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Nutritional and environmental requirements for vegetative growth of edible ectomycorrhizal mushroom *Tricholoma terreum*

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Abstract

This study, providing basic and valuable information on nutritional and environmental requirements of *Tricholoma terreum*, was carried out between 2005 and 2008 to determine the most suitable medium and culture conditions such as temperature, pH, carbon and nitrogen sources for good mycelium growth. Different inoculum media containing peat and peat:vermiculite mixtures (1:4, 1:6, 1:8 and 1:10 v:v) were also investigated for vegetative inoculum production. The effects of potato dextrose agar, biotin aneurin folic acid agar, malt extract agar, modified Melin-Norkrans agar and modified M40 culture media on mycelial growth of *T. terreum* were found not to be significant. The best temperature and pH values for the mycelial growth were 25°C and 4.5–6.0, respectively. All carbon sources except for sucrose having statistically significant effects on mycelium growth gave the best results in terms of mycelial growth. Malt extract, peptone and yeast extract were the most suitable nitrogen sources. The poorest mycelial growth was recorded in sucrose as a carbon source, and NH₄NO₃ and (NH₄)₂HPO₄ as nitrogen sources. Among vegetative inoculum media, the best mycelial growth was found in 1:4 and 1:6 (v:v) peat:vermiculite mixtures.

Key words: *Tricholoma terreum*, vegetative growth, culture media, pH, temperature, carbon, nitrogen.

Introduction

Tricholoma terreum (Schaeff.:Fr.) Kummer is a gray-capped edible mushroom commonly known as Grey Knight or Dirty Tricholoma, due to its discoloured gills. In Turkey, it is variously called as “Karaoğlan”, “Karakiz” and “Karaca”. *T. terreum* is an edible ectomycorrhizal (ECM) fungus, which belongs to the class basidiomycetes, the order Agaricales and the family Tricholomataceae (Phillips, 1994). The cap is 4–7 cm wide and is covered in fine grey scales. Cap is also convex with a low broad umbo, light to dark grey downy to felty. The stipe is 3–8 cm high and 1.5 cm wide, without ring or volva. The flesh is whitish grey, thin and easily broken. The gills are widely spaced and unattached to the stipe. It is regarded as a good edible mushroom with pleasant mild smell and taste.

T. terreum is naturally and widely present in the macro flora of Turkey. It is a late-season mushroom that forms large groups with the first cold spell. A large quantity of *T. terreum* is collected by mushroom hunters who

visit the area regularly and usually have low income from forests during rainy periods and sell in the local markets. This mushroom is an important source of income and also valuable human food for rural population due to its nutritional properties. *T. terreum* contains 8.2% ash, 18.0% proteins, 8.8% fats, 19.8% cellulose, 60.0% carbohydrate, 2.4% common sugar and 730 mg Fe, 139 mg Zn, 19.2 mg Cu, 15.7 mg Mn in every 100 g dry weight (Song, Wang, 2009 a). Diez and Alvarez (2001) reported that *T. terreum* has high protein content and quality when compared with other edible mushrooms, many vegetables and wild plants. Potential economic importance of *T. terreum* is not only a valuable food, but also a good enzyme source in industry (Ertunga et al., 2009).

Isolation of ECM fungi and their maintenance in pure cultures, preparation of fungal inoculum, and inoculation of seedlings are several steps of artificial mycorrhization. Preparation of vegetative inoculum is an important process to supply inoculum required for inoculation and to guarantee ectomycorrhizal formation on seedling roots.

Many researchers have studied the effect of temperature, pH, carbon and nitrogen sources on the mycelial growth of ectomycorrhizal fungi (Han et al., 1993; Jonathan, Fasidi, 2003; Daza et al., 2006). There is limited information on the nutritional requirements and the cultivation conditions to improve mycelial growth and the feasibility of mushroom production of *T. terreum* (Yamada et al., 2007; Song, Wang, 2009 b). The mycelial growth depends on some factors such as culture media, pH, temperature and nutrient elements (Calam, 1971). These factors greatly affect the formation and growth of ectomycorrhizal fungi both in the field and laboratory conditions (Lilleskov et al., 2002). For this reason, it is very important to evaluate the factors for the optimum mycelia growth of *T. terreum*. The aim of this study was to determine the suitable medium and culture conditions such as temperature, pH, carbon and nitrogen sources for good mycelium growth of *T. terreum*, edible ectomycorrhizal mushroom. The study was also aimed to determine the most suitable media for vegetative inoculum production.

