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Responses of the *Corylus avellana* Colonized by the *Tuber Melanosporum* Mycorrhiza to Short-Term Rhizosphere Disturbance

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Abstract

We hypothesized that *Tuber melanosporum* colonization enhances growth and photosynthetic performance in *Corylus avellana* seedlings. Forty-eight seedlings were assessed for root colonization (stereomicroscopy, ITS sequencing) and photosynthetic traits (Li-6800F) under short-term disturbed and undisturbed rhizosphere conditions. Mycorrhizal colonization was found in 97.9% of seedlings (47/48). The mean colonization was 33.1% (SD = 16.1), 16.7% of seedlings showed more than 50% colonization per seedling, and 65.0% showed more than 30% colonization per seedling. Colonization declined with root depth and correlated with seedling length (r = 0.371, $p = 0.01$). In disturbed roots, longer root length predicted higher Gsw (r = 0.60), $PhiCO_2$ (r = 0.77), and PhiPSII (r = 0.70), while collar diameter negatively affected transpiration (r = −0.60). In undisturbed roots, collar-proximal colonization improved PhiPSII (r = 0.69, $p = 0.02$). Undisturbed seedlings showed ~2× higher CO_2 assimilation, stomatal conductance, quantum yield, and transpiration. These findings confirm that *T. melanosporum* enhances seedling physiology, especially under undisturbed conditions.

Keywords: cultivation; fungal inoculation; host plant; mycorrhizal disruptions; symbiosis

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1. Introduction

Tuber melanosporum, also known as the Périgord black truffle, is one of the world's most valuable truffle species. It is native to France, Italy, Spain, Slovenia, and Croatia, and it is of great importance in the European truffle industry because of its culinary value and high market demand [1–3]. Truffles were long collected from the wild, but systematic cultivation started in the early 19th century when Joseph Talon successfully transplanted seedlings mycorrhized with truffle [4]. Later, in the late 1960s and early 1970s, advanced techniques for artificially inoculating truffle spores into greenhouse plants were developed. Publicly, commercial production of mycorrhized plants began in 1974 and this marked the start of a truffle renaissance [4,5]. As a result, truffle plantations expanded globally in the late 20th century [6–8]. This history of truffle plantations demonstrates how truffle farming gained popularity due to its economic benefits and its potential to support rural agroforestry [9,10].

Despite considerable advances in truffle cultivation, the symbiotic relationships between truffles and their host plants remain not fully understood. Researchers continue

to investigate these interactions, as addressing these knowledge gaps is essential for improving truffle production and achieving consistent, reliable harvests. In advanced truffle farming, both the host plant and the fungi below ground need to stay healthy to support optimal growth [8,11]. This indicates the importance of using high quality seedlings for successful truffle cultivation [12]. Therefore, assessing the quality of the mycorrhizal symbiosis is important, as higher levels of mycorrhization are linked to the production of high-quality seedlings [6]. While a well-developed root system can typically contribute to the formation of high levels of mycorrhization, and other factors like nursery practices, inoculum dose, soil type, container size, and the choice of host plants play a significant role in determining the quality of mycorrhization [13–15].

One of the common host plants used for *T. melanosporum* cultivation is the European hazelnut (*Corylus avellana*), which is economically and ecologically important. It is widely cultivated in Europe because of its well-developed root system that helps to form effective mycorrhizal partnership with truffle [14]. In Transylvania, hazelnuts are not as widely used as a host plant as compared to other species like Oak, but it is still favored because it produces seeds faster and has a dense root system that supports truffle growth [16]. Recently, the cultivation of this plant species has expanded across Europe, Asia, Africa, and North and South America, driven by growing demand from the food industry [17]. However, in truffle farming, disturbing the root system can reduce mycorrhizal colonization and harm the host plant's roots [9]. From a mutualistic perspective, mycorrhization with *T. melanosporum* alters both the physiology and root structure and it shortens primary roots, enhances lateral root growth, and stimulates overall root development [18]. In this partnership, some fungi release volatile compounds that modify plant root architecture under laboratory conditions, shortening primary roots and elongation root hairs [19]. Fungi also produce hormones, such as auxins, cytokinin, and gibberellic acids, which help regulate plant growth and stress responses [20].

