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Short communication

Physiological characteristics of pure cultures of a white-colored truffle *Tuber japonicum*

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ABSTRACT

A white-colored truffle *Tuber japonicum*, indigenous to Japan, is an ascomycetous ectomycorrhizal fungus. To clarify the physiological characteristics of this fungus, we investigated the influence of culture medium, temperature, and sources of nitrogen (N) and carbon (C) on the growth of five strains. *Tuber japonicum* strains grew better on malt extract and modified Melin–Norkrans medium, and showed peak growth at 20 °C or 25 °C. This fungus utilized inorganic (NH_4^+ and NO_3^-) and organic N sources (casamino acids, glutamine, peptone, urea, and yeast extract). Additionally, this fungus utilized various C sources, such as monosaccharide (arabinose, fructose, galactose, glucose, and mannose), disaccharide (maltose, sucrose, and trehalose), polysaccharide (dextrin and soluble starch), and sugar alcohol (mannitol). However, nutrient sources that promote growth and their effects on growth promotion widely varied among strains. This can result from the strain difference in enzyme activities involved in the assimilation and metabolism of these sources.

Keywords: carbon, culture medium, nitrogen, strain-specific variation, temperature

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Truffles, primarily belonging to the genus *Tuber*, are ascomycetous ectomycorrhizal (EM) fungi that form symbiotic associations with diverse tree species (Pacioni & Comandini, 1999; Gryndler, 2016). Several *Tuber* species, such as *T. magnatum* Picco (Italian white truffle), *T. melanosporum* Vittad. (Périgord black truffle), *T. aestivum* Vittad. (summer truffle), and *T. borchii* Vittad. (Bianchetto truffle), produce edible fruiting bodies with unique aromas (Hall & Haslam, 2012), and they are globally traded at high prices (Hall, Brown, & Zambonelli, 2007). Various trials have been conducted to develop the proper management of truffle production in fields (Delmas, 1978; Pacioni & Comandini, 1999; Hall et al., 2007). To date, artificial cultivation of several truffle species is accomplished using EM plants inoculated with ascospores or pure-cultured mycelia of these species (Iotti, Piattoni, Leonardi, Hall, & Zambonelli, 2016; Zambonelli & Bonito, 2012; Zambonelli, Iotti, & Murat, 2016; Bach et al., 2021).

Tuber japonicum Hir. Sasaki, A. Kinosh. & Nara is a white-colored truffle, indigenous to Japan (Kinoshita, Sasaki, & Nara, 2016). As its fruiting bodies are promisingly edible with pleasant aromas (Shimokawa et al., 2020), attempts to develop artificial cultivation techniques for this Japanese truffle are currently in progress in Japan (Kinoshita, Obase, & Yamanaka, 2018; Furusawa et al., 2020; Nakano et al., 2020), and biological and ecological information have been accumulated; for example, the fruiting bodies of *T. japonicum* occur beneath a broad range of tree species, such as *Abies*, *Carpinus*, *Lithocarpus*, *Pinus*, and *Quercus*, from autumn to winter (Kinoshita, Sasaki, & Nara, 2011). In addition, habitats in which fruiting bodies of *T. japonicum* occur abundantly are characterized by acidic soils (pH 5.6–6.0) with poor nutrient status, and the mean annual temperature is between 13.9–15.8 °C (Furusawa et al., 2020). However, the data related to the physiology of *T. japonicum* are limited (Nakano et al., 2020). Understanding how abiotic factors, such as pH, temperature, and nutrients, influence the growth of *T. japonicum* should contribute to designing the proper growth conditions of this fungus, which will facilitate the development of cultivation techniques for this Japanese truffle. *Tuber* species, including *T. japonicum*, generally grow very slowly on nutrient media. Therefore, we first examined the growth response to the culture medium and temperature of *T. japonicum* to determine its suitable growth conditions. We further investigated the influence of sources of nitrogen (N) and carbon (C) on mycelial growth of *T. japonicum*.

