlings were several times shorter (Yamada 1999b, 2006; Guerin-Laguette et al. 2004). Mycelial growth (Kawai and Abe 1976; Kawai and Ogawa 1976; Kusuda et al. 2008) and enzyme activities that catalyze carbohydrate transformation (Terashita et al. 1995; Vaario et al. 2002, 2012; Kusuda et al. 2006, 2008) differ among *T. matsutake* strains and likely affect the mycorrhizal host relationship. If procedures for obtaining better mycelial growth of *T. matsutake* on agar can also achieve better mycorrhization in vitro, these methods would facilitate the mycorrhization process in vitro. Any exploration of new procedures should incorporate soil condition as a key factor that influences fungus-plant growth. Thus, we aimed to determine how the *T. matsutake-P. densiflora* ectomycorrhizal system is affected by soil conditions in vitro.

2. Materials and Methods

2.1. Tricholoma matsutake *strains, host plant, and soil types*

Four *T. matsutake* cultured strains that differed in origin of isolation were included in our experiments (Table 1). The strains were isolated and subcultured on Modified Norkrans' C (MNC) agar medium (Yamada and Katsuya 1995) until used. The sampling sites in Nagano Prefecture were located in a mountainous region where this mushroom species is highly productive and is harvested for commercial purposes. The Y1 strain promotes *P. densiflora* seedling growth in vitro by developing ectomycorrhizal structures (Yamada et al. 2006; Murata et al. 2013). Seeds of *P. densiflora* (the host for *in vitro* mycorrhization) used in our study were collected in the experimental forest of Ibaraki Prefectural Forestry Institute, Japan, and stored in a deep freezer until used. Each of the four strains previously cultured on MNC agar plates were used for the mycelial growth test on MNC medium. A 5 × 5 mm colony segment of each strain was inoculated on fresh MNC medium and incubated at 20 °C in the dark. Colony diameters

were measured under a dissecting microscope 10, 20, and 30 d post inoculation. Seven replicates were established for each of the four strains. In addition, a 5 × 5 mm colony segment was inoculated into 10 mL of MNC liquid medium (without agar) in a wide-mouth glass bottle (75 mL volume; closed with a transparent polycarbonate screw cap) and incubated at 20 °C in the dark. Cultured mycelium was harvested from each of the liquid media 10, 20, and 30 d post inoculation and dried at 70 °C for 24 h, then weighed. Seven replicates were established for each of the four strains in each harvest period (Supplementary Table S1).

To compare soil effects in the in vitro experiments, four soil samples were obtained from soil B-layers of different montane areas in Nagano ("Chino," "Nakagawa" and "Matsumoto") and "Ibaraki" (Hitachi-ohmiya, Hitachi-ohta) Prefectures (Table 2). All of the soil samples were dried at 50 °C, sieved through a standard mesh (mesh size: 5 mm), and stored in the laboratory until used. Small amounts of these soil samples were used to measure pH and the contents of water-soluble inorganic phosphorus (PO_4^{3-}) and nitrogen (NH_4^+, NO_3^-, NO_2^-). A 100 g dried soil sample was mixed with 100 mL of distilled water in a flask and stirred for 1 h with a magnetic stirrer, after which the sample was filtered; the pH of the filtrate was determined. To measure water-soluble phosphate and nitrogen, a 50 g soil sample was mixed with 100 mL of distilled water in a flask and stirred for 1 h with a magnetic stirrer. The soil solution was centrifuged at 3000 rpm for 10 min; the supernatant was filtered and subjected to the following tests: PACKTEST WAK-PO4 (D), WAK-NH4, WAK-NO3, and WAK-NO2 by the KYORITSU CHEMICAL-CHECK Laboratory (Tokyo, Japan) to measure the concentrations of PO_4^{3-}, NH_4^+, NO_2^-, and NO_3^-, respectively. The concentrations were measured by absorption spectroscopy (JASCO V-530 UV/VIS spectrometer, Tokyo, Japan) and standard curves were prepared using

standard solutions of KH_2PO_4, NH_4Cl, KNO_3, and KNO_2, respectively. Three measurements on single samples were averaged and used in the analyses (Table 2).

2.2. Effects of fungus strain/soil type combinations on mycorrhizal colonization and host plant growth

Four soil types (Table 2) and four *T. matsutake* isolates (Table 1) were combined in a crossed design to produce 16 treatments. Of the soils sampled, "Matsumoto" and "Nakagawa" in Nagano Prefecture were selected for the experiments, as *T. matsutake* has been commercially harvested from these regions and their soils are expected to develop mycorrhizae in vitro. In contrast, "Chino" soil was categorized as an unsuitable habitat for *T. matsutake* populations in nature (Hamada 1974; Ogawa 1978), and almost no harvest sites occur on such areas of parent rock in Nagano Prefecture. The mixed "Ibaraki" soil was prepared following Kobayashi et al. (2015). Each treatment combination was tested on five replicate seedlings. *Pinus densiflora* seedlings inoculated with *T. matsutake* were incubated for 90 d, inspected for mycorrhization status, and measured for increases in dry weight, as described below. In addition, a non-inoculated control treatment was established on the "Nakagawa" soil to evaluate whether *T. matsutake* would exhibit symbiotic effects on host pine.