It is concluded that both *C. avellana* and *T. melanosporum* hold significant cultural, ecological, and economic values. Therefore, continuous year-round study of their association is important to obtain up-to-date scientific information, which can contribute to improving truffle cultivation and increasing productivity. This study aimed to assess the levels of mycorrhizal colonization and examined the impact of short-term rhizosphere disturbance on the host plants, as the active stage of mycorrhization process are often disrupting during field transplantation and microscopic examination. Many published articles studied the relationship between *C. avellana* and *T. melanosporum*, but direct studies on the impact of rhizosphere disturbance on this association remain limited.

Therefore, the study laid the groundwork for understanding how short-term disturbances affect the mycorrhizal relationship. We hypothesized that short term rhizosphere disturbance would negatively affect both mycorrhization levels and photosynthetic activity in *C. avellana* seedlings. Conversely, seedlings with undisturbed rhizospheres were expected to show better photosynthetic efficiency and stronger correlations between root health and plant performance. To test this, the study aimed to (1) evaluate *T. melanosporum* mycorrhization levels in *C. avellana* seedlings and (2) assess its effects on plant morphology and photosynthetic performance under disturbed and undisturbed rhizosphere conditions.

2. Materials and Methods

2.1. Host Plant

In this study, 48 hazelnut (*C. avellana*) seedlings were grown from surface-sterilized seeds, rather than propagated vegetatively. The seeds were obtained from the local market in Hungary and disinfected by soaking in a 0.15% sodium hypochlorite (NaOCl) solution for 20 min, followed by rinsed in distilled water to remove any undesired organisms or

pathogens [21]. The seeds were then sown in a growing medium consists of a carefully balanced mix of peat moss, vermiculite, perlite, and steam-sterilized garden soil, with a pH of about 7.2–7.3. Each seedling was planted in a 0.8 L plastic pot filled with this substrate, which supported a high germination rate and vigorous sprouting. Deliberately, a small space was maintained at the top of each plastic pot to avoid overflow during watering. This cultivation method, aimed at achieving successful mycorrhizal colonization under controlled conditions in the greenhouse, aligned with the previous methods as described in the published literature [22].

2.2. Spore Inoculation

The inoculant used for inoculation was obtained from mature and intact *T. melanosporum* fruiting bodies, which was morphologically characterized by brownish-black, compact ascomata with small warts on the surface, a blackish to brown peridium, and grayish to brownish-black gleba containing light to mid-brown, spiny spores [23]. These mature and intact fruiting bodies used for inoculation were obtained from recognized experts and clearly identified based on their morphological characteristics. Therefore, additional molecular confirmation was not necessary.

Therefore, the inoculant solution was prepared from 300 g of fruiting bodies by chopping them with a Waring blender, Stamford, CT, USA and mixing them with 600 mL of distilled water to create spore suspensions. Each seedling received four ml of this suspension into the root of each seedling using a syringe. After inoculation, the seedlings were grown in controlled environment inside a foil house and watered regularly to maintain optimal growth conditions, ensure healthy development and effective mycorrhization.

2.3. Estimation and Identification of Mycorrhization

In the study, 48 *C. avellana* seedlings were assessed to estimate mycorrhization one year after inoculation (Table S1). The entire root systems were carefully removed from the plastic pots, washed with cold water, and observed under a microscope [24,25]. Morphological examination of the mycorrhizal root tips was performed using a Nikon SMZ-U stereomicroscope, while the structural of the fungal mantle were examined using a Nikon Optiphot-2 research microscope (Nikon Co., Tokyo, Japan). Additionally, height, root length, and collar diameter of the seedlings were measured. The study also considered some variations in root architecture, such as root length and hair distribution, which can differ depending on plant species, soil type, and nutrient availability [26].