Five strains of *T. japonicum* were used in this study (Supplementary Table S1). Vegetative mycelia were obtained by putting the

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gleba tissues of each fruiting body on the modified Melin–Norkrans agar medium (MMN: Marx, 1969) containing (L^{-1}) the following: $(NH_4)_2HPO_4$, 250 mg; KH_2PO_4, 500 mg; $MgSO_4 \cdot 7H_2O$, 150 mg; $CaCl_2$, 50 mg; NaCl, 25 mg; 1% $FeCl_3$, 1.2 mL; thiamine HCl, 0.1 mg; glucose, 10 g; malt extract (ME), 3 g; agar, 15 g. These strains were deposited at the Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Japan and maintained on MMN agar medium in a Petri dish (90 mm diam) in darkness at 23 °C before use.

The fungal inoculum was prepared on the MMN agar medium in a Petri dish at 23 °C in darkness for 45–60 d of incubation. Mycelial disks (4.0 mm diam) of each strain were obtained with a cork borer from actively growing colonies on the MMN agar media. Each mycelial disk was placed in a 100 mL flask with 30 mL liquid medium prepared for the following incubation tests: culture medium, temperature, and sources of N and C. Based on the observation by Nakano et al. (2020) that *T. japonicum* grows well at medium pH 5.0–6.0, before autoclaving for 20 min at 121 °C, the pH value of the prepared media was adjusted to 5.5 with 1 N KOH after the addition of 50 mM 2-morpholinoethanesulfonic acid (Dojin Kagaku, Kumamoto, Japan) in these media. Five replicates were prepared for each treatment. The cultures were statically incubated at 23 °C in darkness unless otherwise noted.

Five different liquid media were used; MMN medium, ME medium (Difco; Becton Dichinson Co., Sparks, MD, USA), Murashige–Skoog (MS) medium (M-5519; Sigma-Aldrich Co., St. Louis, MO, USA) containing 30 g/L sucrose (Murashige & Skoog, 1962), potato dextrose broth (PDB) medium (Difco), and Hagem–Modess (HM) medium (Modess, 1941) containing (L^{-1}) the following: KH_2PO_4, 500 mg; $MgSO_4 \cdot 7H_2O$, 500 mg; NH_4Cl, 500 mg; 1% $FeCl_3$, 0.5 mL; glucose, 5 g; ME, 5 g.

The effect of temperature on the growth of *T. japonicum* strains was examined in the MMN liquid medium using a Multi Thermo Incubator (MTI-202B; EYELA, Tokyo, Japan) at 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C.

The basal liquid medium as the control treatment for N source was prepared by MMN medium, from which 250 mg di-ammonium hydrogen phosphate [$(NH_4)_2HPO_4$] was excluded, and 1.0 g instead of 3.0 g ME was added for 1,000 mL media. The N sources tested were $(NH_4)_2HPO_4$, ammonium chloride (NH_4Cl), ammonium sulfate [$(NH_4)_2SO_4$], ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$), asparagine, glutamine, glycine, phenylalanine, urea, casamino acids (N content: 8%) (Daigo; Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), peptone (N content: 15.5%) (Difco), and yeast extract (N content: 10.9%) (Daigo). After sterilizing by filtration using a 0.2 μm membrane filter (Sartorius, Gottingen, Germany), each N source was added to the basal medium autoclaved at 121 °C for 20 min at the final N concentration of 53.0 mg/L. When each amino acid and urea were added to the basal medium, the amount of glucose in the medium was adjusted to give the final C:N ratio of 75:1 in the medium.

The basal liquid medium as the control treatment for C source was prepared by MMN medium, from which 10 g glucose was excluded, and 1.0 g instead of 3.0 g of ME was added for 1,000 mL media. To this medium, one of the following C sources was added to produce the final concentration of 4.0 g/L; arabinose, fructose, galactose, glucose, mannose, maltose, sucrose, trehalose, and mannitol. For dextrin and soluble starch (Difco), 3.0 g/L of each source was used. After sterilizing by filtration using a 0.2 μm membrane filter, each C source was added to the basal medium autoclaved at 121 °C for 20 min.

