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FAVOURABLE CULTURE CONDITIONS FOR MYCELIAL GROWTH OF *HYDNUM REPANDUM*, A MEDICINAL MUSHROOM

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Abstract

In this study, factors such as pH, temperature, carbon and nitrogen sources that affect mycelial growth of *Hydnum repandum*, a medicinal mushroom, were investigated. Different inoculum media for vegetative inoculum production were also examined. The best suitable pH for mycelial growth was found to be 5.5. Among constant temperatures, the best mycelial growth was obtained at 20 and 25°C. The mycelial growth drastically decreased at 15°C, and no mycelia were obtained at 30°C. Glucose and mannitol were found to be the most suitable carbon sources. $Ca(NO_3)_2$ as a nitrogen source gave the best results for mycelial growth. The poorest mycelial growth was noted in sucrose and xylose as carbon sources and in NH_4NO_3 and $(NH_4)_2HPO_4$ as nitrogen sources. Peat and peat: vermiculite mixtures (1:4, 1:6, 1:8 and 1:10, v:v) were the best media to use in producing the vegetative inoculum of *H. repandum*

Keywords: *Hydnum repandum;* pH; temperature; carbon; nitrogen; vegetative growth

Introduction

Hydnum repandum (L. ex Fr.) S.F. Gray is an edible, ectomycorrhizal and medicinal mushroom, which belongs to the class Basidiomycetes, the order *Cantharellales* and the family *Hydnaceae* (Phillips, 1994). It is commonly known as the Wood Hedgehog or Hedgehog mushroom. This mushroom is easily recognised because of its spore-bearing structures which are shaped like teeth or spines rather than gills.

H. repandum is an important source of income and also valuable human food for rural population due to its nutritional and medical properties. An extract of the culture mycelia showed 70% inhibition against Sarcoma 180 solid cancer in mice, while extracts from the fruit bodies showed 90% inhibition against both Sarcoma 180 and Ehrlich solid cancer in mice (Ohtsuka et al., 1973). Repandiol isolated from *H. repandum* and *H. repandum* var. *album* displayed potent cytotoxic activity against various tumour cells (Takahashi et al., 1992). *H. repandum* contains 9.16% crude ash, 27.07% crude protein, 7.60% crude cellulose, 3.16% crude fat, and 53.01% carbohydrates (Ertan and Gulyavuz, 1993). Murcia et al. (2002) stated that *H. repandum* has antioxidant activity. It is also reported that *H. repandum* is an important nutrient for diet (Alonso et al., 2003).

H. repandum is widely distributed in North America and Europe, found single or in groups in summer or fall, and forms ectomycorrhizal association with hardwoods or conifers (Wikipedia, 2012). This mushroom is also naturally and widely present in the macro fungi flora of Turkey. It is widely distributed in the pine, hornbeam, beech and oak forests in Adana, Antalya, Artvin, Balikesir, Bolu, Eskisehir, Giresun, Gumushane, Istanbul, Izmir, Manisa, Ordu, Samsun and Trabzon provinces of Turkey (Solak et al., 2007). It is sold in the local markets and has high export potential (Peksen and Karaca, 2000) because it is a good delicious edible mushroom, having a sweet, nutty taste, mild odour and a crunchy texture.

There is limited information on mycelial growth conditions of *H. repandum.* The fastest mycelium growth was recorded in MEPA and PDA among 8 different media including PDA, ME, BAF, MMN, M40, MEPA, PDYA and MYPA. Mycelium type and form in MEPA and PDA media were radial and radial-floccose, respectively (Kibar and Peksen, 2007). However, the determining of a suitable media requires a detailed investigation to establish the most suitable medium that meets certain basic requirements. To find optimal nutritional and environmental conditions for mycelial growth of *H. repandum* and to produce its vegetative inoculum are necessary and have a great importance from the point of cultivation of this species. The aim of this study was to determine the best mycelial growth conditions (pH, temperature, carbon and nitrogen sources) of *H. repandum,* and to realise its vegetative inoculum production.

Materials and Methods

The study was conducted in the mycelial production laboratory of Department of Horticulture, Faculty of Agriculture, Ondokuz Mayis University between 2008 and 2009.

Collection, identification and isolation

The fruiting bodies of *H. repandum* were collected from the pine forests in Sinop province of Turkey in autumn 2008. Identification of mushroom was done using conventional methods (Phillips, 1994). Pure mycelial cultures of *H. repandum* were obtained using tissue culture method as described by Jonathan and Fasidi (2003). The mycelial culture was maintained on Potato Dextrose Agar (PDA) at 25°C in complete darkness, and the cultures were stored at 4°C.