A preliminary observation indicated that the seedlings showed dense root architecture, so examinations were conducted at five root spots from three root zones: proximal (5 cm from the collar), middle (>5–10 cm), and distal (10–15 cm), as shown in (Figure S1). This study slightly modified methodologies used from previous research [21,24,27,28] and at least 350 root tips per seedling were checked under a microscope. Mycorrhizal identification followed standard morphological descriptions for *Tuber* species [29], with the extent of mycorrhization expressed as a percentage of mycorrhizal root tips relative to the total number examined [30]. The general estimation Equation is as follows.

$$\text{Mycorrhization}(\%) = \left(\frac{\text{Observed mycorrhized roots}}{\text{Total observed roots}(\text{Mycorrhized} + \text{non} - \text{mycorrhized})} \right) \times 100$$

Molecular confirmation was performed by extracting DNA from typical *T. melanosporum* mycorrhizal morphotypes using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The internal transcribed spacer (ITS) region was amplified through PCR using the ITS1F/ITS4 [31,32] and thermal cyclers (Techne TC-312 and BIOER Little Genius, Shenzhen, China). After amplification, the DNA was

purified using the QIAquick®, Hilden, Germany, PCR Purification Kit, as per the manufacturer's guidelines. The purified DNA samples were then sent for sequencing to BIOMI Ltd. (Gödöllő, Hungary). The sequenced data were stored in the database under accession numbers PV053854, PV053855, and PV053856, which are scheduled for public release on 29 January 2029. These sequences were analyzed using BLAST, version 2024 and compared with the GenBank databases based on query coverage and percentage identity.

2.4. Photosynthetic Activities

To investigate the effects of mycorrhizal disturbance on *Corylus avellana* seedlings, we conducted a controlled experiment with 48 inoculated seedlings. Due to logistical constraints, 22 seedlings were randomly selected for physiological analysis and divided into two treatment groups of 11 each. Group I (Disturbed) underwent rhizosphere disturbance during initial mycorrhizal observations, with photosynthetic measurements taken two weeks later. Group II (Undisturbed) served as the control, with photosynthetic activity measured first, followed by mycorrhizal assessment. All seedlings were kept under identical conditions, receiving consistent light and 50 mL of water daily for two weeks. While the reduced sample size was necessary due to experimental limitations, we acknowledge that it may affect the statistical power of our findings, and this limitation will be discussed further.

Photosynthetic performance was assessed using a Li-6800F portable photosynthesis system (Li-Cor, Lincoln, NE, USA) equipped with a 2 cm^2 aperture, following the manufacturer's protocols [33]. Measurements were conducted on healthy, fully expanded leaves (typically the second from the apex) between 9:00 A.M. and 7:00 P.M. under controlled conditions: CO_2 concentration of 400 µmol mol^{-1}, relative humidity of approximately 60%, flow rate of 800 µmol s^{-1}, and air temperature maintained between 23 and 25 °C. Actinic light at 300 µmol photons m^{-2} s^{-1} was applied to stimulate photosynthesis. The parameters recorded after stabilization included maximum photochemical efficiency of photosystem II (Fv/Fm), effective quantum yield of PSII (ΦPSII), stomatal conductance (gsw), CO_2 assimilation rate (A), transpiration rate (E), quantum yield of CO_2 (ΦCO_2), and non-photochemical quenching (NPQ).

2.5. Statistical Analysis

A simple linear regression and Pearson's correlation coefficient ($p < 0.05$) were used to examine the relationships between photosynthesis, plant growth parameters, and mycorrhization levels. Differences in photosynthetic activities between disturbed and undisturbed rhizospheres were analyzed using Minitab (V. 21.2) and SPSS (V. 20). Descriptive statistics (means and standard deviations) summarized the dataset, and graphical representations were used to visually illustrate correlations and trends.

3. Results

In this study, mycorrhization was observed from the roots of 47 out of 48 seedlings, while one seedling showed no mycorrhizal presence. Most importantly, no contaminants were observed or detected in any of the seedlings, even though the mean root length (15.9 ± 2.7 cm) exceeded the container depth of 14 cm as depicted in Figure S1. The mycorrhizal structure displayed simple branching with a puzzle-like mantle, bifurcate cystidia extended from the surface, and mycorrhizal coloration ranging from reddish-brown to dark brown (Figure 1). Molecular analysis confirmed the mycorrhizal fungus as *T. melanosporum* through sequence comparison with the GenBank database. Mycorrhizal colonization rates were generally high with 16.66% of seedlings had over 50% root colonization, 64.96% showed strong colonization (>30%), and nearly all (97.92%) had some level of colonization.

