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*Original Scientific Paper*

# Optimising *in vitro* culture conditions for the truffle *Tuber brumale*

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**ABSTRACT:**
The vegetative propagation of ectomycorrhizal truffle fungi is limited by their slow mycelial growth. Many factors including media, isolate genotypes and environmental conditions can alter fungal mycelial growth rates. This study aimed to improve the *in vitro* growth rate of *Tuber brumale* by determining the optimal carbohydrate and nitrogen sources, temperature and pH. After 8 weeks, the highest level of growth and densest hyphal branching were recorded in the medium containing glucose as the main carbohydrate. For nitrogen, glutamine (200 mg N $l^{-1}$) provided the greatest hyphal growth and density compared to the other amino acid treatments. Regarding temperature, 16°C proved to be optimal for *T. brumale* growth and branching. Media of pH 6 and pH 7 were most favourable for the growth of *T. brumale*. The results from this research provide baseline data on the vegetative nutrition of *T. brumale* and have implications for the *in vitro* culture of winter truffle hyphae.

**Keywords:**
growth medium, hyphal branching, hyphal extension, mycelium, winter truffle

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## INTRODUCTION

Hypogeous fungi belonging to the genus *Tuber* establish ectomycorrhizal symbiosis with diverse host plants. Species of *Tuber* are among the most valuable and esteemed fungal fruiting bodies (Donnini *et al.* 2014; Hilszczańska *et al.* 2016). The pungent and distinctive aroma released by truffles, especially when mature, attracts animals and entices them to consume the truffles. Unbeknownst to these animals, they act as vectors of truffle spore dispersal, thus playing a crucial role in the life cycle of these fungi (Pacioni *et al.* 2014; Qin & Feng 2022). Truffle cultivation is considered an arduous, expensive and long-term venture. However, the establishment of commercial farms is rapidly expanding as the truffle harvests from such farms have overtaken that of natural harvests (Stobbe *et al.* 2013; Molinier *et al.* 2016; Moser *et al.* 2017).

The first mycorrhizal symbiosis under controlled conditions between *Tuber* and plants used spore inoculum and was carried out in Italy in the late 1960s; successes were followed by the commercial development of spore-inoculated seedlings by AGRITRUFFE in 1973 (Murat 2015). Iotti *et al.* (2016) demonstrated success in producing fruiting bodies of *T. borchii* via seedlings inoculated with pure mycelium. However, most commercially available truffle-inoculated (*Tuber*) seedlings are still established by inoculating seedlings with a suspension of truffle spores (Nakano *et al.* 2020). Pure culture mycorrhizal synthesis of plants with *Tuber* may offer certain advantages over spore inoculations. First, this method can allow for the continuous production of ectomycorrhizal seedlings of consistent quality. Second, pure mycelium inoculation allows for a higher speed of seedling colonization compared to seedlings inoculated with ascospores (Iotti *et al.* 2012). Finally, inoculation

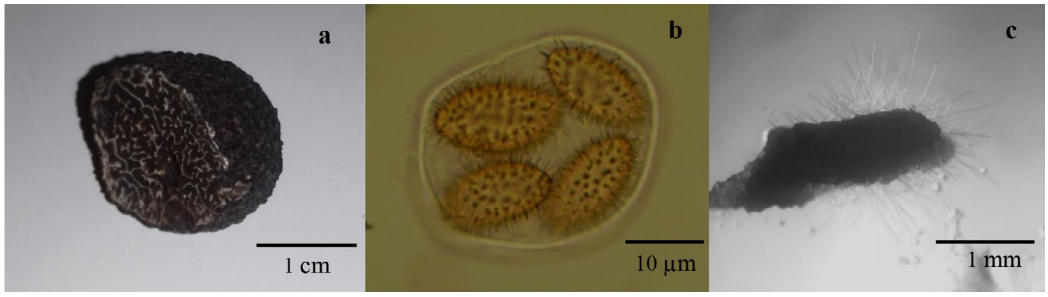


**Fig. 1.** The morphological properties of the ascocarp, ascospores and hyphae of *Tuber brumale*: a) The ascocarp of *T. brumale*; b) The ascus with ascospores of *T. brumale* (1000X); c) The primary hyphal growth of *T. brumale* in the PDA medium.

with mycelium opens up the possibility for selecting improved strains in terms of sexual compatibility, efficiency of mycorrhizal infectivity, and adaptability to local soil and climate conditions (Zambonelli *et al.* 2015; Leonardi *et al.* 2017).

*Tuber brumale* is a sought after and commercial European truffle species (Strojnik *et al.* 2020), which was recently reported from natural habitats in the Mazandaran Province of Iran (Puliga *et al.* 2021). Previous studies showed that the growth of *Tuber* species in controlled conditions is very slow (Iotti *et al.* 2002; PAcioni *et al.* 2007). Based on data released by Iotti *et al.* (2002), the mean growth rate of various *Tuber* species in *in vitro* culture after 40 days ranged from 616 to 896 µm. In fact, *T. borchii* and *T. maculatum* showed the highest hyphal growth (896 and 842 µm, respectively), while *T. brumale* was the slowest growing truffle species of all of the species tested in the study (616 µm). Given the commercial value of *T. brumale,* improving its mycelial growth *in vivo* has real-world applications, and strategies to improve its growth rate may be translatable to other truffle species. For these reasons, research was carried out to optimise the growth of *T. brumale* in pure culture by testing the carbohydrate, amino acid, pH and temperature preferences of this truffle species.