2.3. Mycorrhizal synthesis in vitro

Stored *P. densiflora* seeds were washed, surface sterilized, and germinated on MNC agar plates (Yamada et al. 2010); 7–10-d-old seedlings were transplanted into prepared soil in culture bottles, as described below. Two mycelial segments (5 × 5 mm) of each *T. matsutake* strain that had been previously cultured on a MNC agar plate for 2 mo 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 mo to

prepare a mycelial inoculum for mycorrhization. Previously dried and stored soil was dried again at 70 °C overnight to reach a relative water content of 0%. The dried soil was saturated with distilled water to produce a relative water content of 100%. Approximately 240 mL water was required to increase the water content of 1 kg dried soil to 100%. Dried and fully water-saturated soils were mixed in appropriate ratios to produce a relative water content of 75%. A 200 mL sample of the prepared soil was autoclaved at 121 °C for 45 min in a 250 mL polycarbonate wide-mouth jar (No. 2116-0250; Thermo Scientific Inc., Rochester, NY, USA). The cap of the autoclaved jar was removed (under a sterile hood), and the soil was inoculated with a liquid-cultured *T. matsutake* mycelium (wet volume equivalent to ~30 mg dry weight); the inoculum was divided into several segments before being dispersed through the soil in the polycarbonate jar. An axenically germinated *P. densiflora* seedling was transplanted into each polycarbonate jar. A second (open), autoclaved polycarbonate jar was inverted and placed over the top of the planter jar; the two jars stood mouth to mouth (Kobayashi et al. 2007). Then the necks of the jars were sealed with transparent polyvinyl chloride film (Riken Tape; Kyoei Plastic MGF Co. Ltd., Tokyo), after which the whole assembly was weighed. Four 6 mm diam aeration holes were punched through the top jar, and each hole was covered with a fluorocarbon membrane filter (pore size 0.45 mm; Milliseal, Millipore, Yonezawa, Japan). These jar assemblies were incubated in a growth chamber at 20 °C under continual florescent illumination at a photon flux of 100 μmol/m^2/s for 90 d. Each experimental unit comprised five replicate seedlings. Sterilized distilled water was supplied weekly to the soil substratum in each jar under a sterile hood to maintain a constant jar weight.

2.4. Harvest and measurement of grown seedlings

After incubations of 90 d, a small volume of the soil particles in each jar was inoculated onto a MNC agar plate to check for the presence/absence of contaminating microbes and the growth of inoculated *T. matsutake* mycelium. After this procedure, each seedling was removed from the jar and separated into shoot and root systems. The shoot was dried at 60 °C for 24 h, after which the dry weight was measured. The root system was washed in flowing tap water and dissected into segments < 1 cm long. Total root length and mycorrhizal root length were measured at the same time using the grid-line intersect method (Brundrett et al. 1996). We examined small samples of sound ectomycorrhizal tips microscopically to check the morphological and anatomical characteristics of ectomycorrhizal development (Yamada et al. 2010). After measurements were completed, the root system was also dried to determine its dry weight. With these data, we calculated total seedling weight and shoot/root (S/R) biomass ratios.

2.5. Data analyses

One-way ANOVA was used to test for significant differences among means in each experiment using Kaleidagraph ver. 4.1.2 software (Synergy Software, Reading, PA, USA). Tukey's HSD post hoc test or Dunnett post hoc test was used for multiple comparisons ($P < 0.05$) of treatment means. If necessary, t-test was secaondarily used for the comparison between selected two treatments. Two-way ANOVA was used to detect significant effects of the inoculated fungal strains, soil type, and their interaction on host pine growth. S/R ratios were arcsine transformed for ANOVA. Regression analyses were also conducted to determine the effects of ectomycorrhizal development on plant growth parameters as well as how soil nutrient nitrogen and phosphorus affect ectomycorrhizal development and plant growth parameters.

3. Results

3.1. Effect of T. matsutake *inoculation on pine seedling growth on "Nakagawa" soil*

All four *T. matsutake* strains exhibited significant positive effects on pine root length and root dry weight compared to non-inoculated controls (Table 3). In the total dry weight, AT-0707 and AT-0781 inocularions exhibited significant positive effects compared to non-inoculated controls. In the Y-1 and AT-0742 inoculations, such positive effect was briefly detected ($P < 0.1$). However, shoot dry weight was not affected by *T. matsutake* inoculation. Therefore, S/R ratios were significantly lower in the inoculated pine seedling than controls. Inoculated pine seedlings exhibited ectomycorrhizal formations (Supplementary Fig. S1), with the exception of AT-0707 (Table 3), for which inoculated mycelia grew in the soil (with no accompanying microbial contamination; Supplementary Table S2) and on the fine roots. Among the inoculated seedlings, no significant differences were observed among strains in terms of root length, shoot dry weight, root dry weight, total dry weight, and S/R ratio (Tukey HSD post hoc test of one-way ANOVA at $P < 0.05$).

3.2. Effect of soil types on mycorrhizal formation and host plant growth

On the "Chino," "Matsumoto," and "Ibaraki" soils, all of the *T. matsutake* strains showed ectomycorrhizal formation (Table 4). Host pine growth and root colonization levels differed among *T. matsutake* strains in the limited soil conditions. On the "Chino" soil, the S/R ratio significantly differed between Y1 and AT-0781, whereas total root length only marginally differed ($P < 0.10$) between Y1 and AT-0707. On the "Matsumoto" soil, root dry weight and total dry weight significantly differed between Y1 and AT-0742 and the S/R ratio significantly differed between AT-0707 and AT-0742. Mycorrhizal root length significantly differed among all of the *T. matsutake* strains on "Matsumoto" soil and between AT-0742 and AT-0781 on

the“Chino” soil. The colonization ratio significantly differed among several combinations of *T. matsutake* strains on “Matsumoto” and “Chino” soils and between AT-0742 and AT-0707 on the “Ibaraki” soil. The properties of these *T. matsutake* strains on pine hosts with different soil types could not be inferred from mycelial growth trends on MNC medium (i.e., AT-0707 and AT-0781 grew significantly better in terms of biomass and colony diameter compared to Y1 and AT-0742) (Supplementary Table S1). Two-way ANOVA indicated that soil type had significant effects on all of the plant growth parameters and mycorrhization (Table 5); however, the *T. matsutake* strain and its interaction with soil type had significant effects on only mycorrhizal root length and the root colonization ratio.