Two weeks after the disturbance, some mycorrhizal root tips exhibited signs of shrinkage and desiccation.

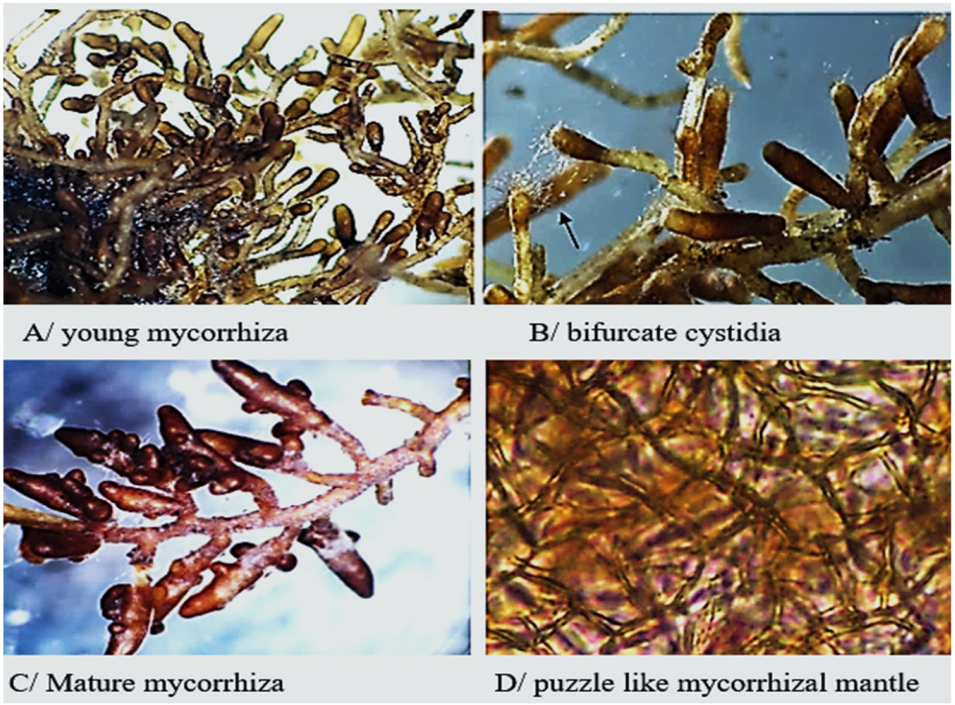


Figure 1. *Tuber melanosporum* mycorrhization in *C. avellana* seedling roots, (**A**,**C**) showing ramification, (**B**) cystidia, and (**D**) mantle structure under a stereomicroscope (scale bars: (**A**–**C**) = 500 µm; (**D**) = 50 µm). The arrow highlights the observed massive cystidia of Tuber melanosporum located at the tip of the swollen region of the root.

The mean mycorrhizal colonization across all seedlings was 33.1%, showing the highest variability with a wide range (SD = 16.1). Among seedling traits, collar diameter was the most stable (SD = 0.94), while seedling length (SD = 8.5) and root length (SD = 2.7) exhibited moderate variability, with root length having a narrower range compared to seedling length (Figure 2a). Colonization levels decreased from the root base toward the tips as depth increased (Figure 2b). Statistical analysis indicated a positive correlation between mycorrhization and seedling length (r = 0.371, p = 0.01), while seedling traits showed strong (length–diameter, r = 0.584, $p < 0.001$) and moderate (diameter–root length, r = 0.312, p = 0.033) correlations, with other relationships being weak and non-significant (Table 1).