Materials and methods

Some properties of soil collected from T. terreum sampling area and T. terreum mushroom samples. The forest soil, collected from *T. terreum* sampling area, was analyzed for pH values (pH – 7.6) and electrical conductivity (EC, 412 micromhos cm^{-1}) (Rowell, 1996) and organic matter (OM, 6.47%), organic carbon (OC, 3.75%) and total nitrogen (N, 0.26%) content (Kacar, 1994) and ratio of OC to organic N (OC:N – 14.42) was also calculated. The texture of forest soil was clay. The OM, OC and N contents of dried *T. terreum* mushroom samples were determined and the crude protein content of the mushrooms was calculated by multiplying the total nitrogen content by a factor of 6.25. OM, OC and protein contents of the mushrooms were found to be 82.22%, 47.68% and 27.69%, respectively.

Sporocarp isolation. *T. terreum* mushrooms were collected from the pine forest in Samsun, Turkey during autumn season and identified using conventional methods (Phillips, 1994). *T. terreum* mycelia were obtained by tissue culture method on modified Melin-Norkrans medium (Jonathan, Fasidi, 2003), the cultures were stored at 4°C.

Effect of culture media on mycelial growth. To determine the effect of different culture media on the mycelial growth, the following culture media were used: 1) potato dextrose agar (PDA) (200 g potato, 20 g dextrose, 20 g agar, 1 l distilled water); 2) biotin aneurin folic acid agar (BAF) (30.0 g glucose, 2.0 g peptone, 0.2 g yeast extract, 0.5 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 10.0 mg $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 1.0 mg $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 5.0 mg MnSO_4 , 100.0 mg $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 50.0 μg thiamine HCl, 1.0 μg biotin, 100.0 μg folic acid, 50.0 μg inositol, 15 g agar, 1 l distilled water); 3) malt extract agar (ME) (20 g malt extract, 15 g agar, 1 l distilled water); 4) modified Melin-Norkrans agar (MMN) (10 g glucose, 3 g malt extract, 0.25 g $(\text{NH}_4)_2\text{HPO}_4$, 0.025 g NaCl, 0.5 g KH_2PO_4 , 0.05 g CaCl_2 , 0.15 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.012 g $\text{FeCl}_3 \cdot$

6 H_2O , 0.003 g thiamine, 15 g agar, 1 l distilled water); 5) modified M40 medium (10 g glucose, 5 g malt extract, 0.25 g $(\text{NH}_4)_2\text{HPO}_4$, 66.8 mg $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 500 mg KH_2PO_4 , 25 mg NaCl, 150 mg $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 10 mg thiamine, 0.5 ml FeCl (1%), 15 g agar, 1 l distilled water).

Prepared media were autoclaved at 121°C and 15 psi pressure for 20 min. The media were dispensed into the 9 cm diameter sterile Petri dishes. Mycelial agar discs (diameter – 5 mm) were placed on each media and then inoculated media were incubated at 25°C in the dark. The experimental design was a completely randomized design (CRD) with 6 replications.

Effect of initial pH and temperature on mycelial growth. The effects of different temperatures and pH on mycelial growth of *T. terreum* were investigated on potato dextrose agar (PDA) plates. The pH values of the media were adjusted to 4.0, 4.5, 5.0, 5.5 and 6.0 by adding NaOH or HCl, then the media were autoclaved at 121°C for 20 min. PDA medium was poured into the 9 cm diameter Petri dishes. Mycelial discs with 5 mm diameter were used as inoculum and inoculated plates were incubated at 15, 20, 25 and 30°C in the dark. The experiment was set in a completely randomized design with 5 replications.

Effect of carbon and nitrogen sources on mycelial growth. Glucose, lactose, maltose, dextrose, mannitol, xylose and sucrose were carbon sources and each of them were added to the MMN medium separately as a carbon source (10 g l^{-1}) to test their effects on mycelial growth. The MMN medium without carbon was used as C-free control medium. Nitrogen sources were malt extract, yeast extract, peptone, $(\text{NH}_4)_2\text{HPO}_4$, NH_4NO_3 and $\text{Ca}(\text{NO}_3)_2$. Nitrogen sources were separately added into the MMN medium at a concentration of 3.25 g l^{-1} instead of 3 g malt extract and 0.25 g $(\text{NH}_4)_2\text{HPO}_4$ present in the MMN medium. N-free MMN medium was used as control medium. The media prepared with different carbon and nitrogen sources were autoclaved at 121°C for 20 min and each medium was poured into 9 cm Petri dishes. The plates were inoculated with an agar-mycelium disc (5 mm in diameter) and incubated at 25°C in the dark with 8 replications.