3.3. Relationships between mycorrhizal root length and pine host growth of the four soil types

Although symbiotic effects of *T. matsutake* inoculation on pine host growth in vitro has been previously reported (Guerin-Laguette et al. 2004; Yamada et al. 2006; Murata et al. 2013), this study is the first to examine the correlation between mycorrhizal root length and host pine growth. Pine growth parameters other than the S/R ratio were significantly positively correlated with mycorrhizal length (Fig. 1); however, the correlation coefficients were relatively low.

3.4. Relationships between soil nutrients and pine host growth and mycorrhizal colonization

Plant growth parameters were positively correlated with ectomycorrhizal root length (which can determine soil nutrient adsorption by the host plants); we therefore analyzed the relationships between soil nutrients (nitrogen and phosphorus) and (i) pine host growth, and (ii) mycorrhizal root length. Total and mycorrhizal root length, mycorrhizal colonization ratio, shoot dry weight, root dry weight, and total dry weight were significantly positively correlated with concentrations of water-soluble nitrogen (mostly NH_4^+) in the soil (Fig. 2). In contrast, ectomycorrhizal root

length, mycorrhizal colonization ratio, root dry weight and total dry weight were significantly negatively correlated with water-soluble phosphorus concentrations (PO_4^{3-}) in the soil (Fig. 3). However, the correlation coefficients were very low.

4. Discussion

We measured host pine growth at an early stage of ectomycorrhizal colonization with *T. matsutake* under different soil types in vitro. This is the first report showing that ectomycorrhization between strains of *T. matsutake* and the pine host is affected by (i) soil type and (ii) their interactions with fungal strains. We were able to detect different mycorrhization properties of *T. matsutake* strains in vitro within a short (90 d) incubation period; additionally, these properties could not be inferred from their mycelial growth on nutrient medium. Our protocol should enable efficient strain selection of *T. matsutake* (as a mycorrhizal symbiont of pine) under selected soil conditions in vitro.

The ectomycorrhization of *T. matsutake* in vitro has been reported previously for a system comprising a 1 L soil volume and host pine plants more than 1-y-old (Kobayashi et al. 2007); this system produced a tennis ball-sized *shiro* with perhaps thousands of mycorrhizal root tips. This *shiro* survived for 2 y under natural pine forest conditions after the colonized seedlings were outplanted (Kobayashi et al. 2015). Production of *T. matsutake shiro* in vitro within a shorter incubation period would facilitate outplantation trials of this fungus-colonized pine seedlings, but the effects of environmental and biological factors that may regulate host pine growth and mycorrhizal development during this shorter incubation period require clarification. We therefore selected smaller mycorrhization system in a 250-mL soil volume. However, although the system developed ectomycorrhizal root tips, a distinct *shiro* structure did not form due the limited incubation period and low seedling biomass.

This study demonstrated the symbiotic effects of four *T. matsutake* strains on the pine host in "Nakagawa" soil collected from a productive habitat of this fungus. These results provide additional support to those reporting similar symbiotic traits of *T. matsutake* in vitro (Guerin-Laguette et al. 2004; Murata et al. 2013). On the "Nakagawa" soil, although all four *T. matsutake* strains showed root growth promotion in both length and biomass compared to non-inoculated controls, shoot growth promotion was not observed in any strain. In addition, AT-0707 inoculation did not exhibit ectomycorrhizal development. Because this strain colonized the soil area and the root surface (Supplementary Table S1), these results suggest that *T. matsutake* first promotes host plant growth in the belowground root system, even at the pre-Hartig net developmental stage in vitro. Guerin-Laguette et al. (2004) also documented enhanced plant growth by *T. matsutake* on small pine seedlings (75 d after fungal inoculation on 12 d-old seedlings), but the symbiotic effect was detected in both the shoot and root. Further research is necessary to determine how such variation in carbon allocation patterns occur. One probable explanation is variation in soil type: we used relatively nutrient-poor granite-based mineral soil in contrast to the organic-based soil used by Guerin-Laguette et al. (2004).

The four strains of *T. matsutake* differentially affected pine host responses under different soil types. Soil type was a more important determinant of host growth compared to the type of *T. matsutake* strain (Table 5). AT-0707 produced no ectomycorrhization in "Nakagawa" soil, and its mycorrhization level tended to be lower in the remaining three soil types (Table 4). Therefore, this strain has a low potential for ectomycorrhizal formation with pine hosts in vitro. In contrast, AT-0742 formed ectomycorrhiza with all of the 20 pine seedlings tested, and its mycorrhization level was generally high among strains across soil conditions. "Matsumoto" soil produced the highest level of mycorrhization, whereas "Chino" soil produced the lowest. The higher nitrogen concentrations of the "Matsumoto" soil may have led to the enhanced

mycorrhizal colonization. “Chino” soil is a weathered volcanic mafic rock that is characterized by higher concentrations of elements, such as iron and magnesium; this soil can be categorized as an unsuitable habitat for *T. matsutake* (Hamada 1974; Ogawa 1978). Because we did not analyze these mineral elements, future studies should examine the effects of soil properties on the mycorrhizal development of *T. matsutake*.