## MATERIAL AND METHODS

Winter truffles (*Tuber brumale*) were harvested from the Yanesar region of Iran located at 36° 37′ 11.6″ latitude and 53° 43′ 8″ longitude, Behshahr, Iran in the autumn of 2019 (Fig. 1a, b). After collecting truffles from natural areas, all of the samples were transferred to the Sari Agricultural Sciences and Natural Resources University. Intact ascocarps were selected and washed under tap water and cleaned with a soft bristle brush. Their peridia were then disinfected with ethanol 70% (V/V). Tissue isolation was carried out with the aid of a stereomicroscope to choose ascocarps with intact peridia to minimise the growth of unwanted fungi including soil born and other saprotrophic fungi. For *in vitro* culturing, the truffle ascocarps were cut with a sterile razor and small fragments of less than 5 mm were separated from the inner part of the ascocarp tissue. These fragments were soaked in streptomycin (100 µg/ml) for 7–10 min, then the sterile samples were dried on filter paper and transferred to Petri dishes containing potato dextrose agar (PDA) medium (Potato infusion: 250 $g.l^{-1}$, Dextrose: 20 $g.l^{-1}$, Agar: 20 $g.l^{-1}$). A total of 19 truffle strains were isolated in this manner. Subcultures of the primary hyphae which emerged from the plated tissues were then transferred to a secondary plate after 20–30-days of growth and were used to carry four independent experiments. The size of the transferred plugs was 3–5 mm (Fig. 1c). To evaluate hyphal growth, the length of a single hypha was measured weekly with a graduated binocular. In order to assess the branching density, the average distances between the branches and the *G* index were also calculated. Five isolates were measured for each of the described assays below with 10 replicate plates per assay.

**Calculating the *G* index.** *G* is an indicator for evaluating the hyphal branching density and it shows the average length of the hyphae subtending from a primary hypha, according to the following equation:

$$G = \frac{Lt}{Nt}$$

Lt: total hyphal length; Nt: the total number of tips

**The morphological and molecular identification of winter truffle ascocarps and pure hyphae.** The morphological identification of the truffles was carried out by evaluating the width, length and shape of the asci and ascospores (Fig. 1b). These data and observations were compared with the descriptions provided by Dimitrova & Gyosheva (2008) to assess the morphological characters and species designations. For molecular characterisation, DNA was isolated from the selected ascocarps and the mycelia were grown for 30 days on PDA following the protocols described by Pacioni *et al.* (2007). The internal transcribed spacer (*ITS*) nuclear rDNA was PCR amplified with the primer pair ITS5/ITS4 (White *et al.* 1990) and was used for molecular identification. The generated sequences have been deposited in GenBank (Table 1). The

**Table 1**. Identification of the truffle ascocarps and pure culture isolates according to molecular methods.

| Source | Isolate name | Sequences length (bp) | Accession number | Blast match | |
|---|---|---|---|---|---|
| | | | | Species | Accession number |
| Truffle ascocarp | TB12 | 869 | OL669325 | *Tuber brumale* | MW829420 |
| Truffle ascocarp | TB13 | 868 | OL669326 | *Tuber brumale* | MT495426 |
| Truffle ascocarp | TB14 | 863 | OL669327 | *Tuber brumale* | MT495426 |
| Truffle ascocarp | TB15 | 869 | OL669322 | *Tuber brumale* | MT495426 |
| Truffle ascocarp | TB16 | 862 | OL672491 | *Tuber brumale* | MT495426 |
| Pure hyphae | TB12ph | 863 | OL669331 | *Tuber brumale* | MW829420 |
| Pure hyphae | TB13ph | 857 | OL669329 | *Tuber brumale* | MT495426 |
| Pure hyphae | TB14ph | 860 | OL669330 | *Tuber brumale* | MT495426 |
| Pure hyphae | TB15ph | 868 | OL669323 | *Tuber brumale* | MT495426 |
| Pure hyphae | TB16ph | 860 | OL672490 | *Tuber brumale* | MT495426 |

**Table 2.** The media ingredients used for investigating the carbohydrate effect on the hyphal growth of *Tuber brumale*

| Medium name | Carbohydrates sources | Compounds |
|---|---|---|
| PA | - | Potato infusion + Agar |
| PDA | Dextrose (Glucose) | Glucose + Potato infusion + Agar |
| PFA | Fructose | Fructose + Potato infusion + Agar |
| PMA | Maltose | Maltose + Potato infusion + Agar |
| PSA | Sucrose | Sucrose + Potato infusion + Agar |
| PDexA | Dextrin | Dextrin + Potato infusion + Agar |
| PMtlA | Mannitol | Mannitol + Potato infusion + Agar |

phylogenetic relationships were further evaluated based on the maximum likelihood method using MEGA7.

**Carbohydrate assays**. To determine the carbohydrate preferences of the truffle isolates, vegetative growth on a potato extract (250 $g.l^{-1}$) reference medium was compared to that of six media containing different carbohydrates (glucose, fructose, maltose, sucrose, dextrin and mannitol) at a concentration of (20 $g.l^{-1}$), in addition to a potato infusion (250 $g.l^{-1}$) reference medium (Table 2). A twenty-day old primary hypha (mother culture) was used to establish this experiment. Colony growth was measured weekly over an 8-week period.

**Amino acids assays.** A PDA medium was used as the basal medium. In this experiment, amino acids glutamine, L-asparagine, methionine, and phenyl alanine as well as a mixture of all these amino acids were compared. Assays were carried out at two concentrations, 100 and 200 mg of nitrogen per litre. The amino acids solutions were sterilised with a 0.22-micron filter and then added to the PDA medium which was autoclaved at 120°C for 20 min and cooled. A twenty-seven-day old primary hypha (mother culture) was used for this experiment. Colony growth was measured weekly over an 8-week period.

**pH assays.** To determine the pH preference of *T. brumale*, PDA was chosen as the basal medium and was prepared at different pH levels. To stabilise the pH, two buffers comprising N-Tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid and 2-morpholinoethanesulfonic acid were used. The media were pH adjusted with 1M NaOH and KOH to target pH levels of 5, 6, 7 and 8 following the protocols of Nakano *et al*. (2020). Colony growth was measured weekly over an 8-week period.