For all treatments, the cultured mycelia after 50 d of incubation were collected by vacuum filtration using a 5.0 μm pore size membrane filter (Omnipore; Merck, Darmstadt, Germany), freeze-dried at −85 °C, and weighed. The dry weight of mycelia was obtained by subtracting the dry weight of an inoculum agar disk from that of the collected mycelial dry weight. The final pH value of the medium was measured with a glass electrode pH meter (SevenCompact pH meter S220; Mettler-Toledo, Greifensee, Switzerland).

All statistical analyses were performed using BellCurve for Excel (Social Survey Research Information, Tokyo, Japan). Two-way analysis of variance (ANOVA) was used to examine the significant interaction for the mycelial dry weight of *T. japonicum* between strain and each incubation condition: culture medium, temperature, and sources of N and C. To evaluate the N and C utilization ability of each strain, we used Dunnett's test to compare the mycelial dry weight of the control and each N or C treatment. Tukey–Kramer honestly significant difference tests were conducted to compare the mycelial dry weight of each strain at different culture media, temperatures, and inorganic N sources [e.g., KNO_3, $NaNO_3$, NH_4Cl, and $(NH_4)_2SO_4$]. For these tests, the significance threshold was set at 0.05.

A significant interaction for mycelial growth was observed between the culture medium and strain (Supplementary Fig. S1, two-way ANOVA: culture medium, $F = 67.72$, $p < 0.001$; strain, $F = 65.32$, $p < 0.001$; interaction, $F = 18.46$, $p < 0.001$). Except for the strain FFPRI 460514, which showed a similar dry weight in different culture media, *T. japonicum* grew better on ME and/or MMN media compared with the other media tested. The strain FFPRI 460515 had the greatest dry weight on MMN medium, the strains FFPRI 460516 and FFPRI 460517 on ME medium, and the strain FFPRI 460518 on ME and MMN media.

The mycelial dry weight of all strains was high at 20 °C and/or 25 °C and significantly lower at 15 °C, 30 °C, and 35 °C (Supplementary Fig. S2). The growth patterns at different temperatures varied among strains (two-way ANOVA: temperature, $F = 143.7$, $p < 0.001$; strain, $F = 10.80$, $p < 0.001$; interaction, $F = 13.08$, $p < 0.001$). The optimal growth temperatures were 20 °C and 25 °C for the strains FFPRI 460514 and FFPRI 460517, 20 °C for the strains FFPRI 460515 and FFPRI 460516, and 25 °C for the strain FFPRI 460518.

Tuber japonicum utilized inorganic (NH^{4+} and NO^{3-}) and organic N sources (glutamine, urea, casamino acids, peptone, and yeast extract), except for asparagine, glycine, and phenylalanine. However, the pattern of N utilization was different, depending on the strains used (Table 1, two-way ANOVA: N source, $F = 69.05$, $p < 0.001$; strain, $F = 141.1$, $p < 0.001$; interaction, $F = 18.57$, $p < 0.001$). In inorganic N sources, the mycelial dry weight of the strain FFPRI 460517 was significantly increased by all N sources [KNO_3, $NaNO_3$, NH_4Cl, and $(NH_4)_2SO_4$], whereas that of the strain FFPRI 460518 was significantly increased by only NH_4Cl. The dry weight of other strains was significantly increased by all inorganic N sources except for KNO_3 in the strain FFPRI 460514, NH_4Cl in the strain FFPRI 460515, and $(NH_4)_2HPO_4$ in the strain FFPRI 460516. For organic N sources, the dry weight of the four strains (FFPRI 460514–460517) was significantly increased by urea and casamino acid, and other N sources (i.e., glutamine, peptone, and yeast extract) showed different effects on these strains. By contrast, the dry weight of FFPRI 460518 was not significantly increased by any organic N sources (Table 1).

