

Article

Short-Term Physiological Responses of Black Locust Saplings to *Trichoderma*-Based Root Priming Under Field Drought Conditions

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Abstract

Black locust (*Robinia pseudoacacia* L.) has exceptional growth capacity in nutrient-poor environments and is therefore widely used for afforestation and land reclamation on degraded soils. However, drought stress can restrict sapling growth, which undermines the success of their establishment. The effect of a product containing two endophytic strains (*Trichoderma afroharzianum* P. Chaverri, F.B. Rocha, Degenkolb & Druzhinina TR04 and *Trichoderma simmonsii* P. Chaverri, F.B. Rocha, Degenkolb & Druzhinina TR05) was studied on a black locust sapling stand under severe drought in eastern Hungary. The two-year-old saplings were root-soaked before planting in sandy soil. The growth of *Trichoderma*-treated plants improved by late spring. Compared to the control trees, average height increased by 25.75%, and root collar diameter was 21.96% larger. Treated plants also showed 9.1% higher chlorophyll content and 11.1% Normalized Difference Vegetation Index (NDVI). The reduced intercellular CO₂ concentration, together with slightly lower stomatal conductance and increased transpiration rate, suggests tighter stomatal regulation and altered water-use dynamics under drought conditions. These responses indicate improved short-term drought acclimation rather than enhanced carbon assimilation capacity. Pre-planting inoculation with endophytic *Trichoderma* strains provides a sustainable method to enhance the early establishment and drought resilience of black locust, thereby increasing the efficacy of forest restoration by improving the survival of black locust on challenging degraded sites.

Keywords: *Robinia pseudoacacia* L.; degraded soil restoration; endophytic *Trichoderma*; pre-planting treatment; afforestation; sapling priming



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1. Introduction

Black locust (*Robinia pseudoacacia* L.), originally native to North America, is one of the most widely planted tree species in the world [1]. Its widespread use is attributed to its vigorous growth and economic significance. As a fast-growing leguminous tree, it produces substantial wood yields even on marginal soils, with its biomass being highly suitable for bioenergy generation [2–4]. It is also an important commercial timber species, as it has highly durable and strong hardwood suitable for several purposes [5]. Moreover, it is also a valuable pioneer for restoring degraded lands worldwide, promoted by a lack of natural competitors, as well as tolerance toward drought and erosion [6,7]. Its symbiotic N₂-fixation characteristic also supports its growth on degraded soils [8]. Recognizing these characteristics, it was extensively planted in the early 19th century to stabilize the shifting sand areas of the Great Plain in Hungary [9]. Despite its socio-economic value, the species is also regarded as a challenging invasive species, as prolific seeding and aggressive vegetative spreading via root suckers enable its rapid colonization [10–12]. However, this invasive characteristic can be reduced by applying strict cultivation practices. Some studies propose a stratified, site-specific management approach, in which black locust may be tolerated or even favored in selected areas, while being strictly eradicated from habitats of high natural value. Therefore, the evaluation of the role of black locust in forestry should consider both the ecological and economic factors related to its distribution among diverse habitat types [12,13].

Since black locust is typically planted on degraded soils under extreme ecological conditions, the application of biostimulants may offer a significant advantage, particularly during the early stages of post-establishment development [9,14]. *Trichoderma* spp. are among the most widely applied biostimulant fungi, enhancing growth under both abiotic and biotic stress. They promote development through the production of phytohormones, secondary metabolites, siderophores, and enzymes [15,16]. Additionally, they facilitate nutrient uptake via phosphate solubilization and organic matter decomposition, while exerting biocontrol through parasitism, competition, antagonism, or inducing acquired resistance in the plant [17,18].