**Temperature assay.** To determine the optimum temperature for the growth of *T. brumale* mycelium, a 27-day old hypha of *T. brumale* was placed on a PDA medium and the plates were incubated at three different temperatures including 8°C, 16°C and 24°C. Colony growth was measured weekly over an 8-week period.

**Statistical analysis.** Analysis of variance was determined using SAS ver. 9.1. Duncan's tests were used for mean comparisons.

## RESULTS

The results from the morphological (Fig. 2a) and phylogenetic analyses demonstrate that the winter truffles collected for this study belong to *T. brumale* haplogroup II of clade A, based on Merényi *et al*. (2014) (Fig. 2b). We further tested the carbohydrate, amino acid, pH and temperature preferences of the obtained isolates in order improve the rate of *in vitro* hyphal growth.

**The effect of carbohydrate sources on *T. brumale* hyphal growth.** After 8 weeks, the greatest hyphal length (1.1 mm) was observed in the potato dextrose agar (PDA) medium, which contained dextrose (glucose) as the main carbohydrate (Fig. 3a). All the other carbohydrate sources tested significantly improved the hyphal growth of *T. brumale* compared to the potato agar (PA) medium with the exception of mannitol, which inhibit-

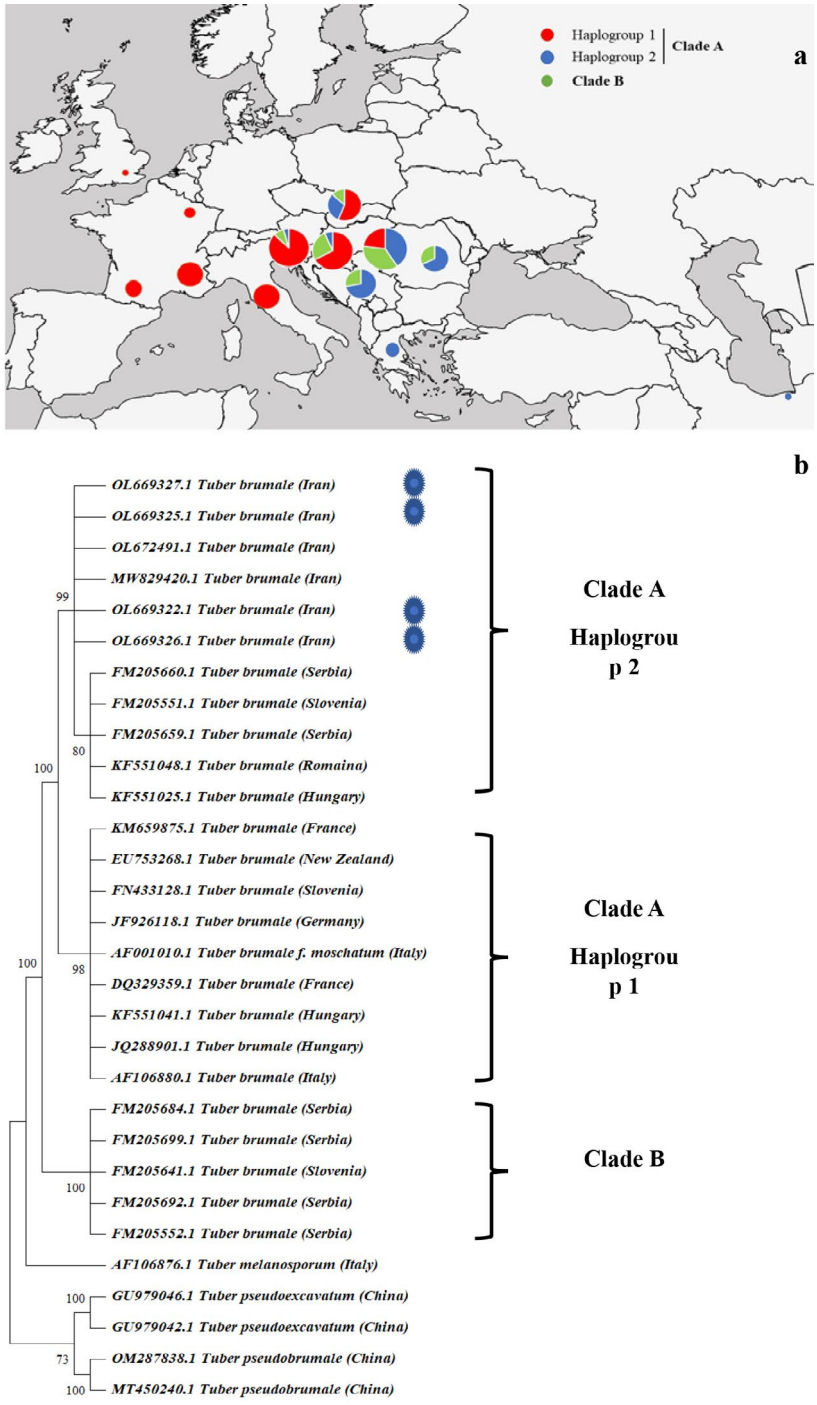


**Fig. 2.** The distribution and phylogenetic relationship of *Tuber brumale* populations: a) The distribution and habitat of *T. brumale* according to the report made by Merényi *et al.* (2014) in addition to our sampling location in Iran (blue dot); b) The phylogenetic relationship between Iranian *T. brumale* (blue asterisk) and populations in Eurasia.

ed hyphal growth (Fig. 3a). In fact, PDA supported the greatest growth of *T. brumale* across all weeks, except for during the eighth week (Supplementary Fig. S1). A comparison of eight weeks of experiments was conducted to shed light on how the mycelium lifespan can affect its growth rate. According to Fig. 4a and Supplementary Fig. S1, the highest and lowest mycelium growth rate was recorded in the first and eighth weeks after *in vitro* culture respectively.