When inorganic N utilization of this fungus was examined using two different salts for each ammonium and nitrate N source (Fig. 1), the mycelial dry weight of the strain FFPRI 460516 was significantly increased by only nitrate N sources (KNO_3 and $NaNO_3$), whereas that of the strains FFPRI 460514 and 460518 was significantly increased by only ammonium N sources [NH_4Cl and/or $(NH_4)_2SO_4$]. On the other hand, the dry weight of the two strains

Table 1 Mycelial dry weight of *Tuber japonicum* on modified Melin–Norkrans liquid medium containing different nitrogen sources.

Strain	Control	Inorganic nitrogen source				Organic nitrogen source							
		$(NH_4)_2HPO_4$	NH_4Cl	NH_4NO_3	KNO_3	Asp	Glu	Gly	Phe	Urea	Cas	Pep	Yea
FFPRI 460514	0.5 (0.1)	1.8 (0.4)***	1.6 (0.2)***	1.2 (0.3)***	0.7 (0.2)	0.7 (0.1)	1.1 (0.2)***	0.4 (0.1)	0.6 (0.1)	0.8 (0.1)*	1.0 (0.2)***	0.6 (0.2)	0.8 (0.2)*
FFPRI 460515	0.6 (0.1)	1.9 (1.1)**	1.0 (0.2)	3.1 (0.5)***	6.7 (0.9)***	0.9 (0.1)	1.8 (0.5)**	0.8 (0.1)	1.0 (0.3)	1.9 (0.5)**	2.0 (0.8)***	1.6 (0.3)*	1.4 (0.1)
FFPRI 460516	0.8 (0.3)	1.2 (0.4)	1.5 (0.5)*	4.0 (0.7)***	3.7 (0.5)***	0.9 (0.2)	1.3 (0.3)	0.7 (0.2)	0.8 (0.1)	3.7 (0.9)***	1.7 (0.3)*	2.3 (0.2)***	1.8 (0.3)**
FFPRI 460517	0.4 (0.2)	4.5 (0.4)***	4.7 (1.3)***	3.5 (0.5)***	6.5 (1.3)***	0.8 (0.2)	3.9 (2.1)***	0.7 (0.2)	1.0 (0.2)	3.9 (0.7)***	1.9 (0.5)*	1.5 (0.4)	2.1 (1.0)*
FFPRI 460518	0.8 (0.1)	1.2 (0.3)	1.4 (0.5)*	0.8 (0.2)	0.7 (0.1)	0.9 (0.1)	0.9 (0.3)	0.7 (0.2)	0.7 (0.1)	0.9 (0.2)	0.9 (0.2)	0.9 (0.2)	1.3 (0.7)

Each value represents a mean with standard deviation (n = 5) of the mycelial dry weight (mg). *, **, and *** indicate significant differences between control and each nitrogen source (Dunnett's tests) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. The final pH value of the medium was ca. 5.5 after 50 d of incubation. Asp: Asparagine; Glu: Glutamine; Gly: Glycine; Phe: Phenylalanine; Cas: Casamino acids; Pep: Peptone; Yea: Yeast extract. Significant interaction was found between nitrogen source and fungal strain (two-way ANOVA: N source, F = 69.05, $p < 0.001$; strain, F = 141.1, $p < 0.001$; interaction, F = 18.57, $p < 0.001$).