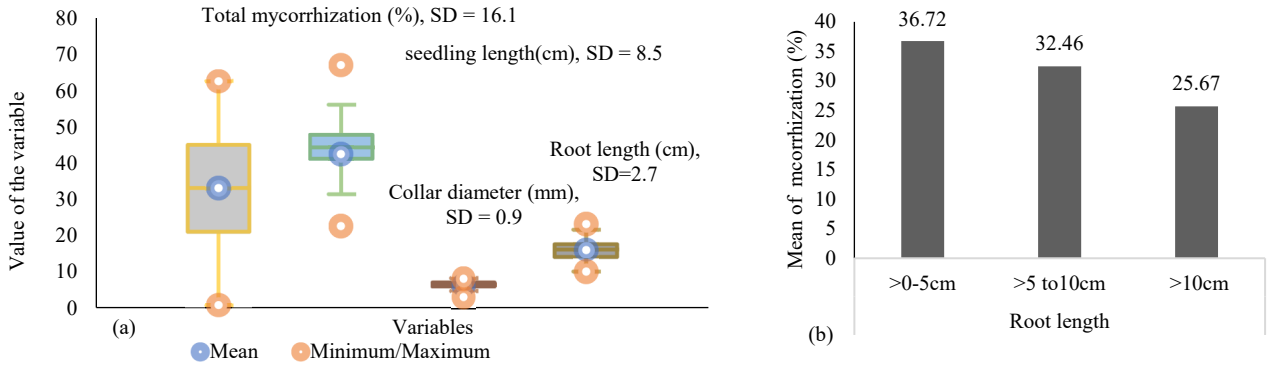


Figure 2. Descriptive statistics of (**a**) mycorrhization, collar diameter, seedling height, and root length of *C. avellana* (47 seedlings), and (**b**) mean of mycorrhization by root length from stem collar.

Table 1. Pairwise comparison among mycorrhization, seedling length, collar diameter, and root length of *C. avellana* (47 seedlings).

Variable 1 vs. Variable 2	Pearson's r	*p*	Lower 95% CI	Upper 95% CI
Mycorrhization (%) vs. Seedling length (cm)	0.371 *	0.01	0.094	0.595
Mycorrhization (%) vs. Collar diameter (mm)	0.17	0.254	−0.123	0.436
Mycorrhization (%) vs. Root length (cm)	0.179	0.228	−0.114	0.444
Seedling length (cm) vs. Collar diameter (mm)	0.584 ***	<0.001	0.357	0.746
Seedling length (cm) vs. Root length (cm)	0.203	0.172	−0.09	0.463
Collar diameter (mm) vs. Root length (cm)	0.312 *	0.033	0.027	0.55

* $p < 0.05$, *** $p < 0.001$.

In the first group (disturbed), where mycorrhizal colonization was assessed two weeks prior to measuring photosynthetic activity, there was no significant impact on the maximum efficiency of photosystem II (Fv/Fm) or its quantum yield (PhiPSII), as shown in Table S2. However, longer roots showed a moderate to strong positive correlation with higher stomatal conductance (gsw, r = 0.6, $p = 0.05$), better CO_2 uptake efficiency ($PhiCO_2$, r = 0.77, $p = 0.006$), and improved photosystem II efficiency (PhiPSII, r = 0.70, $p = 0.02$), with linear regression indicating that root length explained approximately 37%, 59%, and 49% of the variability in these parameters, respectively, as presented in Table S2. On the other hand, a thicker collar diameter showed a moderate negative correlation with transpiration rate (E, r = −0.6, $p = 0.05$), with linear regression indicating it accounted for approximately 38% of the variability, as illustrated in Table S2.

In undisturbed group (II), mycorrhiza found near the stem collar (0–5 cm) improved the efficiency of photosystem II (PhiPSII, r = 0.69, $p = 0.02$); however, mycorrhization did not significantly affect the maximum efficiency of photosystem II (Fv/Fm), as shown in Table S3. In the comparison of the two groups (disturbed and undisturbed), most photosynthetic parameters showed higher in undisturbed, with higher CO_2 assimilation rate (A: 7.8 ± 0.9 vs. 3.03 ± 1.6 µmol m^{-2} s^{-1}), stomatal conductance (gsw: 0.08 ± 0.02 vs. 0.03 ± 0.020 mol m^{-2} s^{-1}), and quantum yield of CO_2 ($PhiCO_2$: 0.02 ± 0.005 vs. 0.009 ± 0.004). Transpiration rate (E) was, also higher in undisturbed (0.002 ± 0.0003 vs. 0.0005 ± 0.0003 mol m^{-2} s^{-1}), about three times higher as compared to disturbed Group, as shown in Table 2. Overall, the undisturbed Group showed higher photosynthetic performance than disturbed.