The “Matsumoto” soil/AT-0742 combination produced marked mycorrhization and host growth. Strain AT-0781 also showed a similar level of pine growth in “Matsumoto” soil, but the mycorrhization level was less than half that observed in the “Matsumoto” soil/AT-0742 combination. Thus, the soil/fungal strain combination has a major determining effect on mycorrhizal development in vitro.

Rich soil conditions, e.g., those in organic andosol, promote extensive in vitro mycelial growth of *T. matsutake* in monoculture and have been used for in vitro mycorrhization in *P. densiflora/T. matsutake* combinations (Guerin-Laguette et al. 2003, 2004). In ectomycorrhizal symbioses, host growth and nutrition may be determined by soil nutrient levels (e.g., nitrogen and phosphorus contents) and by the species and strains of fungi (Gobert and Plassard 2008; Smith and Read 2008). Our work corroborated previous studies on the effects of water-soluble nitrogen in soil, but contradicted those examining the effects of water-soluble phosphorus. We used natural mineral soils sampled from different sites where the phosphorus levels might not have spanned the entire range of concentrations required to estimate phosphorus effects. However, the wild *shiro* structure develops from *T. matsutake* mycelium growing exclusively in mineral soil layers (Ogawa 1975; Yamada et al. 1999a; Lian et al. 2006; Vaario et al. 2015); we therefore included only mineral soil conditions in our experimental design.

In conclusion, we tested mycorrhization and host growth response of inoculated *T. matsutake* strains under different soil types in vitro, and found that fungal strains and soil type

(and the combination thereof) strongly affect mycorrhization and host pine growth. Thus, *T. matsutake* strain selection for the efficient development of large numbers of ectomycorrhizal root tips on pine root systems in vitro must take into account soil type.

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 – Relationships between ectomycorrhizal root length and pine growth parameters. (A) Total root length, (B) shoot dry weight, (C) root dry weight, and (D) total dry weight. The fitted line in each graph was derived from 79 data points of fungus-inoculated seedlings.

Fig. 2 – Relationships between water-soluble nitrogen concentrations in the soil and plant growth parameters. (A) Total root length, (B) ectomycorrhizal root length, (C) mycorrhizal colonization tatio, (D) shoot dry weight, (E) root dry weight, and (F) total dry weight. The fitted line in each graph was derived from 79 data points of fungus-inoculated seedlings.

Fig. 3 – Relationships between water-soluble phosphorus concentrations in the soil and plant growth parameters. (A) Total root length, (B) ectomycorrhizal root length, (C) mycorrhizal colonization tatio, (D) shoot dry weight, (E) root dry weight, and (F) total dry weight. The fitted line in each graph was derived from 79 data points of fungus-inoculated seedlings.

Table 1 – Cultured strains of *Tricholoma matsutake* used for mycorrhizal syntheses.

Strain	Origin of cultured strain					Culture deposit in NBRC [c]	Accession number of ITS sequence
	Isolation source [a]	Sampling site in Japan	Putative ectomycorrhizal host	Parent rock [b]	Year		
Y1	FB	Takaizuri, Hitachi-ohmiya, Ibaraki (N 36˚41', E 140˚24', 230 m above sea level)	*Pinus densiflora*	Accretionary prism, mudstone	1993	33136	AB036890
AT-0707	FB	Matsukawa, Nagano (N 35˚36', E 137˚59', 750 m asl)	*P. densiflora*	Plutonic, felsic, granite	2000		LC120308
AT-0742	M	Kami-hisakata, Iida, Nagano (N 35˚27' E 137˚55', 1060 m asl)	*Tsuga sieboldii* Carrière	Plutonic, felsic, granite	2001		LC120310
AT-0781	FB	Kuwahara, Nakagawa, Nagano (N 35˚37' E 137˚59', 700 m asl)	*P. densiflora*	Plutonic, felsic, granite	2004		LC120312

[a] FB: Fruit body, M: Monotropoid mycorrhizal root tips of *Monotropa hypopithys* L. were used for the isolation. See Yamada et al. (2001) for the isolation procedure.

[b] The mother rock information was obtained from GeomapNavi (https://gbank.gsj.jp/geonavi/).

[c] NBRC, Biological Resource Center, National Institute of Technology and Evaluation.

Table 2 – Characteristics of soil types used for mycorrhizal synthesis.

Sampling site	Forest canopy vegetation [a]	Parent rock [b]	pH	Concentration (mg/kg soil) of:	
				Phosphate	Nitrogen [c]
Kitayama, "Chino", Nagano (N 36˚03' E 138˚ 20', 2105 m asl)	Lk	Volcanic, non-alkali mafic rock	5.13	0.080	2.50
Anazawa, "Matsumoto", Nagano (N 36˚ 20' E138˚01', 855 m asl)	Pd	Sedimentary, sandstone	4.43	0.016	9.66
Tajima-zawa, "Nakagawa", Nagano (N 35˚36' E 137˚00', 620 m asl)	Pd, Qa	Plutonic, felsic, granite	5.75	0.020	2.49
Takaizuri, Hitachi-ohmiya, "Ibaraki"	Pd	Accretionary prism, mudstone*			
Satomi, Hitachi-ohta, "Ibaraki" (N 36˚45' E 140˚31', 570 m asl)	Pd	Plutonic, felsic, granite*	4.31	0.020	1.43

[a] Lk: *Larix kaempferi* (Lamb.) Carrière, Pd: *Pinus densiflora*, Qa: *Quercus acutissima* Carruth.