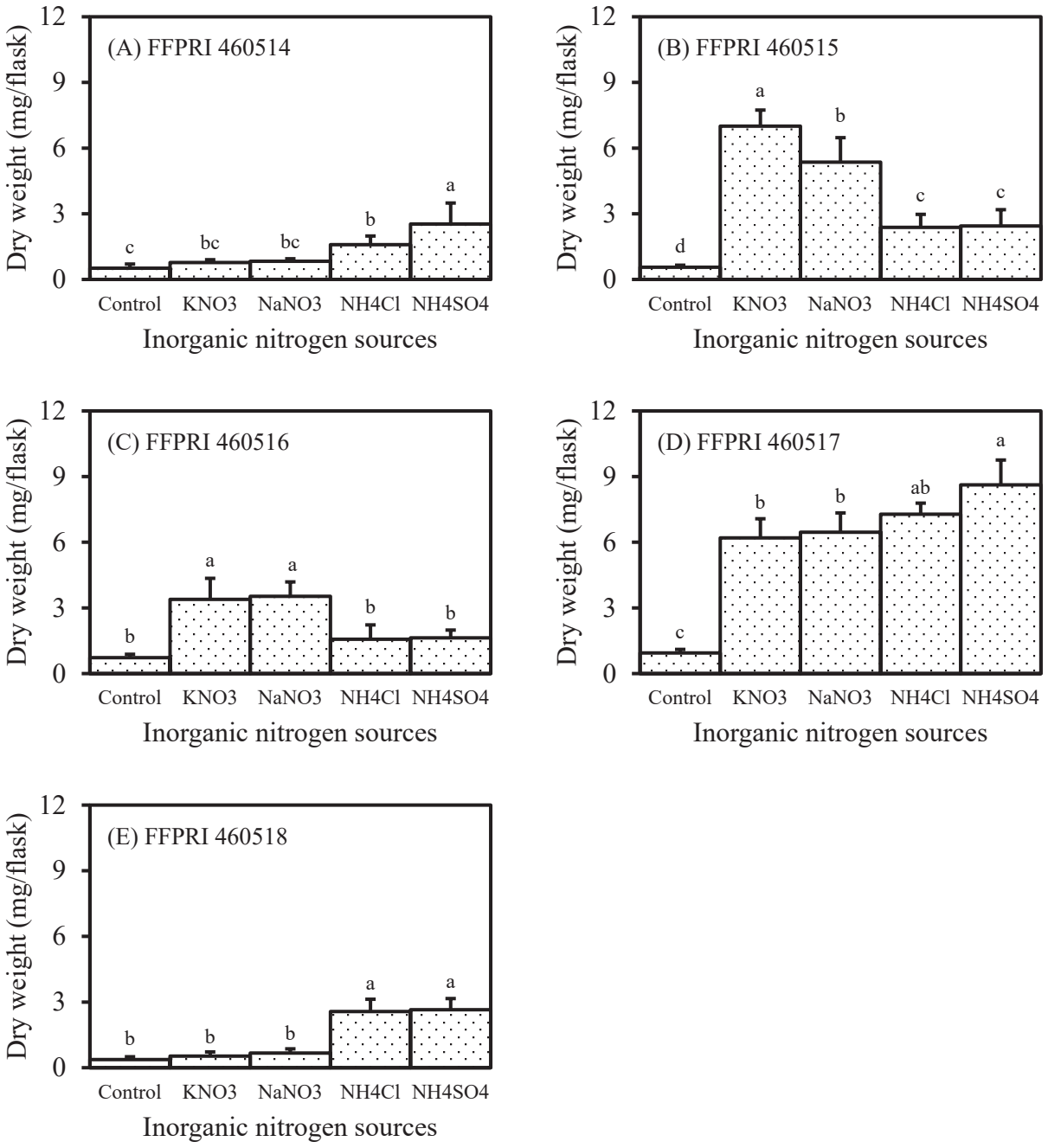

Fig. 1 – Mycelial dry weight of *Tuber japonicum* on modified Melin–Norkrans liquid medium containing different inorganic nitrogen sources. Each value represents a mean with standard deviation (n = 5). Different letters indicate significant differences among inorganic nitrogen sources in each strain (Tukey–Kramer test, $p < 0.05$). The final pH value of the medium was ca. 5.5 after 50 d of incubation.

Table 2 Mycelial dry weight of *Tuber japonicum* on modified Melin–Norkrans liquid medium containing different carbon sources.