Mycelium growth rate and area were measured as mycelial growth parameters in all experiments. Mycelium growth rate (mm day^{-1}) was determined daily by measuring from the two different sections of the colony diameter using a digital caliper. The mycelium growth area (cm^2) covered by mycelia was marked when it was completed in any of Petri dish, and measured with a digital planimeter. Mycelial density was also observed visually as 0) no growth, 1) very sparse, 2) sparse, 3) moderate, 4) dense, 5) very dense.

Vegetative inoculum preparation. In this study, 5 different vegetative inoculum media containing peat and peat:vermiculite mixtures (1:4, 1:6, 1:8 and 1:10 v:v) were tested. Vegetative inoculum media, in 250 ml small culture bottles containing 230 ml of peat or different peat:vermiculite mixtures, were autoclaved at 121°C for

1.5 h. After 24 h, vegetative inoculum media moistened with 80 ml BAF liquid medium were autoclaved again at 121°C for 30 min. After sterilization, the moisture (Kacar, 1994) and pH (Rowell, 1996) of vegetative inoculum media were determined. The small culture bottles were inoculated with 3 mycelial discs of 5 mm diameter. The inoculated bottles were incubated at 25°C in the dark with 5 replications. Mycelium growth rate (cm day⁻¹) was determined by daily measurements from the two different sections of the culture bottles.

Statistical analysis. The data obtained from the experiments were subjected to analysis of variance using SPSS statistical programme and means showing statistical significance were compared by Duncan's multiple range test.

Results and discussion

The effect of culture media on mycelia growth of *T. terreum* is shown in Table 1. No statistically significant differences were found among the culture media for both mycelium growth rate (mm day⁻¹) and mycelium growth area (cm²). Mycelium growth was completed within 6–7 days (Table 1). However, mycelial growth on ME, MMN and M40 media were slender than that on PDA and BAF media according to visual observations. Culture media are important as they supply required nutrients for mycelia growth. Therefore, the effect of culture media on the mycelial growth varies according to mushroom species. MMN has been referred as the most commonly used medium and it is usually offered for the best results in studies on ectomycorrhizal fungi (Kumar, Satyanarayana, 2002). Santiago-Martinez et al. (2003) stated that the MMN, ING and HG media produced the lowest values for diameters and biomass production of *Laccaria bicolor*. In many studies, it has been reported that PDA promotes growth of mushroom mycelia (Fasidi, Akwakwa, 1996; De Araujo et al., 1998; Fasola et al., 2007). Our findings were in agreement with these results.

Table 1. Effect of culture media on mycelial growth of *Tricholoma terreum*

Media	Mycelium growth rate mm day ⁻¹	Mycelium growth area cm ²
ME	7.01ns	49.95ns
M40	6.22	40.44
MMN	6.47	41.64
PDA	7.10	48.39
BAF	7.56	49.83

Note. Mycelial growth area was measured when it was completed in any of Petri dishes, at the end of 6th day for the current study; ns (non significant) there are no statistically significant differences among means in the column by Duncan's multiple range test.

The analysis of variance revealed that there were significant differences among temperatures, pH and temperature × pH interactions for both mycelium growth

rate and mycelium growth area (Table 2). The optimum temperature for the best mycelial growth was found to be 25°C. Many researchers stated that the most suitable temperature for mycelial growth of a lot of mushroom species was 25°C (Imtiaj et al., 2007; Kalyoncu et al., 2009). The mycelium growth decreased drastically at 20 and 15°C. The reduction of the mycelial growth at below 25°C may be sourced from the reducing metabolic activities of the fungus that allow the absorption of essential nutrients needed for growth (Garraway, Evans, 1984). The mycelial growth was found to be slender and the pellet of mycelia was compact under unfavourable growth conditions such as high or low temperature and poor nutrient media (Papagianni, 2004). In the present study, no mycelial growth was observed at 30°C (Table 2). It is probable that preventing factor of mycelium growth at 30°C is denaturation of important enzymes which catalyze fungal metabolic processes (Jonathan et al., 2004).