Table 2. Summarized the mean ± SD of mycorrhization levels and photosynthetic activity in disturbed group (I) and undisturbed group (II) seedlings.

Parameters	Mean ± SD		Minimum		Maximum	
	I	II	I	II	I	II
Total mycorrhization (%)	35.7 ± 18.5	37.7 ± 18.6	1.89	6.4	62.5	**61.4**
Mycorrhization near to collar (0–5 cm) (%)	37.9 ± 23.7	41.6 ± 20.5	0	8.3	70	**70.2**
seedling length (cm)	36.5 ± 9.5	46.6 ± 7.9	22.5	35.5	48.5	**67**
Collar diameter (mm)	5.9 ± 1.3	6.2 ± 0.4	3	5.7	7.8	**7.1**
Root length (cm)	16 ± 2.3	14.8 ± 2.3	13.5	12	21.5	**19**
Fv/Fm	0.8 ± 0.01	0.8 ± 0.05	0.8	0.8	0.8	**0.8**
PhiPS2	0.1 ± 0.03	0.2 ± 0.02	0.06	0.2	0.2	**0.2**
NPQ	2.9 ± 0.4	2.6 ± 0.3	2.3	2.3	3.5	**3.2**
E (mol m^{-2} s^{-1})	0.0005 ± 0.0003	0.002 ± 0.0003	0.0002	0.001	0.001	**0.002**
A (µmol m^{-2} s^{-1})	3.03 ± 1.6	7.76 ± 0.9	0.9	6.6	6.2	**9.2**
Gsw (mol m^{-2} s^{-1})	0.03 ± 0.02	0.08 ± 0.02	0.007	0.06	0.06	**0.1**
$PhiCO_2$ (µmol CO_2 µmol photons^{-1})	0.009 ± 0.004	0.02 ± 0.005	0.005	0.01	0.02	**0.03**

4. Discussion

In this study, the mycorrhization was identified based on key morphological markers following previous studies [9,29,34]. The morphotype of the mycorrhizas observed in this study, including their shape, branching structure, and mantle characteristics, corresponded to previously described *T. melanosporum* morphotypes. Molecular analysis further confirmed this identification by comparing ITS sequences with those available in GenBank, confirming that the mycorrhizal type belonged to *T. melanosporum* (Table S4). Importantly, no contamination by other ectomycorrhizal fungi was detected in the one-year-old seedlings, indicating that the controlled conditions during growth were successful in maintaining clean and healthy mycorrhizal associations. Similar results were previously reported for vigorous one-year-old *Q. ilex* seedlings without contamination [10]. According to European standards for truffle cultivation, seedlings should have at least 30% colonization by the target truffle species and a contamination rate of less than 5% [35]. The seedlings used in this study met those standards and were therefore suitable for field transplantation.

Regarding the spatial distribution of mycorrhizas within the root system, the results revealed a clear gradient in colonization intensity. Mycorrhizal presence was varied widely among seedlings, ranging from 0% to 62.5% of the root system and showed a clear gradient along the root system. Higher colonization was observed in the proximal roots, while lower colonization occurred in the distal roots. This pattern may reflect differences in metabolic activity and environmental conditions such as moisture and aeration in the upper soil layers. This gradient pattern aligns with previous studies showing a negative correlation between soil depth and mycorrhizal colonization, with highest colonization often observed within the first 10 cm from the stem collar [36]. These findings highlight the importance of planting depth and root zone conditions for optimizing mycorrhizal development. However, colonization can also vary significantly depending on the host species and environmental factors [37], and seasonal or annual fluctuations have also been documented, including in *T. melanosporum* colonization of pecan seedlings [34].