Trichoderma treatments positively influenced the height, stem diameter, biomass and root development of various tree seedlings in controlled settings like nurseries and greenhouses [19–22]. Specifically, studies have shown that *Trichoderma asperelloides* Samuels & J.P.Z. Ligoxig and *Trichoderma harzianum* Rifai treatments increased not only height and root collar diameter (RCD), but also the leaf area of the leguminous Brazilian orchid tree (*Bauhinia forficata* Link) [23]. Likewise, *Trichoderma strigosellum* P. Chaverri, F.B. Rocha, Degenkolb & Druzhinina increased height, number of leaves and leaf thickness of timor white gum (*Eucalyptus urophylla* S.T. Blake) [24]. The influence of *Trichoderma* species on legume growth, physiology, and their symbiotic relationships with nitrogen-fixing bacteria has also been demonstrated. Fungal interaction affected plants' hormonal modulation, the enhancement of nutrient status, and occasionally the modification of the signaling pathways that regulate nodulation [25,26]. The application of a biostimulant containing two *Trichoderma* strains (TR04 and TR05) significantly increased the fresh biomass yield of the legume *Medicago sativa* L. (alfalfa) particularly in the later cutting phases, and generally improved growth and chlorophyll content (SPAD) [27]. These two strains, *Trichoderma afroharzianum* P. Chaverri, F.B. Rocha, Degenkolb & Druzhinina TR04 and *Trichoderma simmonsii* P. Chaverri, F.B. Rocha, Degenkolb & Druzhinina TR05 were isolated from grapevine vascular tissues in 2014, along with eight other endophytic strains [28]. While they belong to the common Harzianum clade used in many biocontrol agents, their selection was based on a critical differential: their long-lasting colonization capability within woody tissues beyond the root system [29]. This superior systemic colonization differentiates them from standard,

commercially available *Trichoderma* species. The outstanding growth characteristics as well as biostimulant and biocontrol potential of *T. afroharzianum* TR04 and *T. simmonsii* TR05 strains led to their registration as a commercial product [28]. The application of these strains resulted in enhanced grapevine growth and increased maize drought tolerance [29,30]. Therefore, the formulated product of these strains (Tricho Immun) was tested on black locust saplings during their first year to determine the formulation's potential for early-stage biostimulation on growth parameters, vegetation index, chlorophyll content, and photosynthetic activity under drought stress on sandy soil.

2. Materials and Methods

2.1. Experimental Site and Plant Material

The newly established black locust plantation was located on the outskirts of Debrecen-Haláp, Hungary, within the South Nyírség Mesoregion of the Great Hungarian Plain. The central coordinates of the site are 47°30'10.30" N, 21°49'36.40" E, with an average elevation of 128 m above sea level. The site officially falls within the forestry climate zone characteristic of sessile oak (*Quercus petraea* (Matt.) Liebl.) woodlands, according to the Hungarian forest site classification [31]. The area is characterized by a free-draining hydrological regime, unaffected by surface or groundwater surplus. The soil type of the research field is humic sand with high organic matter content and a moderately deep effective layer. The plasticity limit according to Arany (P_A) ranges between 25 and 30 and the settleable particulate matter (SPM) ranges between 15 and 25%. Kuron's hygroscopicity (hy) value of the soil is between 0.5 and 1%. Capillary water rises over a five-hour timeframe of over 300 mm.

The experiment was conducted in a randomized complete block design (RCBD) in five repetitions. The blocks were placed with minimum five buffer rows between them. To eliminate spatial gradients and edge effects, all the pots were placed on the central, homogenous part of the field. Originally, each plot was constructed with 20 saplings, but after early-stage wildlife damage, there was a steady, uniform decline in final counts. Consequently, the modal plant number per plot was 18, with a minimum of 13 plants recorded. The planting material consisted of two-year-old black locust saplings. Seeds were harvested in 2022, then cultivated in a nursery in 2023. The saplings were planted at the experimental site in 2023 November. Prior to planting, the saplings were root-soaked up to the collar in a 1% tap water suspension of the Tricho Immun biostimulant (Danuba, Szentendre, Hungary) for 24 h. The inoculum was prepared by dissolving 500 g of product in 50 L of water, providing approximately 0.5 L of suspension per plant. This formulation contains a combination of *T. afroharzianum* strain TR04 and *T. simmonsii* strain TR05, with a minimum viable count of 2×10^8 CFU/g. The suspension temperature was maintained at 6–8 °C. At the time of treatment, saplings were in a dormant, leafless state, precluding any risk to developing foliage. Due to the species' thorns, staff wore protective gloves during all handling procedures. Control saplings were treated identically but soaked for 30 min after the *Trichoderma*-treated group in fresh tap water only. Soaking was performed in separate, labeled plastic vessels and the controls were maintained on the opposite side of the room during the entire process (Figure 1). Single-stem selection thinning (cutting to a single leader) was conducted as part of the cultivation technology on 28 August 2024.