[b] The mother rock information was obtained from GeomapNavi (https://gbank.gsj.jp/geonavi/). *The two soils were mixed in a 1:1 (v/v) ratio before measurements were made. See Kobayashi et al. (2015).

[c] Nitrogen contents are summed values of NH_4^+, NO_3^-, and NO_2^- concentrations. In most cases, NH_4^+ was the dominant ion; NO_2^- and NO_3^- were minor components.

Table 3 – Effects of *T. matsutake* inoculation on pine seedlings grown on "Nakagawa" soil.

Strain	Mean (standard error) [a]; $N = 5$ in each treatment						
	Total root length (cm)	Mycorrhizal root length [b] (mm)	Colonization ($\times 10^{-2}$%) [b]	Shoot dry weight (mg) [b]	Root dry weight (mg) [b]	Total dry weight (mg)	S/R ratio (w/w) [b]
Non-inoculated control	99 (17)	0	0	67 (5)	30.0 (4.4)	97 (9)	2.37 (0.29)
Y1	157 (15) s	7.0 (3.2)	43 (19)	64 (6)	51.3 (3.5) s	115 (9)	1.25 (0.10) s
AT-0707	175 (10) s	0	0	71 (8)	62.8 (2.8) s	134 (11)*	1.13 (0.10) s
AT-0742	176 (16) s	5.9 (2.5)	33 (13)	67 (5)	58.5 (4.6) s	126 (9)	1.16 (0.07) s
AT-0781	176 (14) s	4.7 (2.0)	28 (12)	80 (11)	56.1 (6.3) s	136 (17)*	1.43 (0.09) s

[a] The lower case letter "s" in the columns indicate significant difference of means between non-inoculated control and fungal inoculations by the Dunnett post hoc test of one-way ANOVA at $P < 0.05$.

[b] Raw data of root colonization proportions and S/R ratio were subjected to arcsine transformation before statistical analyses. S/R ratio, seedling shoot dry weight/root dry weight ratio. See Table 1 for strain information.

*Significant difference against non-inoculated control by the t-test at $P < 0.05$.

Table 4 – Effects of soil type/fungal strain combinations on pine growth and root colonization.

Soil/strain [a]	Mean (standard error)[b]; $N = 5$ in each treatment						
	Total root length (cm)	Mycorrhizal root length (mm)	Colonization ($\times 10^{-2}$%) [c]	Shoot dry weight (mg)	Root dry weight (mg)	Total dry weight (mg)	S/R ratio (w/w) [c]
C /Y1[d]	134 (17) a***	4.4 (3.5) a	30 (24) a	67 (6) a	36.1 (3.5) a	103 (8) a	1.87 (0.15) a*
C /AT-0707	193 (20) a***	1.2 (1.2) a	8 (8) a**	75 (9) a	45.3 (4.6) a	120 (12) a	1.68 (0.23) a
C /AT-0742	148 (20) a	5.3 (1.1) a*	35 (3) a*, **	63 (9) a	40.9 (6.2) a	104 (15) a	1.57 (0.10) a
C /AT-0781	168 (12) a	0.6 (0.6) a*	3 (3) a*	69 (7) a	47.4 (4.1) a	117 (11) a	1.47 (0.07) a*
(mean; $N = 4$)	161 A	2.9 A	19 A	69 BC*	42.4 BC	111 BC*, **	1.64 A
M /Y1	166 (28) a	11.7 (4.6) b	105 (67) b	85 (10) a	52.3 (7.1) a*	137 (17) a*	1.66 (0.1) a
M /AT-0707	175 (18) a	2.3 (1.1) b**	15 (6) b*	108 (11) a	63.2 (8.1) a	172 (19) a	1.74 (0.07) a*
M /AT-0742	219 (11) a	69.3 (13.1) a*	308 (51) a	110 (8) a	76.7 (4.6) a*	187 (12) a*	1.44 (0.08) a*
M /AT-0781	221 (17) a	28.8 (3.6) ab*, **	128 (10) b*	107 (8) a	67.0 (3.9) a	174 (11) a	1.60 (0.06) a
(mean; $N = 4$)	195 A	28.0 A	139 A	103 A	64.8 A	168 A	1.61 A
I /Y1	116 (19) a	5.3 (1.7) a	42 (13) a	50 (5) a	40.3 (5.7) a	91 (11) a	1.29 (0.10) a
I /AT-0707	135 (15) a	1.8 (1.2) a	11 (7) a***	57 (9) a	47.0 (7.1) a	104 (16) a	1.22 (0.03) a
I/AT-0742	109 (16) a	13.5 (7.1) a	113 (44) a***	54 (10) a	34.8 (5.1) a	89 (15) a	1.52 (0.07) a
I /AT-0781	106 (24) a	7.6 (5.0) a	55 (30) a	53 (11) a	37.6 (11.1) a	91 (22) a	1.61 (0.25) a
(mean; $N = 4$)	117 B	7.1 A	55 A	54 C*	39.9 C	94 C**	1.41 AB
(mean; "Nakagwa" $N = 4$)	171 A	4.4 A	26 A	71 B	57.2 B	128 B*	1.24 B

[a] C: "Chino", M: "Matsumoto", I: "Ibaraki". The mean value of Nakagwa soil experiment is based on the data in Table 3.