The pH of medium is a very important factor for mycelial growth. The mycelial growth decreased at pH below 4.5. It was found that the suitable pH range for mycelial growth was 4.5 to 6.0 (Table 2). Mycorrhizal fungi favor acidic conditions (Han et al., 1993). Sundari and Adholeya (2003) reported that the ectomycorrhizal members of *Agaricales*, except for *Laccaria laccata*, favored neutral to near neutral pH.

The growth of fungi is promoted by temperature and pH of the media (Garraway, Evans, 1984). The maximum mycelial growth was determined in pH between 4.5 and 6.0 at 25°C (Table 2).

In *T. terreum*, the effects of carbon sources, except for sucrose showing the lowest mycelium growth, on mycelium growth rate ($P < 0.01$) and the mycelium growth area ($P < 0.05$) were not different (Table 3). However, C-free control media have low mycelial density when compared to the others. Contrary results have been obtained from the studies on the use of sucrose as a carbon source in *Suillus* and *Boletus* (Murata, 1993; Hatakeyama, Ohmasa, 2004). Yeast extract was the most suitable nitrogen source for the mycelial growth rate of *T. terreum*. For the mycelium growth area, malt extract, yeast extract, peptone and N-free control media, not statistically different from each other, gave the highest values (Table 4). However, mycelial density observed in the control media was very low and mycelial growth was weak and loosely woven. This case could be explained by limited nutrition in the growth media. The lowest mycelial growth was determined in NH₄NO₃ and (NH₄)₂HPO₄ (Table 4). Ammonium is the most suitable source of N for the most of ECM fungi (Rangel-Castro et al., 2002; Sangtjean, Schmidt, 2002). In *Amanita caesarea*, the greatest mycelium dry weight yields were obtained from ammonium when it was used as N source (Daza et al., 2006), but our findings from the present study were not in agreement with these results. The presence of nitrate ion has a negative effect on the development of some ECM fungi (Griffin, 1994). The effect of nitrogen source on the mycelia growth depends on species, culture media and conditions (Lin, Yang, 2006).

Table 2. Effect of temperature and initial pH on mycelial growth of *Tricholoma terreum* on PDA

Properties	pH	Temperature °C				Mean
		15°C	20°C	25°C	30°C	
Mycelium growth rate mm day ⁻¹	4.0	3.63d**	6.25b	11.04a	0.00e	5.23b*
	4.5	3.37d	4.80bcd	12.06a	0.00e	5.06b
	5.0	4.62bcd	4.52bcd	13.07a	0.00e	5.55ab
	5.5	4.96bcd	4.21cd	13.19a	0.00e	5.59ab
	6.0	5.85b	5.49bc	12.41a	0.00e	5.94a
	Mean		4.49b**	5.05b	12.36a	0.00c
Mycelium growth area cm ²	4.0	11.54d**	20.20bc	27.02b	0.00e	14.69b**
	4.5	10.96d	21.70b	44.80a	0.00e	19.37a
	5.0	13.04d	26.98b	49.34a	0.00e	22.34a
	5.5	14.46cd	22.30b	53.16a	0.00e	22.48a
	6.0	13.42d	23.56b	47.46a	0.00e	21.11a
	Mean		12.68c**	22.95b	44.36a	0.00d

Note. Mycelial growth area was measured when it was completed in any of Petri dishes, at the end of 8th day for the current study; *means followed by different letters are statistically different by Duncan's multiple range test ($P < 0.05$), **means followed by different letters are statistically different by Duncan's multiple range test ($P < 0.01$).

Table 3. Effect of carbon sources on mycelial growth of *Tricholoma terreum*

Carbon sources	Mycelium growth rate mm day ⁻¹	Mycelium growth area cm ²
Xylose	9.77a**	63.60a*
Lactose	9.52ab	63.60a
Sucrose	9.30b	62.89b
Maltose	9.65a	63.60a
Mannitol	9.46ab	63.60a
Glucose	9.51ab	63.60a
Dextrose	9.79a	63.60a
Control (C-free)	9.48ab	63.60a

Note. Mycelial growth area was measured when it was completed in any of Petri dishes, at the end of 5th day for the current study; *means followed by different letters in the columns are statistically different by Duncan's multiple range test ($P < 0.05$), **means followed by different letters in the columns are statistically different by Duncan's multiple range test ($P < 0.01$).