On the other hand, in this study, because a non-mycorrhizal control group was not included in the experiment, it is not possible to confirm that mycorrhization directly caused the observed growth enhancement. Instead, the results can only modestly suggest that the presence of functional mycorrhizas may have supported improved seedling performance by facilitating more efficient nutrient and water uptake. Ecologically, mycorrhizas are known to improve plant growth and physiology, including shoot growth, stem diameter, photosynthetic efficiency, transpiration rate, and CO_2 assimilation [38]. For example, *T. melanosporum* has been shown to increase photosynthesis and carbon assimilation in *Q. mongolica* seedlings without affecting stomatal conductance [39]. In this study, active mycorrhization was associated with healthy seedling growth, likely due to improved nutrient and water absorption through mycorrhizal roots and possibly increased carbon allocation from the host to the fungus, enhancing nutrient exchange. Similar trends were reported over two decades ago, where *T. melanosporum* mycorrhization enhanced photosynthetic activity in *Q. ilex* seedlings with undisturbed rhizospheres compared to those with disturbed roots [40]. This emphasizes the importance of maintaining a strong plant–fungal symbiosis to support photosynthetic performance, as ectomycorrhizal fungi can contribute up to 25% of soil CO_2 efflux and 3.5–15% of total plant photosynthesis [41].

After 14 days of disturbance, colonization levels remained unchanged, indicating that fungal structures persisted, but their functional activity may have declined. This suggests that short-term disturbance can reduce the effectiveness of mycorrhizal nutrient exchange and lower photosynthetic capacity. Therefore, maintaining undisturbed root systems is essential for sustaining mycorrhizal function and plant physiological performance in truffle

cultivation. Field studies have further shown that activities such as tilling, transplanting, and root pruning can impair mycorrhizal functioning by reducing fungal survival and disrupting nutrient transfer [42]. These observations highlight the importance of maintaining undisturbed root systems to preserve an active mycorrhizal network, which supports plant growth and physiological stability. Earlier research also suggests that ectomycorrhizal fungi play a role in enhancing CO_2 sequestration within roots, stabilizing internal CO_2 levels, and boosting photosynthesis [43]. Moreover, *T. melanosporum* mycorrhizas in *Q. ilex* can facilitate vertical water transfer [40]. Therefore, in disturbed rhizospheres, mycorrhizal structures may remain present but not fully functional, as the active stage of mycorrhization can become dormant. This emphasizes that root pruning or transplantation can compromise mycorrhizal activity and, consequently, affect the physiological performance of the host plants, underlining the need to minimize root disruption in truffle cultivation.

Finally, this experiment also emphasized the importance of pot size in promoting effective mycorrhization. Larger containers support a healthier and more expansive root system by reducing root curling, clumping, or contamination, all of which can adversely affect mycorrhizal establishment [14]. The use of 22 plants in this experiment was based on the need to maintain uniform conditions while ensuring adequate replication for reliable observation of mycorrhizal development and disturbance effects. Altogether, these results emphasize how root architecture and mycorrhizal efficiency jointly influence plant performance, highlighting the essential ecological role of mycorrhizas in sustaining growth and productivity.

5. Conclusions

Tuber melanosporum mycorrhization supports the growth and physiological function of *C. avellana*, but its success depends on careful planting practices, particularly planting depth and container size, which affect root architecture and colonization efficiency. This insight is important for optimizing nursery protocols and ensuring effective field establishment of truffle host plants. Additionally, short-term soil and root disturbances, especially during the active stage of fungal colonization, can impair the functional relationship between fungus and host without visibly reducing colonization levels. Recognizing this is essential for improving mycorrhizal assessment methods and guiding field management to minimize disruption and preserve photosynthetic performance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol5040133/s1, Table S1 presents the raw data for 48 seedlings, including their mycorrhization levels and morphological measurements such as seedling length (cm), collar diameter (mm), and root length (cm). Table S2 represents the correlation between parameters such as mycorrhization levels, morphological traits, and photosynthetic activities of 11 seedlings, with mycorrhization assessed two weeks before measuring photosynthetic activities. Table S3 represents the correlation between parameters such as mycorrhization levels, morphological traits, and photosynthetic activities of 11 seedlings, with photosynthetic parameters being assessed before any disturbance. Table S4 provides details on the similarity of ectomycorrhizal sequences obtained from Corylus avellana to those identified through BLAST analysis. Figure S1. depicts the root length of *C. avellana* seedlings (mean of 48 seedlings). Observation was taken from: A-proximal region of the root (>0–5 cm from collar), B-middle region (>5–10 cm from the collar), C-the distal region of the root (>10–15 cm from the collar).

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