[b] Different lower case letters in the columns indicate significant difference of means between inoculated strains in each soil condition by the Tukey HSD post hoc test of one-way ANOVA at $P < 0.05$.

Different capital letters in the columns indicate significant difference of means between soil types by the Tukey HSD post hoc test of one-way ANOVA at $P < 0.05$.

[c] Raw data of root colonization proportions and S/R ratio were subjected to arcsine transformation before statistical analyses. S/R ratio, seedling shoot dry weight/root dry weight ratio. See Table 1 for strain information.

[d] The number of replicate was 4, because one seedling dead in the incubation period. Please see supplementary Table S2.

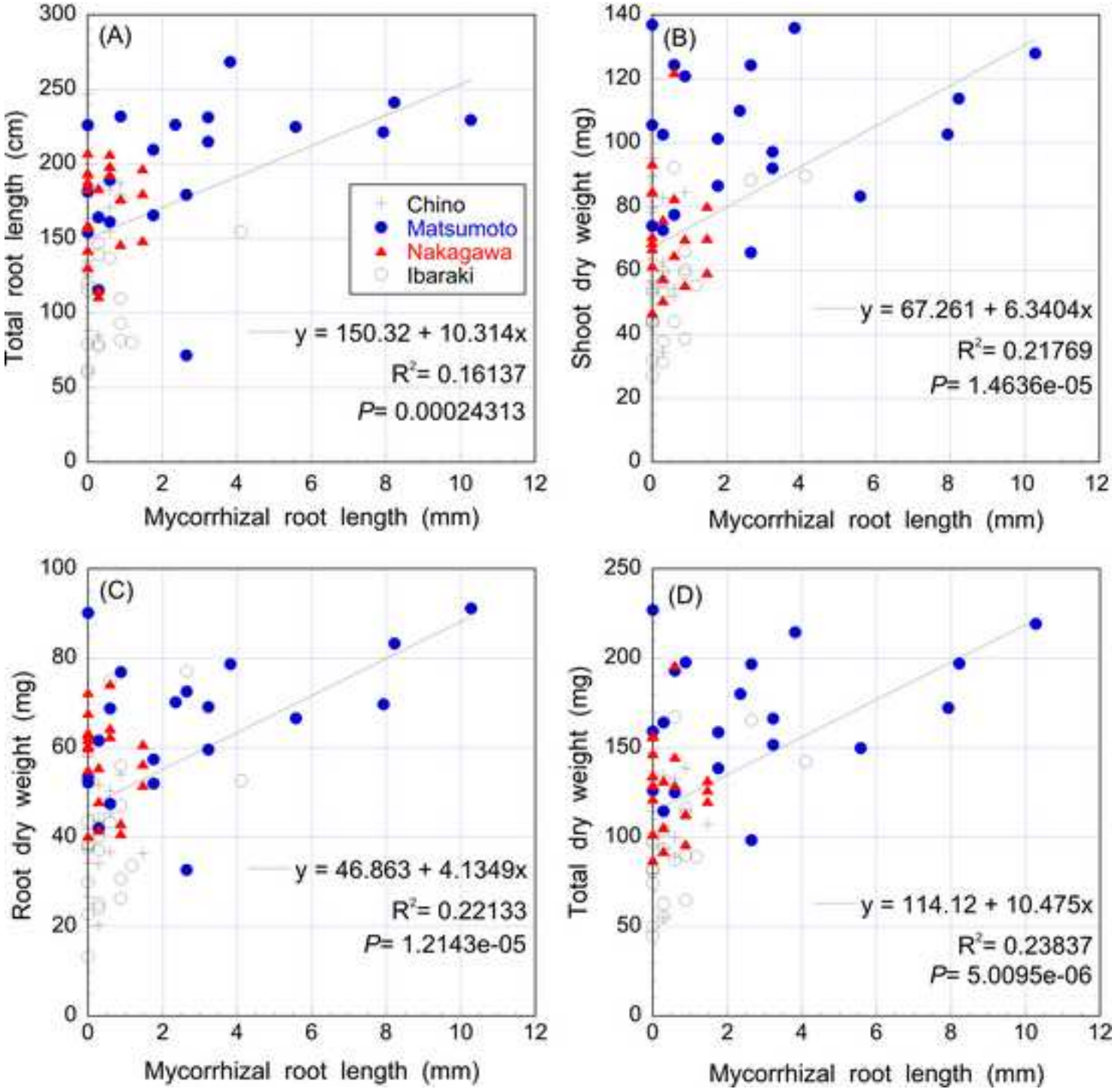
*, ** Significant difference between the two mean values by the t-test at $P < 0.05$, in the soil type.