2.2. Meteorological Data

Homogenized gridpoint meteorological data used to characterize the climatic conditions (e.g., temperature-minimum, maximum, average, total precipitation, average global radiation) of the study area were sourced from the Debrecen station of the HUNGAROMET. The analysis included long-term climatic trends and annual records for the period of

1994–2023, as well as monthly data for 2024. To evaluate interannual variability and climatic extremes, the Forestry Aridity Index (FAI) was applied, providing a relevant metric for assessing aridity conditions in forested environments. FAI was calculated from the sum of mean temperatures (T) of the critical months (July–August) by the total precipitation (P) for the period from May to August, representing the main growing season of tree species, where July, as the hottest month, is weighted by 2 [32].

$$FAI = \frac{T_{\text{July–August}}}{P_{\text{May+June+(2×July)+August}}} \quad (1)$$

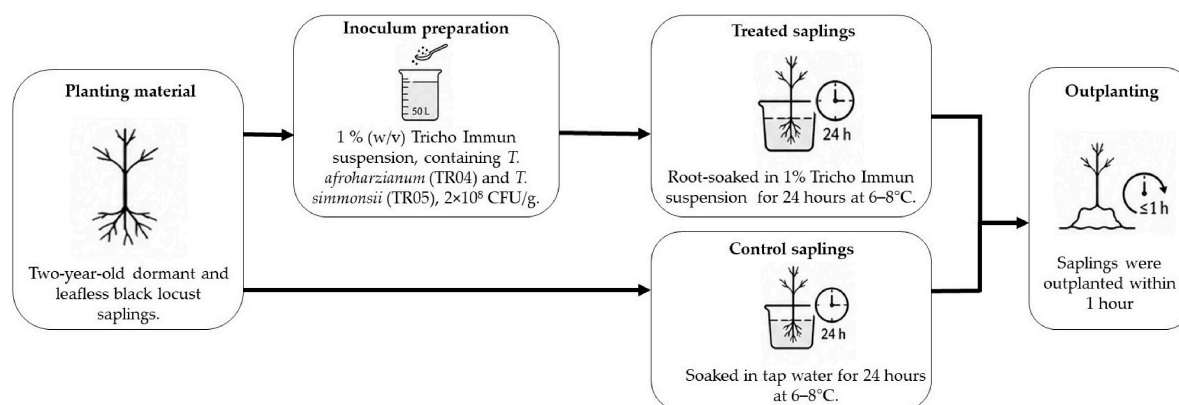


Figure 1. Schematic overview of the *Trichoderma*-based root priming and planting process.

2.3. Data Collection

RCD (cm) and plant height (cm) were measured with an analog caliper and a calibrated Bosch GR 500 leveling staff (Robert Bosch Tools GmbH, Leinfelden-Echterdingen, Germany), respectively, on 28 May 2024 to assess the early post-planting biostimulant effects of the *Trichoderma* treatment. The same parameters were measured again on 24 September 2024 at the end of the growing season. Chlorophyll content was measured on 28 May 2024 using a SPAD 502Plus meter (Konica Minolta Sensing Inc., Osaka, Japan). The protocol involved four readings per leaf on four leaves for every sapling in the study plots.

Normalized Difference Vegetation Index (NDVI) data were collected on 6 June 2024. All plants within the experimental plots were individually assessed based on the absorption and reflection of near-infrared and visible red light [33] using a Trimble GreenSeeker handheld crop sensor (Trimble Inc., Westminster, CO, USA). The sensor operates with active illumination at wavelengths of 656 nm and 774 nm. The device was kept at a constant 30 cm height above the saplings. For phytophysiological measurements on 6 June 2024, a LI-6800 Portable Photosynthesis System (LI-COR Environmental, Lincoln, NE, USA) was used to measure parameters as net assimilation (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration rate (Tr , $\text{mmol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_{tc} , $\text{mol m}^{-2} \text{s}^{-1}$) and intercellular CO_2 concentration (C_i , $\mu\text{mol mol}^{-1}$). Light conditions within the leaf chamber were precisely controlled, with the photosynthetic photon flux density (PPFD, $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) maintained at $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, comprising 90% red (625 nm) and 10% blue (475 nm) light. The Li-6800-01A multiphase flash fluorometer head was used as a light source; the aperture was 2 cm^2 . The CO_2 concentration was controlled in the chamber: $400 \mu\text{mol mol}^{-1}$ using an injector and CO_2 canisters. Light-adapted leaves were measured six times per leaf on three plants per plot. Readings were logged when the measured parameters stabilized, but after a minimum of 120 s.