Strain	Control	Monosaccharide					Disaccharide			Sugar alcohol	Polysaccharide	
		Arabinose	Fructose	Galactose	Glucose	Mannose	Maltose	Sucrose	Trehalose	Mannitol	Dextrin	Starch
FFPRI 460514	1.0 (0.3)	1.2 (0.6)	1.2 (0.5)	1.1 (0.2)	1.9 (0.4)**	1.4 (0.2)	1.3 (0.2)	1.2 (0.3)	1.1 (0.6)	1.3 (0.2)	3.3 (0.5)***	2.7 (0.5)***
FFPRI 460515	2.1 (0.5)	4.3 (1.3)*	6.4 (2.1)***	3.9 (0.9)	4.3 (0.5)*	7.8 (1.1)***	4.0 (0.9)	5.7 (0.8)***	5.1 (0.6)**	9.7 (2.6)***	10.1 (1.7)***	8.8 (0.7)***
FFPRI 460516	1.4 (0.9)	3.4 (1.2)**	3.2 (0.7)*	1.8 (1.1)	5.0 (0.7)***	1.7 (0.7)	4.0 (1.1)***	2.9 (0.8)*	4.8 (1.0)***	2.7 (1.0)	4.9 (0.9)***	4.8 (1.3)**
FFPRI 460517	2.1 (0.7)	5.4 (1.4)**	5.9 (1.7)***	4.6 (1.6)*	5.5 (1.6)**	5.3 (2.4)**	7.4 (1.9)***	7.6 (0.3)***	5.0 (0.4)**	5.9 (0.7)***	10.4 (1.3)***	8.9 (0.9)***
FFPRI 460518	0.6 (0.2)	0.4 (0.2)	1.2 (0.6)	0.6 (0.1)	3.2 (0.4)***	0.9 (0.1)	2.4 (0.4)***	3.1 (0.5)***	3.7 (0.4)***	2.4 (0.5)***	2.4 (0.2)***	2.1 (0.3)***

Each value represents a mean with standard deviation (n = 5) of the mycelial dry weight (mg). *, **, and *** indicate significant differences between control and each carbon source (Dunnett's tests) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. The final pH value of the medium was ca. 5.5 after 50 d of incubation. Significant interaction was found between fungal strain and carbon source (two-way ANOVA: C source, F = 40.60, $p < 0.001$; strain, F = 296.4, $p < 0.001$; interaction, F = 8.660, $p < 0.001$).

FFPRI 460515 and 460517 was significantly increased by ammonium and nitrate N sources, and the greatest growth was recorded in KNO_3 for FFPRI 460515 and in $(NH_4)_2SO_4$ for FFPRI 460517.

Tuber japonicum utilized various C sources, such as monosaccharide (arabinose, fructose, galactose, glucose, and mannose), disaccharide (maltose, sucrose, and trehalose), polysaccharide (dextrin and soluble starch), and sugar alcohol (mannitol) (Table 2). The dry weight of all strains was significantly increased by glucose, dextrin, and starch. However, the growth response to other C sources varied among strains (Table 2, two-way ANOVA: C source, F = 40.60, $p < 0.001$; strain, F = 296.4, $p < 0.001$; interaction, F = 8.660, $p < 0.001$). The dry weight of the strain FFPRI 460517 was significantly increased by all C sources tested. A similar tendency was observed in the strain FFPRI 460515, whereas that of the strain FFPRI 460514 was significantly increased by glucose, dextrin, and starch. The dry weight of the strain FFPRI 460516 was not increased by galactose, mannose, and mannitol, and that of the strain FFPRI 460518 was not increased by monosaccharide, except for glucose.

Growth conditions such as culture medium and temperature of *Tuber* species that mainly originate from Europe have been studied (Bonfante & Fontana, 1973; Michaels, 1982; Mamoun & Olivier, 1991; Pirazzi, 1988; Mischiati & Fontana, 1993; Giomaro, Sisti, & Zambonelli, 2005; Leonardi et al., 2017). The MMN medium is suitable for the mycelial growth of *T. magnatum* (Mischiati & Fontana, 1993), whereas the potato dextrose basal medium supports the growth of *T. borchii* (Michaels, 1982). In addition, *T. aestivum*, *T. borchii*, *T. melanosporum*, and *T. rufum* Picco can grow within the temperature range of 5–34 °C with the optimal temperature between 15–26 °C (Bonfante & Fontana, 1973; Michaels, 1982; Leonardi et al., 2017). In this study, although the growth pattern of the *T. japonicum* strain varied on the different media tested, the ME and MMN media were suitable for the mycelial growth of this fungus. The optimal growth temperatures of *T. japonicum* were 20 °C and 25 °C.