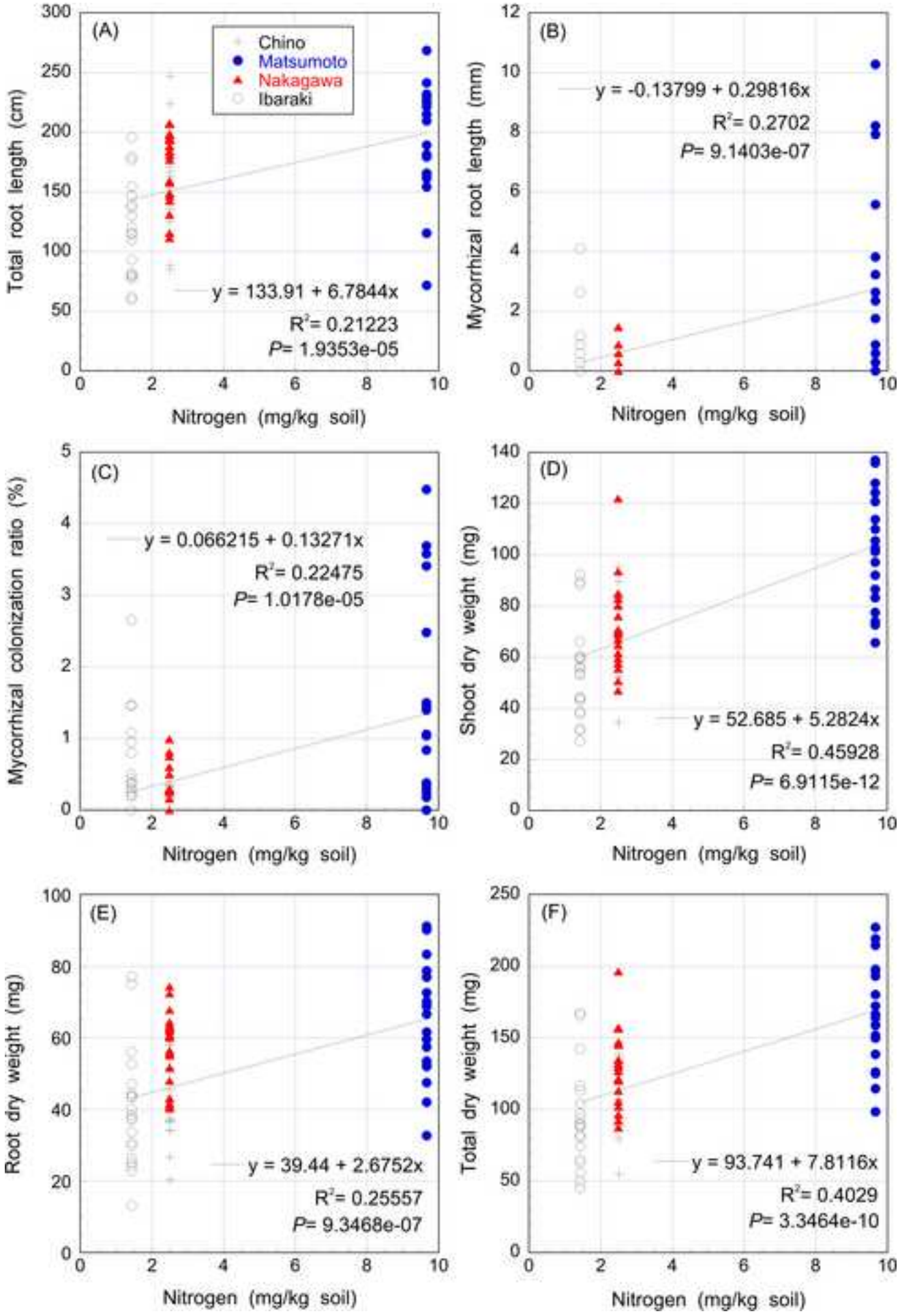
*** Significant difference between the two mean values by the t-test at $P < 0.1$, in the soil type.

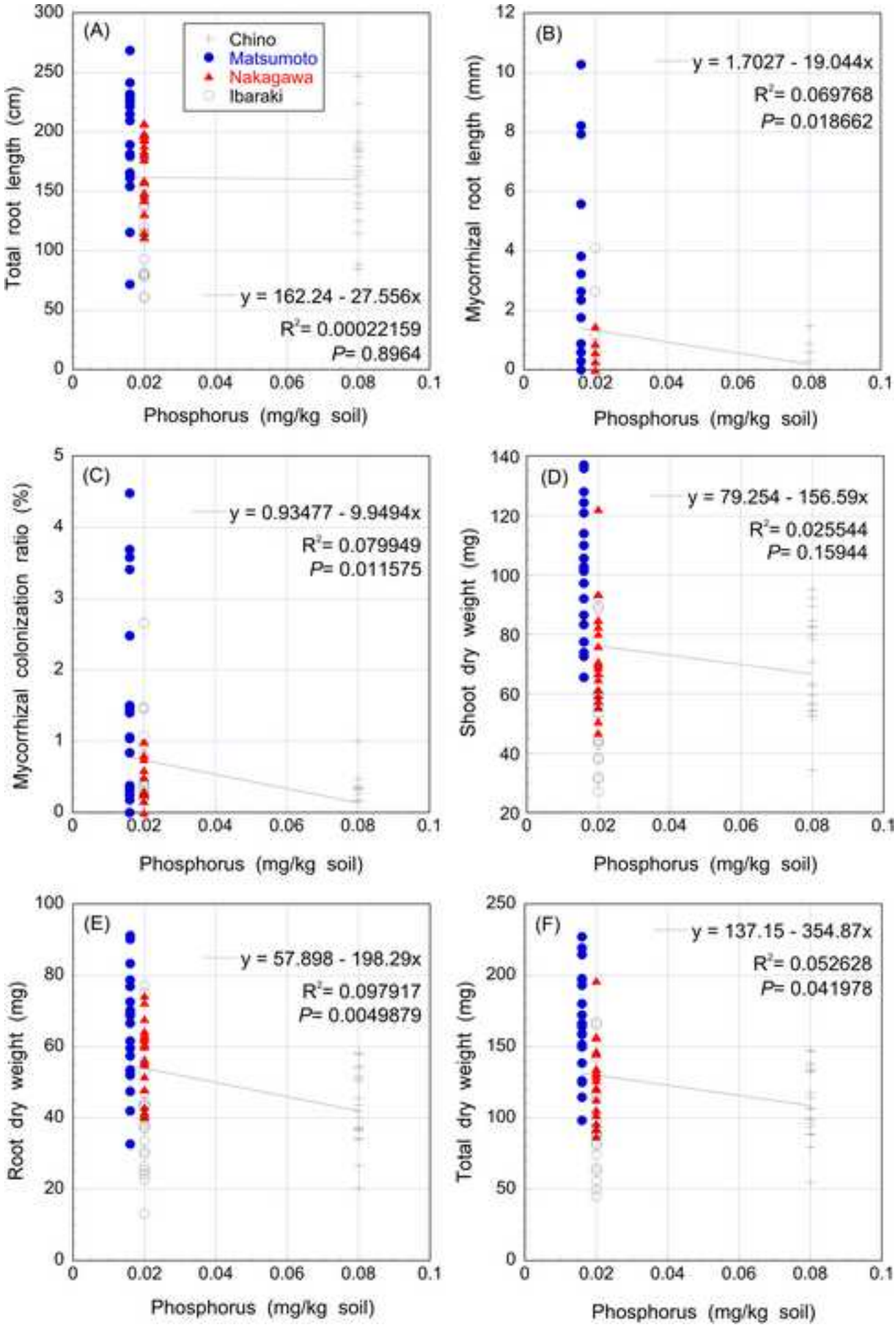
Table 5 – Effects of soil type, *Tricholoma matsutake* strain, and their interaction on seedling biometric traits and mycorrhization (2-way ANOVA).