2.4. Methods for Endophytic *Trichoderma* Isolation and Molecular Identification

To detect existing endophytes, five randomly chosen saplings were tested for endophytic *Trichoderma* colonization prior to treatment. For both this initial analysis and the subsequent re-isolation one year later, tissue samples were collected exclusively from vascular and internal woody tissues after surface sterilization, following the protocol described by Kovács and co-workers [28]. Initial samples were taken as cross-sections from three woody parts of the plants: the root, underground stem, and upper stem. In June 2025, one year after planting, a single representative plant was excavated from each of the five *Trichoderma*-treated plots, as well as from the five control plots, to assess the persistence of *Trichoderma* strains. For the re-isolation, cross-sections were sampled from the roots (1), belowground woody tissues (2–4), and the trunk (5) (Figure 2).

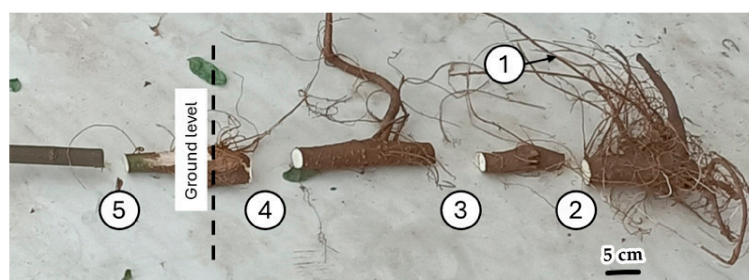


Figure 2. Sampling sites of black locust plants for *Trichoderma* re-isolation (1: roots, 2–4: belowground woody tissues, 5: trunk).

Fungal isolates exhibiting *Trichoderma*-like morphology were subcultured on potato dextrose agar (PDA) for purification and subsequent morphological and molecular identification. Identification was confirmed based on ITS sequences and microsatellite profiling. DNA isolation was performed as previously described in [28]. ITS regions were amplified and sequenced as previously described in [28]. Sequences were deposited in GenBank (PZ039495 and PZ039496). Four microsatellite primers (ThSSR4, ThSSR6, TvSSR1, TvSSR5) [34] were used for amplification of *Trichoderma*-specific microsatellites in a 25 μ L reaction volume, containing 12.5 μ L DreamTaq Green Master Mix (Thermo Scientific, Vilnius, Lithuania), 0.5 μ L of each primer (10 pmol/ μ L), 10.5 μ L of nuclease-free water, and 1 μ L of DNA template (10 ng/ μ L). Amplification began with an initial denaturation step at 95 $^{\circ}$ C for 3 min, followed by 35 cycles of 30 s at 95 $^{\circ}$ C, 30 s at 50 $^{\circ}$ C, and 30 s at 72 $^{\circ}$ C. A final extension step was performed at 72 $^{\circ}$ C for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel (Bioline, Memphis, TN, USA) stained with 4 μ L of EcoSafe (Pacific Image Electronics, New Taipei, Taiwan) and run at 100 V for 60 min using a Bio-Rad electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A 5- μ L GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific, Vilnius, Lithuania) was loaded for size determination.

2.5. Data Analysis

To compare the means of the assessed data, non-parametric Mann–Whitney U tests were used since the assumptions of parametric tests (normal distribution and homoscedasticity) were not fulfilled. Data processing was performed in MS Excel 365, and statistical analysis was conducted in SPSS 29.

3. Results

3.1. Meteorological Background

The FAI of the experimental site, calculated using homogenized gridpoint meteorological data from the HUNGAROMET database, exhibited considerable interannual variability

between 1994 and 2023 (Figure 3). The long-term mean FAI was approximately 7.8, with annual values ranging from 3.9 to 19.1.