Effect of initial pH and temperature on mycelial growth

The effect of initial pH and temperature on mycelial growth of *H. repandum* was investigated on Potato Dextrose Yeast Agar (PDYA) medium. PDYA medium was composed of potato 200 g, dextrose 20 g, yeast extract 2 g, agar 20 g and 1000 ml of distilled water. pH of PDYA medium was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 by means of pH metre with the addition of 1 N NaOH or HCl prior to autoclaving and then

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The media were autoclaved at 121°C for 30 minutes. After cooling, PDYA media were poured into the 9 cm diameter Petri dishes. Mycelial agar discs of 5 mm diameter cut from the margin of an actively growing colony of subcultures were carefully placed in the centre of new Petri plates filled with PDYA. Inoculated Petri plates were sealed with parafilm and placed into separate incubators to incubate at 15, 20, 25 and 30°C under dark conditions. The experimental design was a completely randomised design with 5 replications.

Effect of carbon and nitrogen sources on mycelial growth

To screen the effect of different carbon and nitrogen sources on the mycelial growth of *H. repandum*, 7 carbon and 6 nitrogen sources were tested. Glucose, lactose, maltose, dextrose, mannitol, xylose and sucrose were used as carbon sources and each of them was added into PDYA medium at the rate of 20 g/l. The medium without carbon served as the control (C-free) (Kadiri and Fasidi, 1994). Malt extract, yeast extract, peptone, $(NH4)_2HPO_4$, NH_4NO_3 and $Ca(NO_3)_2$ were used as nitrogen sources and each of them was added into PDYA medium at a concentration of 2 g/l. The control (N-free) medium was constituted without nitrogen (Daza et al., 2006). Sterilisation and inoculation were carried out as described for the pH and temperature above. The inoculated plates were incubated at 25°C under dark conditions. The experiment was carried out in completely randomised design with 9 replications.

Duration of complete mycelium running, mycelial growth area and colony diameter was measured as mycelial growth parameters in the experiments. Duration of complete mycelium running was determined as the number of days from the mycelium inoculation to time that petri plate was completely covered by mycelium. Mycelial growth area (cm^2) covered by mycelia was marked when the mycelia growth was completed in any of the Petri dishes, and measured with a digital planimeter. Mycelial colony diameter (mm) was determined by daily measurements in four different point of mycelial colony in the plates with a digital calliper. The average values which were calculated from these measurements were multiplied by two.

Vegetative inoculum preparation

To produce vegetative inoculum of *H. repandum*, 5 different vegetative inoculum media consisting of peat and peat: vermiculite mixtures (1:4, 1:6, 1:8 and 1:10 v:v) were used. Vegetative inoculum media, in 1000 ml glass culture bottles containing 800 ml of peat or different peat: vermiculite mixtures, were autoclaved at 121°C for 1.5 h. After 24 h, vegetative inoculum media was moistened with 200 ml MEPA liquid medium and autoclaved again at 121°C for 30 min. After cooling, the culture bottles were inoculated with 4 mycelial discs of 5 mm diameter. The inoculated bottles were incubated at 20±2 °C under dark conditions. The experiment was carried out in completely randomised design with 4 replications. pH of vegetative inoculum media was determined both after sterilisation and after mycelial growth was completed (Rowell, 1996). Mycelial growth rate (cm day^{-1}) was determined by daily measurements from the two different sections of the culture bottles. Mycelial growth (cm) on the 18th day was also determined in the trial.

Statistical analysis

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

Results and Discussion

Means of mycelial growth characteristics obtained from different temperature and pH applications are presented in Table 1. Statistical analysis of the data on the mycelial growth parameters revealed that significant differences were found among temperatures, pH and temperature × pH interactions with regards to duration of complete mycelium running, mycelial growth area and mycelial colony diameter. The best temperatures for mycelial growth of *H. repandum* were found to be 20 and 25°C. The mycelial growth drastically decreased at 15°C (Table 1). It may be sourced from the reducing metabolic activities of the fungus that allow the absorption of essential nutrients needed for growth (Garraway

Table 1: Effect of temperature and initial pH on the mycelial growth of *H. repandum* on PDYA[1]