Measured parameters	*P*-values of the following factors		
	Soil type	*Tricholoma matsutake* strain	Interaction
Root length	< 0.0001	0.390	0.280
Mycorrhizal length	< 0.0001	< 0.0001	< 0.0001
Root colonization ratio	< 0.0001	< 0.0001	0.004
Shoot dry weight	< 0.0001	0.217	0.787
Root dry weight	< 0.0001	0.232	0.309
Total dry weight	< 0.0001	0.223	0.676
S/R ratio	< 0.0001	0.142	0.076

The data analyzed were the same with Tables 3 and 4 with *T. matsutake* inoculation ($N = 60$ in total); data of "Chino" soil ($N = 19$ in total) on Table 4 were deleted from the analysis to balance the experimental design.


(A)
Total root length (cm)
Mycorrhizal root length (mm)
Chino
Matsumoto
Nakagawa
Ibaraki
y = 150.32 + 10.314x
R²= 0.16137
P= 0.00024313
(B)
Shoot dry weight (mg)
Mycorrhizal root length (mm)
y = 67.261 + 6.3404x
R²= 0.21769
P= 1.4636e-05
(C)
Root dry weight (mg)
Mycorrhizal root length (mm)
y = 46.863 + 4.1349x
R²= 0.22133
P= 1.2143e-05
(D)
Total dry weight (mg)
Mycorrhizal root length (mm)
y = 114.12 + 10.475x
R²= 0.23837
P= 5.0095e-06


(A)
Chino
Matsumoto
Nakagawa
Ibaraki
Total root length (cm)
y = 133.91 + 6.7844x
R²= 0.21223
P= 1.9353e-05
Nitrogen (mg/kg soil)
(B)
Mycorrhizal root length (mm)
y = -0.13799 + 0.29816x
R²= 0.2702
P= 9.1403e-07
Nitrogen (mg/kg soil)
(C)
Mycorrhizal colonization ratio (%)
y = 0.066215 + 0.13271x
R²= 0.22475
P= 1.0178e-05
Nitrogen (mg/kg soil)
(D)
Shoot dry weight (mg)
y = 52.685 + 5.2824x
R²= 0.45928
P= 6.9115e-12
Nitrogen (mg/kg soil)
(E)
Root dry weight (mg)
y = 39.44 + 2.6752x
R²= 0.25557
P= 9.3468e-07
Nitrogen (mg/kg soil)
(F)
Total dry weight (mg)
y = 93.741 + 7.8116x
R²= 0.4029
P= 3.3464e-10
Nitrogen (mg/kg soil)


(A)
Chino
Matsumoto
Nakagawa
Ibaraki
Total root length (cm)
y = 162.24 - 27.556x
R²= 0.00022159
P= 0.8964
Phosphorus (mg/kg soil)
(B)
Mycorrhizal root length (mm)
y = 1.7027 - 19.044x
R²= 0.069768
P= 0.018662
Phosphorus (mg/kg soil)
(C)
Mycorrhizal colonization ratio (%)
y = 0.93477 - 9.9494x
R²= 0.079949
P= 0.011575
Phosphorus (mg/kg soil)
(D)
Shoot dry weight (mg)
y = 79.254 - 156.59x
R²= 0.025544
P= 0.15944
Phosphorus (mg/kg soil)
(E)
Root dry weight (mg)
y = 57.898 - 198.29x
R²= 0.097917
P= 0.0049879
Phosphorus (mg/kg soil)
(F)
Total dry weight (mg)
y = 137.15 - 354.87x
R²= 0.052628
P= 0.041978
Phosphorus (mg/kg soil)