Observations of the hyphal branching showed that the branching of hyphae only occurred in PDA, PFA, PSA and PMA media, as lateral branches (Fig. 5a; Supplementary Fig. S5a). In addition to hyphal length extension, glucose also supported the densest hyphal branching growth. The lowest average distances between the hyphal branches (0.373 mm) were measured in the PDA medium (Supplementary Fig. S5a). The lowest score in the hyphal branching *G* index (0.385) was related to the PSA medium. No

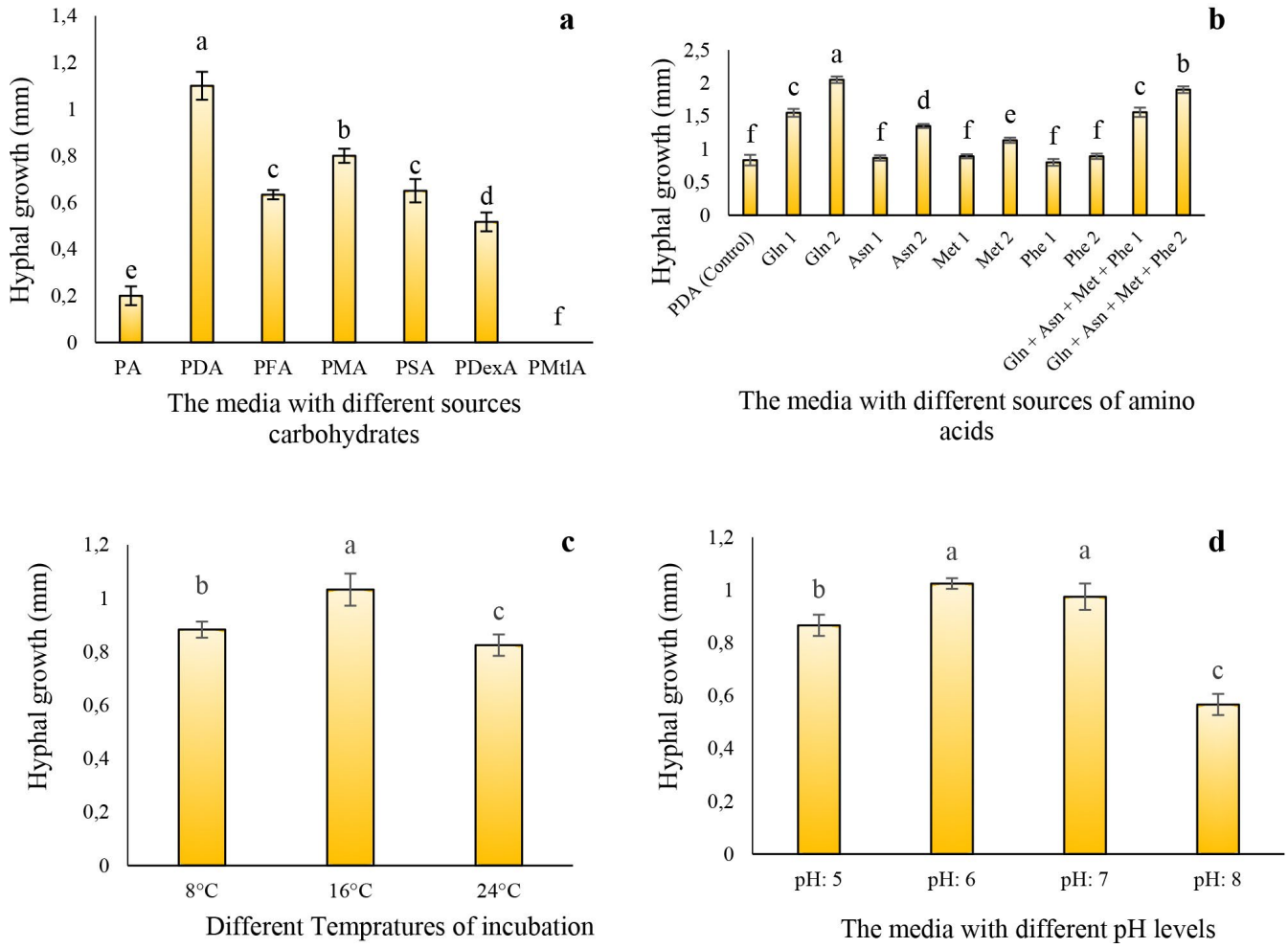


**Fig. 3.** The hyphal growth of *Tuber brumale* after 8 weeks: a) The hyphal growth of *T. brumale* as affected by different carbon sources after 8 weeks. The specific carbon source of different media: (PA: non carbohydrates), (PDA: Dextrose (Glucose)), (PFA: Fructose), (PMA: Maltose), (PSA: Sucrose), (PDexA: Dextrin), (PMtlA: Mannitol); b) The hyphal growth of *T. brumale* as affected by different amino acid treatments after 8 weeks (Basal medium: PDA). Abbreviations: Gln: glutamine, Asn: Asparagine, Met: Methionine, Phe: Phenylalanine. Suffixes1 and 2 are related to the concentration of amino acids (100 and 200 mg N $l^{-1}$, respectively); c) The hyphal growth of *T. brumale* as affected by different temperatures after 8 weeks (Basal medium: PDA); d) The hyphal growth of *T. brumale* as affected by different pHs after 8 weeks (Basal medium: PDA). a, b, c and d - statistically significant at 1% level. The error bars depict the standard deviation. In each column, the means followed by the same letter are not significantly different according to Duncan's tests.

statistically significant differences in the *G* index were noted between the PDA, PMA and PSA media (Fig. 5a).