Properties		Temperature °C				
	pH	15 °C	20 °C	25 °C	30 °C	Mean
Duration of complete mycelium running (day)	4.0	37.00a*	19.80c-f	36.40a	0.00g	23.30a**
	4.5	32.40ab	20.40c-f	26.20bcd	0.00g	19.75ab
	5.0	22.60c-f	21.00c-f	26.80bc	0.00g	17.60abc
	5.5	22.80c-f	18.40def	15.40f	0.00g	14.15c
	6.0	33.80ab	17.60ef	27.00bc	0.00g	19.60ab
	6.5	25.00b-e	19.80c-f	22.80c-f	0.00g	16.90bc
	Mean	28.93a**	19.50b	25.77a	0.00c	
Mycelial growth area (cm^2)	4.0	9.86j**	13.60ıj	20.08f-ı	0.00k	10.89c**
	4.5	11.02j	23.30e-h	25.02d-g	0.00k	14.84bc
	5.0	17.82g-ı	32.74a-e	27.38b-f	0.00k	19.49a
	5.5	14.44ıj	37.78ab	35.98abc	0.00k	22.05a
	6.0	13.54ıj	29.52a-e	27.08c-f	0.00k	17.54ab
	6.5	16.22h-j	33.58a-d	38.32a	0.00k	22.03a
	Mean	13.82b**	28.42a	28.98a	0.00c	
Mycelial colony diameter at 15th day (mm)	4.0	48.55h**	65.90ef	65.37ef	0.00ı	44.96c**
	4.5	53.26gh	77.85a-d	75.76bcd	0.00ı	51.72b
	5.0	65.56ef	82.34a-d	74.95cde	0.00ı	55.71ab
	5.5	61.36fg	86.18ab	88.21a	0.00ı	58.94a
	6.0	59.69fg	83.03a-d	74.03de	0.00ı	54.19ab
	6.5	65.48ef	85.28abc	82.96a-d	0.00ı	58.43a
	Mean	58.98b**	80.10a	76.88a	0.00c	

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[1]Data were arcsine-square root transformed prior to statistical analyses.
*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 according to Duncan's multiple range test (P<0.01).

and Evans, 1984). In the present study, no mycelial growth was observed at 30°C (Table 1). Inhibition of mycelium growth at 30°C can be attributed to denaturation of important enzymes which catalyse fungal metabolic processes (Jonathan et al., 2004). The findings of this study were in agreement with Kalyoncu et al. (2009) who found that optimal temperatures for six *Morchella* species were 25 and 20°C.

The most suitable mycelial growth was obtained at pH 5.0-6.5, while the mycelial growth at pH 4.0 and 4.5 evidently decreased (Table 1). These results were in agreement with Stanbury et al. (1995) who stated that ectomycorrhizal fungi grow better at mild acidic conditions. Temperature and pH are accepted to be important environmental factors that control the fungal development (Johansson, 2002). Temperature × pH interactions were significant (P<0.01). The best mycelial growth was determined in pH between 5.0 and 6.5 at 20 and 25°C (Table 1). Kibar and Peksen (2011a) reported that best mycelial growth was determined in pH between 4.5 and 6.0 at 25°C in both *Lactarius pyrogalus* and *L. controversus.*

The effect of carbon sources on mycelia growth of *H. repandum* was significant. Among carbon sources, the best mycelial growth area and mycelial colony diameter were obtained in the media including mannitol and glucose (Table 2). Glucose and mannitol were the most suitable carbon sources for the mycelial growth of *A. caesarea* (Daza et al., 2006) and *L. pyragolus* and *L. controversus* (Kibar and Peksen, 2011a). Guler and Ozkaya (2008) reported that malt extract agar, wheat agar, potato dextrose agar and complete medium yeast extract agar media containing glucose, sucrose, maltose and starch were the best vegetative mycelial growth of *Morchella conica*.

The lowest mycelial growth area and colony diameter was determined in C-free control medium and media supplemented with sucrose and xylose (Table 2). This result was parallel with the suggestion of Hatakeyama and Ohmasa (2004) who found that the mycelial growth of *Suillus* and *Boletinus* was poor in sucrose contained medium.