Table 4. Effect of nitrogen sources on mycelial growth of *Tricholoma terreum*

Nitrogen sources	Mycelium growth rate mm day ⁻¹	Mycelium growth area cm ²
(NH ₄) ₂ HPO ₄	3.02c**	10.99c**
NH ₄ NO ₃	2.42c	8.03c
Ca(NO ₃) ₂	9.25b	55.53b
Malt extract	9.60b	63.60a
Yeast extract	10.98a	63.60a
Peptone	9.16b	63.60a
Control (N-free)	9.72b	63.60a

Note. Mycelial growth area was measured when it was completed in any of Petri dishes, at the end of 5th day for the current study; **means followed by different letters in the columns are statistically different by Duncan's multiple range test ($P < 0.01$).

Table 5. pH values and moisture contents of the vegetative inoculum media and their effects on mycelial growth

Media	Moisture %	pH after sterilization	Mycelium growth rate cm day ⁻¹	Days to complete mycelium
Peat	51.8	5.25	1.07b**	11.20a**
Peat:vermiculite (1:4)	48.2	5.65	1.40a	8.60b
Peat:vermiculite (1:6)	47.5	5.80	1.30a	9.20b
Peat:vermiculite (1:8)	47.1	5.95	1.15b	10.40a
Peat:vermiculite (1:10)	42.9	6.00	1.07b	11.20a

Note. **means followed by different letters in the columns are statistically different by Duncan's multiple range test ($P < 0.01$).

Conclusions

1. The effect of culture media on mycelial growth was found to be significant. When the mycelial density was considered, PDA and BAF media showed better growth than that in the other media.

2. Temperature of 25°C and pH values between 4.5 and 6.0 gave the best results for mycelial growth in *Tricholoma terreum*.

3. The best mycelial growth was found in the presence of glucose, lactose, maltose, dextrose, mannitol and xylose. The most suitable nitrogen sources were malt extract, peptone and yeast extract. Mycelial density in the both C-free and N-free control media was low. The lowest mycelial growth was determined in sucrose among C sources, and NH₄NO₃ and (NH₄)₂HPO₄ among N sources.

4. Among vegetative inoculum media, the best mycelial growth was obtained from peat:vermiculite mixtures in the rate of 1:4 and 1:6.

As a result, *Tricholoma terreum* is an important local edible mushroom species because of its wide consumption by the rural population and economic value in the markets. To know growth conditions and nutritional requirements of *T. terreum* would be useful for improving its cultivation technology.

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Valgomojo ektomikorizinio grybo *Tricholoma terreum* vegetatyvinio augimo mitybos ir aplinkos sąlygos

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Santrauka

Tyrimas, suteikęs svarbios informacijos apie *Tricholoma terreum* mitybos ir aplinkos poreikius, atliktas 2005–2008 m. siekiant nustatyti grybienos augimui tinkamiausią terpę ir augimo sąlygas – temperatūrą, pH, anglies ir azoto šaltinius. Įvairios grybienos terpės, sudarytos iš 1:4, 1:6, 1:8 bei 1:10 (v:v) durpių ir vermikulito mišinių, buvo tirtos vegetatyvinės grybienos gamybai. Bulvių dekstrozės agaro, biotino aneurino folinės rūgšties agaro, salyklo ekstrakto agaro, modifikuoto Melino-Norkranso agaro ir modifikuotos M40 auginimo terpės poveikis *T. terreum* nebuvo esminis. Grybienos augimui geriausios temperatūros ir pH vertės buvo atitinkamai +25° C ir 4,5–6,0. Micelio augimo atžvilgiu geriausi rezultatai gauti visų anglies šaltinių, turinčių esminį poveikį grybienos augimui, išskyrus sacharozę. Tinkamiausi azoto šaltiniai buvo salyklo ekstraktas, peptonas ir mielių ekstraktas. Micelis prasčiausiai augo ant sacharozės kaip anglies šaltinio ir NH₄NO₃ bei (NH₄)₂HPO₄ kaip azoto šaltinio. Iš vegetatyvinės grybienos terpių micelis geriausiai augo ant 1:4 ir 1:6 (v:v) durpių ir vermikulito mišinių.

Reikšminiai žodžiai: *Tricholoma terreum*, vegetatyvinis augimas, augimo terpė, pH, temperatūra, anglis, azotas.