Several studies have reported that *T. melanosporum* grows better on NH^{4+} than NO^{3-} as an inorganic N source (Bonfante & Fontana, 1973; Mamoun & Olivier, 1991), but the reverse is also true for this fungus (Michaels, 1982). These studies, however, were conducted by using only one or two *T. melanosporum* strains. In this study, we demonstrated that several utilization patterns existed for inorganic N in *T. japonicum*. The utilization and assimilation of inorganic N in EM fungi, including *Tuber* species, are involved in several enzyme activities (Smith & Read, 2008; Amicucci et al., 2016), and strain-specific variations of these activities have been reported in another EM fungus *Hebeloma cylindrosporum* (Wagner, Gay, & Debaud, 1988, 1989). Our results, therefore, imply that these activities of *T. japonicum* vary at strain-specific levels. Mamoun and Olivier (1991) reported that NH_4NO_3 promoted the growth of *T. melanosporum*, and the same is the case for *T. japonicum*. As an organic N source, *T. japonicum* poorly utilized asparagine, glycine, and phenylalanine, whereas this species utilized glutamine, urea, casamino acids, peptone, and yeast extract. The results showed that *T. japonicum* can utilize various organic forms of N, which is in agreement with the result of a previous study on *T. melanosporum* (Bonfante & Fontana, 1973).

Several studies on the C utilization by several *Tuber* species have been reported (Bonfante & Fontana, 1973; Mamoun & Olivier, 1991; Saltarelli et al., 1998, 1999; Ceccaroli, Saltarelli, Cesari, Zambonelli, & Stocchi, 2001; Bedade et al., 2018). For instance, *T. melanosporum* utilizes various C sources, such as monosaccharide (arabinose, fructose, galactose, glucose, and mannose), disaccharide (lactose, maltose, and sucrose), polysaccharide (dextrin and starch), and sugar alcohol (mannitol) (Bonfante & Fontana, 1973; Mamoun & Olivier, 1991). In addition, the C utilization ability of *T. borchii* varies among different strains (Saltarelli et al., 1999; Ceccaroli et al., 2001). This is true for *T. japonicum*. Several enzymes are involved in the utilization and metabolism of C sources by *Tuber* species (Ceccaroli et al., 2011; Amicucci et al., 2016), and these activities vary among strains; one *T. borchii* strain, which grows well in mannitol, showed all enzyme activities of the mannitol cycle, but another strain, which grows poorly in mannitol, lacks mannitol phosphatase and exhibits low mannitol dehydrogenase activity (Ceccaroli et al., 2001). Among the C sources tested, all *T. japonicum* strains showed relatively good growth with glucose, dextrin, and starch, indicating that these are effective C sources for the growth of *T. japonicum*, along with *T. melanosporum* (Mamoun & Olivier, 1991) and *T. maculatum* Vittad. (Bedade et al., 2018).

In conclusion, we demonstrated the physiological characteristics of *T. japonicum* strains. The data in this study should contribute to the design of proper growth conditions of this fungus. ME medium or modified MMN medium with NH^{4+} and NO^{3-} (e.g., NH_4NO_3) as N sources and with glucose, dextrin, and starch as C sources at between 20 °C and 25 °C can support the good growth of this fungus *in vitro*. In addition, such information can be partially used in greenhouses under controlled conditions. Given that the form of N supplied and temperature in the soil can influence the growth of *T. japonicum*, the growth substrates containing weakly acidic soils supplied with NH^{4+} and NO^{3-} (e.g., NH_4NO_3) at temperatures ranging from 20 °C to 25 °C can also support the good growth of this fungus in nursery containers. This study would lead to the development of cultivation techniques of this Japanese truffle species.

Disclosure

The authors declare no conflicts of interest. All experiments undertaken in this study comply with the current laws of the country where they were performed.

Acknowledgments

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