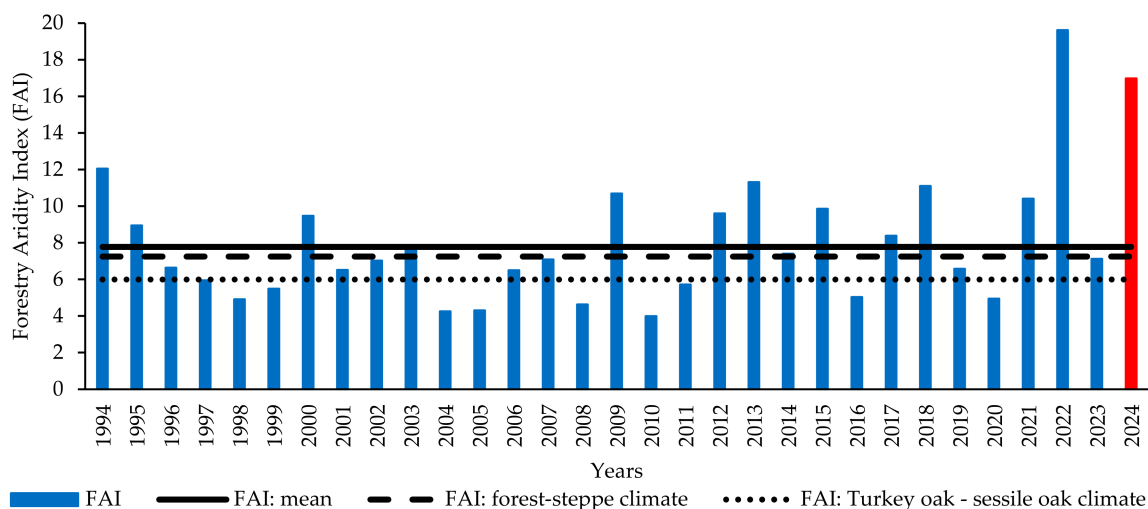


Figure 3. Historical data on the forestry aridity index (FAI) of the experimental site. The red bar highlight the year when the experiments was conducted.

FAI values exceeded the forest-steppe climatic threshold (FAI = 7.3) in 15 out of 29 years, while values surpassed the Turkey oak–sessile oak climate threshold (FAI = 6.0) in 23 years, indicating that the majority of the study period was characterized by moderate to high aridity stress. Notably high aridity was observed in 1994, 2000, 2009, 2012, 2013, 2015, 2018, 2021, and especially in 2022, which showed the highest recorded value (FAI = 19.1). In contrast, FAI values fell below both critical thresholds, indicating relatively wetter conditions in several years (e.g., 2004, 2005, 2008, 2010, 2016, 2020). However, in the past decade, extremely arid periods have become more frequent. In 2022, a historical drought was documented.

In 2024, the cumulative precipitation was also significantly below the long-term average in the whole country. It is noteworthy that the greatest precipitation deficit coincided with the warmest months [35]. During the active growing season (April–October), cumulative precipitation was 108 mm below the 30-year average (274 mm in 2024, compared to 382 mm for the 30-year average from 1994 to 2023, the mean temperature was 1.7 °C higher than the long-term average for the same period (Figure 4). Consequently, the FAI reached a value of 15, indicating severe water scarcity and heat stress during the critical stages of tree development.

3.2. Growth Parameters

Measurement results from 28 May 2024 showed that the *Trichoderma* treatment significantly enhanced early vegetative growth (Table 1, Figure 5). RCD increased by 21.96% compared to control (3.32 ± 0.11 cm vs. 2.72 ± 0.07 cm, $p < 0.001$), while plant height was also significantly greater in the treated group (19.32 ± 0.93 cm vs. 15.37 ± 0.60 cm, $p = 0.001$), reflecting a 25.75% increase (Table 1).

The early height difference between treatments was no longer detectable following the severe summer drought. Although *Trichoderma*-treated plants remained marginally taller (124.46 ± 5.96 cm vs. 122.79 ± 6.30 cm), the difference was not statistically significant ($p = 0.306$). This suggests that the initial growth advantage did not persist by the second measurement on 24 September. Such a bias in growth parameters may have resulted from the applied single-stem selection treatment. Nevertheless, this approach is relevant because single-stem growth matches the structural goals of future forestry.