**The effect of amino acids on *T. brumale* hyphal growth.** Fifty-eight (58) days after *in vitro* culture, the highest hyphal length (2.05 mm) was observed in the medium containing 200 mg $l^{-1}$ nitrogen in the form of glutamine, which was significantly greater than for all the other treatments (Fig. 3b). The application of 200 mg $l^{-1}$ nitrogen in the complex amino acid treatment (contained: glutamine + L asparagine + methionine + phenyl alanine) ranked second for hyphal growth (1.9 mm) (Fig. 3b).

The hyphal growth rates in all the evaluated weeks, with the exception of week 6, showed the highest quantitative values or exhibited no significant differences compared to the optimum treatment (Supplementary Fig. S2). Over the course of the experiment, mycelial growth (0.541 mm) was significantly greater during the first week, after which growth dropped until the fifth week. After week 5, the rate of hyphal growth did not change significantly (Fig. 4b).

There were no statistically significant differences in hyphal branch formation by either glutamine 200 mg $l^{-1}$ or the complex amino acid 200 mg $l^{-1}$ (Fig. 5b; Sup-

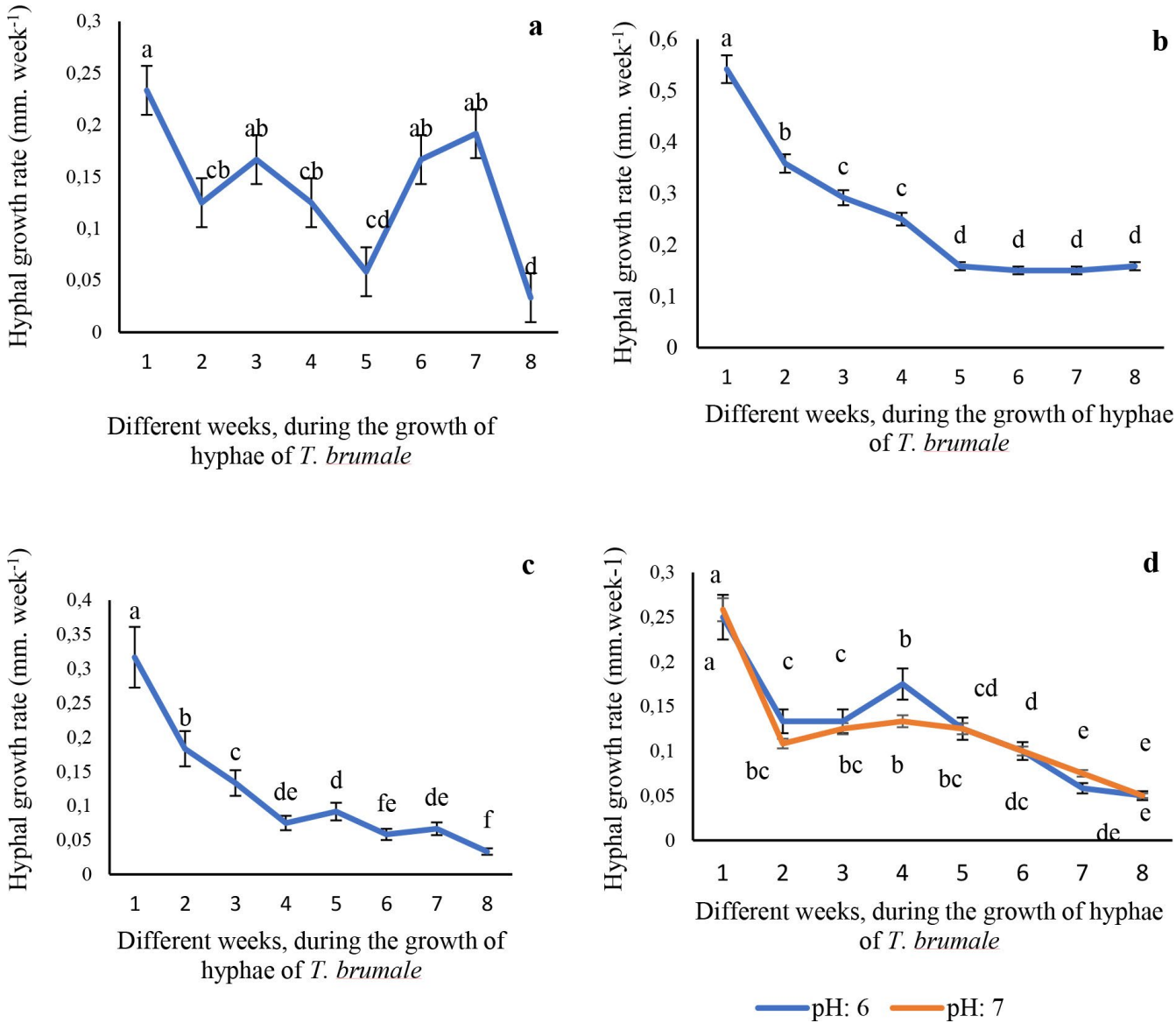


**Fig. 4.** The hyphal growth rate of *Tuber brumale* in the promising media of four carried out experiments: a) The hyphal growth rate of *T. brumale* in the PDA medium during 8 weeks (PDA: the medium which contained dextrose as the main source of carbohydrates); b) The hyphal growth rate of *T. brumale* in glutamine 200 mg N $l^{-1}$ treated medium during 8 weeks; c) The hyphal growth rate of *T. brumale* as affected by 16°C during 8 weeks; d) The hyphal growth rate of *T. brumale* as affected by pH 6 and 7 during 8 weeks. a, b, c and d - statistically significant at 1% level. The error bars depict the standard deviation. In each column, the means followed by the same letter are not significantly different according to Duncan's tests.

plementary Fig. S5b). The average distances between the new hyphal apexes of glutamine 200 mg $l^{-1}$ and complex amino acid 200 mg $l^{-1}$ were 0.275 and 0.285 mm, respectively (Supplementary Fig. S5b). Figure 5b shows that the lowest values for *G* were recorded in the media containing glutamine 200 mg $l^{-1}$ (0.287) and complex amino acid 200 mg $l^{-1}$ (0.285), which show denser hyphal branching than for the other treatments.