Table 2: Effect of carbon sources on the mycelial growth of *H. repandum*

Carbon sources	Duration of complete mycelium running (day)	Mycelial growth area (cm^2)[1]	Mycelial colony diameter (mm)[1]
Xylose	18.43c**	25.41de**	53.98cd**
Lactose	24.43b	29.07d	59.26c
Sucrose	33.00a	23.10de	52.79cd
Maltose	17.71c	37.03c	68.05b
Mannitol	15.00c	51.80a	82.72a
Glucose	14.86c	47.11ab	81.65a
Dextrose	15.71c	43.17bc	73.15b
Control (C-free)	24.14b	19.46e	48.87d

[1]Mycelial growth area and mycelial colony diameter were measured when it was completed in any of Petri plates at the end of the 11th day after mycelium inoculation for the current study;
**Means followed by different letters in the same columns are statistically different according to Duncan's multiple range test (P<0.01).

The results of the study carried out to determine nitrogen requirement of *H. repandum* indicated that $Ca(NO_3)_2$ was the best nitrogen source (P<0.01). This was followed by peptone. Mycelial growth area and mycelial colony diameter of media containing $(NH_4)_2HPO_4$ and NH_4NO_3 were lower than that of the control and the other nitrogen sources (Table 3). It is generally asserted that ammonium is the most commonly used nitrogen source for most ECM fungi (Rangel-Castro et al., 2002; Sangtiean and Schmidt, 2002; Daza et al., 2006), but the results from the present study were contrary to the reports of these researchers.

Table 3: Effect of nitrogen sources on the mycelial growth of *H. repandum*

Nitrogen sources	Duration of complete mycelium running (day)	Mycelial growth area (cm^2)[1]	Mycelial colony diameter (mm)[1]
$(NH_4)_2HPO_4$	17.29ab**	30.10d**	56.63d**
NH_4NO_3	19.86a	36.11cd	66.47cd
Peptone	14.14bc	51.16ab	80.27ab
$Ca(NO_3)_2$	11.00c	56.86a	86.20a
Malt extract	15.29bc	42.60bc	72.59bc
Yeast extract	14.86bc	41.09bc	69.66bc
Control (N-free)	16.29ab	41.43bc	72.69bc

[1]Mycelial growth area and mycelial colony diameter were measured when it was completed in any of Petri plates at the end of the 11th day after mycelium inoculation for the current study;
**Means followed by different letters in the same columns are statistically different according to Duncan's multiple range test (P<0.01).

Initial pH values of the vegetative inoculum media ranged from 5.47 to 7.30. pH of media at the end of mycelial growth was found between 6.08 and 7.22. In vegetative inoculum media studies, the effects of vegetative inoculum media on mycelial growth were found non-significant (Table 4). The best mycelial growth for *T. terreum* was obtained from peat:vermiculite mixtures in the rate of 1:4 and 1:6 (Kibar and Peksen, 2011b). In another study, peat: vermiculite mixture in the rate of 1:4 was found to be the most suitable vegetative inoculum medium for *L. pyrogalus* (Kibar and Peksen, 2011a). In many studies, it was reported that the most appropriate media to obtain the vegetative inoculum of ectomycorrhizal mushrooms were peat, vermiculite, sphagnum moss and perlite mixtures moistened with different liquid nutrient media (Santiago-Martínez et al., 2003; Parlade et al., 2004; Flores et al., 2005).

Table 4: pH values of the vegetative inoculum media and their effects on the mycelial growth of *H. repandum*

Media	Initial pH of media	pH of media at the end of mycelial growth	Mycelial growth rate (cm day^{-1})	Mycelial growth (cm) (18th day)
Peat	5.47[ns]	6.08[ns]	1.08[ns]	14.18[ns]
Peat:vermiculite (1:4)	6.88	7.01	1.07	13.73
Peat:vermiculite (1:6)	7.10	7.22	1.06	13.03
Peat:vermiculite (1:8)	7.24	7.10	1.14	13.64
Peat:vermiculite (1:10)	7.30	7.20	0.94	13.31

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ns: non-significant

In conclusion, the most suitable mycelial growth of *H. repandum* was obtained at 20 and 25°C, pH 5.5, by using glucose and mannitol as carbon sources and $Ca(NO_3)_2$ as nitrogen source. The poorest mycelial growth was recorded in sucrose and xylose as carbon sources and NH_4NO_3 and $(NH_4)_2HPO_4$ as nitrogen sources. It can also be concluded that the vegetative inoculum of *H. repandum* can be produced by cultivating the fungus in media which are prepared by peat and peat:vermiculite mixtures. To know the most suitable nutritional and environmental conditions for mycelial growth of *H. repandum* and to produce its vegetative inoculum would be useful for successful cultivation of this mushroom.

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