**The effect of temperature on *T. brumale* hyphal growth.** The highest growth of *T. brumale* hypha (1.03 mm) after 8 weeks was recorded at 16°C (Fig. 3c). The optimum temperature for hyphal growth in the 1st, 3rd and 8th weeks was 16°C, however, in the other weeks of the experiment no significant differences were observed among the tested temperatures (Supplementary Fig. S3). At 16°C, the highest hyphal growth rate was measured as 0.316 mm in the first week, which was greater than all the other weeks and temperatures (Fig. 4c). The densest hyphal branching was observed at 16°C after 8 weeks (Fig. 5c; Supplementary Fig. S5c). Although there were no statistically significant differences in the *G* index of

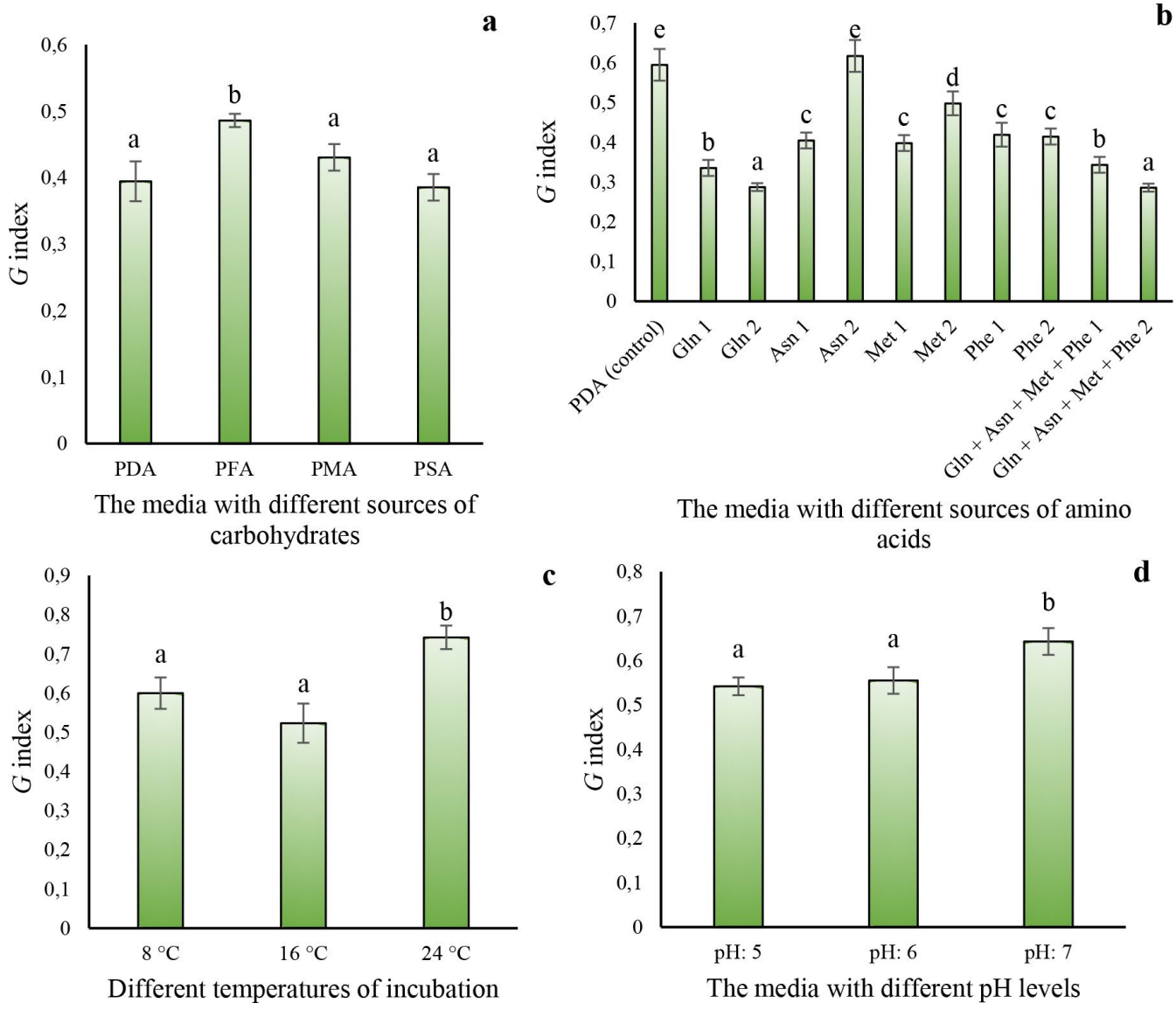


**Fig. 5.** The *G* index of the hyphal branching of *Tuber brumale*: a) The *G* index of the hyphal branching of *Tuber brumale* in certain media with different carbon sources which lead to hyphal branching; after 8 weeks. The specific carbon source of different media: (PDA: Dextrose (Glucose)), (PFA: Fructose), (PMA: Maltose), (PSA: Sucrose); b) The *G* index of the hyphal branching of *T. brumale* as affected by different amino acid treatments after 8 weeks (Basal medium: PDA). Abbreviations: Gln: glutamine, Asn: Asparagine, Met: Methionine, Phe: Phenylalanine. Suffixes1 and 2 are related to the concentration of amino acids (100 and 200 mg N $l^{-1}$, respectively); c) The *G* index of the hyphal branching of *T. brumale* as affected by different temperatures after 8 weeks (Basal medium: PDA); d) The *G* index of the hyphal branching of *T. brumale* as affected by different pHs after 8 weeks (Basal medium: PDA). a, b, c and d - statistically significant at 1% level. The error bars depict the standard deviation. In each column, the means followed by the same letter are not significantly different according to Duncan's tests.

16°C (0.523) and 8°C (0.599) treatments, the average distance between the new hyphal apexes was 0.409 mm at 16°C, which was significantly lower than at the other temperatures (Fig. 5c; Supplementary Fig. S5c).

**The effect of pH on *T. brumale* hyphal growth.** The growth of *T. brumale* hypha was the greatest at pH 6 and 7, leading to hyphal lengths of 1.025 and 0.975 mm, respectively, after 8 weeks with no significant differences between the two pHs (Fig. 3d). The highest hyphal growth rate took place during the first week and the lowest growth rate was recorded in the seventh and eighth weeks (Fig. 4d; Supplementary Fig. S4). The observations of hyphal branching showed that the densest branches formed at pH 6 (Fig 5d; Supplementary Fig. S5d). At the densest hyphal branching junctions, the distance between the branches was 0.400 mm at pH 6. Figure 5d shows that there were no statistically signifi-

cant differences between the *G* index of pH 6 (0.555) and pH 5 (0.541). However, hyphal extension at pH 7 was significantly greater than at pH 5, while acidic conditions increased hyphal branching and branches did not form at pH 8 (Fig. 5d).

## DISCUSSION

In this study we tested the carbohydrate, amino acid, pH and temperature preferences of *T. brumale* in order to improve the hyphal growth rate of this commercial truffle species *in vitro*. We found that *T. brumale* isolates have a nutritional preference for glucose as a carbohydrate source and glutamine as a nitrogen source. Mycelial growth was also improved by culturing on media at a pH of 6 and 7, and incubating at 16°C.

Optimising mycelial growth requires an understanding of fungal nutrition and physiology. The comparison of our results with previous studies indicates that truffle species may differ in their nutritional preferences. For example, Saltarelli *et al*. (1998) reported that glucose and fructose are a superior source of carbon for *T. borchii* strain ATCC 96540. Yet, Ceccaroli *et al*. (2001) concluded that mannose was preferred as a carbohydrate for three strains of *T. borchii* (i.e. 1BO, 17BO and 10RA) in comparison with glucose and mannitol. Interestingly, the hyphal growth of the 17BO strain was curbed in the medium containing mannitol. Oriaifo (2014) evaluated diverse carbohydrate sources on the growth of *T. borchii* and found that the highest growth rate in all the studied strains was observed with starch as the carbohydrate source in comparison with sucrose, fructose, xylose, glucose, mannose, glycerol sorbitol and mannitol. In contrast, research on *T. maculatum* showed that mycelial growth was highest when lactose was used as a carbon source, but the difference between it and other carbohydrate sources including sucrose, fructose, xylose, glucose, dextrose, mannose and maltose was not statistically significant (Nadim *et al*. 2016).

It is well documented that *T. melanosporum* and *T. brumale* have a close phylogenetic relationship (Bonito *et al*. 2013). Thus, our results for *T. brumale* may be generalisable to *T. melanosporum*, and *vice versa*. Although compounds such as cellulose, cellobiose and starch can be used as a carbon source for *T. melanosporum* (Mamoun & Olivier 1991), genomic sequencing showed a limited gene repertoire in this species' coding for carbohydrate active enzymes (CAZymes), which are the enzymes responsible for biodegrading non-living organic matter. For example, just a few glycoside hydroxylase (GH) genes were identified in *T. melanosporum* in contrast to its saprotrophic relatives which contain many genes in the GH6 and GH7 cellulase gene families (Martin *et al*. 2010). These findings provide the genetic basis for the weak saprotrophic ability of *T. melanosporum* observed. However, *T. melanosporum* has an invertase enzyme, enabling it to hydrolyse the sucrose provided by the host plant. Based on these data, it is expected that *T. brumale* grows in a similar way to *T. melanosporum* and prefers to assimilate simple sugars rather than polymer carbohydrates.

The proliferation and growth of mycelium is dependent on hyphal branching patterns. *G* is an efficient index for the assessment of branching conditions. The value of *G* depended on the total length of the mycelium and the number of tips. Thus, *G* is an indicator of growth density and when branching is rare, the *G* index will be higher (Moore *et al*. 2020). We found that *G* was highest with fructose as a carbon source and asparagine as a nitrogen source. The *G* values were also greater at higher temperatures and the pH levels tested. Although many aspects of hyphal branching are not yet known, one interesting hypothesis is that heat shock proteins (polypeptides) are involved in the mechanisms of branching initiation. It is thought that conformational change in the cell wall proteins is required for branch initiation, and that heat shock proteins play a key role in the delivery of initiation polypeptides to the appropriate site (Moore *et al*. 2020). The Spitzenkorper (SPK) is a multicomponent pleomorphic structure which plays a key role in maintaining hyphal extension (Riquelme & Sanchez-Leon 2014). Lateral hyphal branching requires the emergence of a new SPK to support the growth of nascent hyphae (Harris 2019). Previous studies have revealed that the main components of the SPK include an accumulation of vesicles (containing the enzymes required for cell wall synthesis), ribosomes, actin microfilaments, chitosomes and an amorphous material of undefined nature (Riquelme & Sanchez-Leon 2014; Harris 2019). Amino acids are, in terms of actin (as a protein polymer) and enzyme structure, critical to the formation and activity of SPK (Berepiki *et al*. 2011).

Cell division is controlled by core cell cycle genes and transcription factors. Among the core cell cycle genes involved in the biosynthesis of cyclins and their catalytic partners, cyclin-dependent kinases (CDKs) play a key role in the regulation and progression of cell cycles (Hydbring *et al*. 2016). Considering the structure of cyclins and CDKs (proteins), amino acids serve as their monomeric units, thus increasing the amino acid content of the cells would be effective for the progression of cell cycles. Furthermore, the amino acids and amino groups involved in histones and nucleotides play an important role in cell cycle division (Mariño-Ramírez *et al*. 2005). Glutamine is an energetically favoured nitrogen source for fungi, and is involved in the biosynthesis pathways of many secondary metabolites and other amino compounds (Tudzynski 2014). Glutamine serves as an efficient nitrogen source for *T. brumale* hyphal growth. Previous studies on nitrogen metabolism in ectomycorrhizal fungi demonstrated that some amino acids including glutamine, glutamate, arginine and aspar-

agine are abundant in the fungal mycelium (Johansen *et al*. 1996; Bago *et al*. 1999). Ectomycorrhizal fungi are known to have the ability to uptake some forms of organic nitrogen, which they can transfer to the host plant in the form of glycine and glutamate (Hawkins *et al*. 2000). Thus, it is interesting that *T. brumale* utilised and preferred glutamine over the other amino acids, raising the question as to whether *T. brumale* utilises and transfers organic N to host plants in natural systems.

The evaluation of the hyphal growth of *T. sinense* in media with different nitrogen sources showed that the highest growth occurred in yeast extract and peptone treatments, which were preferred as a nitrogen source compared to mineral forms of nitrogen such as ammonium nitrate and potassium nitrate (Liu *et al*. 2008). Alanine was also found to stimulate more hyphal growth of *T. melanosporum* in comparison with asparagine and inorganic sources (Kamal 2011). The formation of D-type cyclins is critical for progression through the G1 phase of the cell cycle to drive DNA synthesis (S phase). The biosynthesis pattern of D-type cyclins changes in response to the intracellular conditions and environmental factors (Hydbring *et al*. 2016). In another study by Oriaifo (2014), the effect of nitrogen sources was investigated on *T. borchii*. The results revealed that the effect of the nitrogen source was genotype-dependent, and the response of each strain to the nitrogen source was different.

According to Zambonelli *et al*. (2016), the habitats of *T. brumale* have a 3.7–15.8°C annual mean temperature. We found that 16°C was the optimal incubation temperature for *T. brumale* growth among all the isolates tested. The optimum temperature for hyphal growth in *T. maculatum* was reported to be 15°C (Nadim *et al*. 2016). In another investigation, Oriaifo (2014) pointed out that most strains of *T. borchii* preferred 20 and 17.5°C, but they noted that temperature preference was isolate-dependent.

*Tuber* species are known to prefer non-acidic environments, which is one of the reasons why truffle orchard soils are often limed or planted on naturally alkaline soils. Previous studies aimed at understanding the ecological parameters of *T. brumale* habitats elucidated that the average water pH in the Carpathian Basin is 6.9 (Zambonelli *et al*. 2016). We found that *T. brumale* mycelium grew significantly better at a pH of 6 and 7 compared to the other pH levels tested. Hyphal growth rates and preferences differ between the different clades of *Tuber*. It was reported that the hyphal growth of *T. japonicum* in *in vitro* culture was observed in pH 5 and 6, while *T. himalayense* and *T. longispinosum* exhibited maximum growth in pH 7 (Nakano *et al*. 2020). Oriaifo (2014) tested a range of pH values (4–9) on *T. borchii* mycelial growth and found pH to be a significant factor for mycelial growth, however, the optimum pH varied by isolate. In another study, the optimum pH hyphal growth of *T. maculatum* was reported to be in the range of pH 5.8–7.6 (Nadim *et al*. 2016). These results demonstrate that the response to the pH medium is both species and genotype dependent. Thus, each species of fungi, and possibly each strain, may need to be tested to determine the optimum pH for growth.

## CONCLUSION

*Tuber brumale* is an important commercial truffle which grows across Europe and was recently reported from Iran. Through this research we were able to improve the culture medium and environmental conditions for the growth of *T. brumale* in pure culture. We showed that glucose and glutamine are the preferred carbon and nitrogen sources, respectively. For the growth of *T. brumale* mycelium, a PDA (potato extract + Dextrose (glucose) + agar) medium containing 20 g/L glucose is an appropriate option, and adding 200 mg $l^{-1}$ nitrogen from a glutamine source is recommended to promote faster growth. We found that the optimal temperature for hyphal growth is around 16°C and the optimal pH medium is 6–7. Importantly, the hyphal growth rates decreased significantly over time, indicating that fresh mycelium should be propagated and sub-cultured frequently to maintain active growth.

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**REZIME**

Botanica SERBICA

## Optimizacija uslova za *in vitro* gajenje tartufa *Tuber brumale*

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Vegetativno razmnožavanje ektomikoriznih gljiva tartufa ograničeno je njihovim sporim rastom micelija. Mnogi faktori, uključujući medijume, izolovane genotipove i uslove životne sredine, mogu promeniti stopu rasta micelija gljiva. Ova studija je imala za cilj da poboljša *in vitro* brzinu rasta *Tuber brumale* određivanjem optimalnih izvora ugljenih hidrata i azota, temperature i pH. Posle 8 nedelja, najveći rast i najgušće grananje hifa zabeleženi su u medijumu koji je sadržao glukozu kao glavni ugljeni hidrat. Za azot, glutamin (200 mg N $l^{-1}$) je obezbedio najveći rast i gustinu hifa u poređenju sa drugim tretmanima amino kiselina. Što se tiče temperature, 16°C je bilo optimalno za rast i grananje *T. brumale*. Rezultati ovog istraživanja daju osnovne podatke o vegetativnoj ishrani *T. brumale* i imaju primenu u kulturi hifa zimskih tartufa *in vitro*.

**Ključne reči:** medijum za gajenje, grananje hifa, izduživanje hifa, micelijum, zimski tartuf