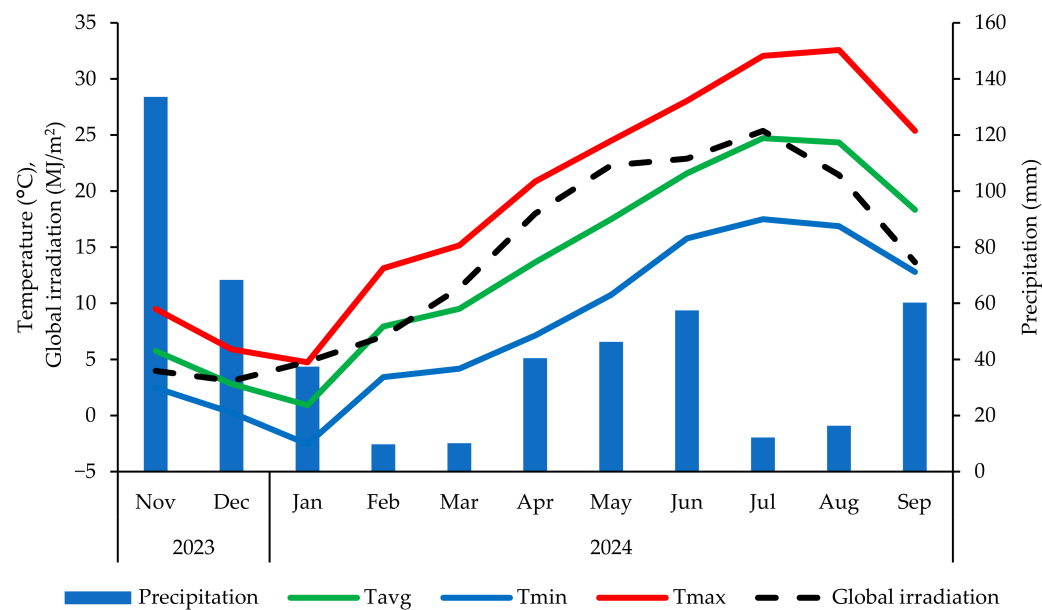


Figure 4. Climatic conditions from planting until the last measurement. Tavg: Average temperature. Tmin: Minimum temperature. Tmax: Maximum temperature.

Table 1. Growth parameters following *Trichoderma* treatment.

Date	Parameter	Control		Trichoderma-Treated		Difference (%) ²	p ³
		N ¹	Mean ± SE	N ¹	Mean ± SE		
28 May 2024	Root collar diameter (cm)	161	2.72 ± 0.07	90	3.32 ± 0.11	21.96	<0.001
	Height (cm)		15.37 ± 0.60		19.32 ± 0.93	25.75	
24 September 2024	Height (cm)	82	122.79 ± 6.30	92	124.46 ± 5.96	1.36	0.306

¹ N: number of measurements. ² Stronger red colors indicate a higher positive relative difference to the control than the lighter one. ³ The p-values of Mann–Whitney U tests are bolded in cases of significant differences.

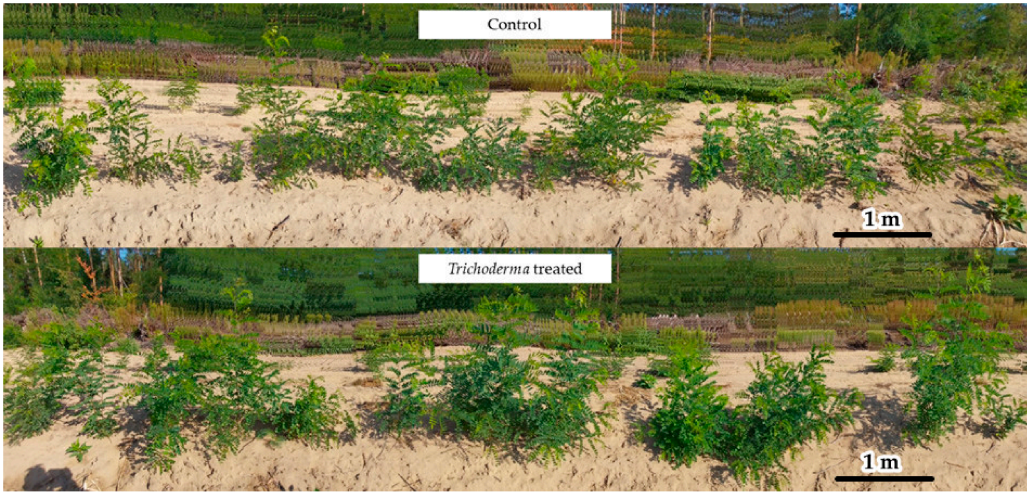


Figure 5. Panoramic images of control and *Trichoderma*-treated plots within a homogeneous block on 8 August 2024.

3.3. Impact of *Trichoderma* Treatment on Plant Physiological Performance

Treated plants had higher chlorophyll levels (34.64 ± 0.61 vs. 31.74 ± 0.5 , $p < 0.001$) on 28 May, and superior NDVI values (0.36 ± 0.01 vs. 0.4 ± 0.01 , $p < 0.001$) on 6 June. These results suggest that the treatment improved both photosynthetic capacity and canopy health (Table 2).

Table 2. The physiological and spectral responses after *Trichoderma* treatment.

Date	Parameter ¹	Control		<i>Trichoderma</i> -Treated		Difference (%) ³	<i>p</i> ⁴
		N ²	Mean ± SE	N ²	Mean ± SE		
28 May 2024	SPAD	161	31.74 ± 0.5	90	34.64 ± 0.61	9.14	<0.001
	NDVI	84	0.36 ± 0.01	89	0.4 ± 0.01	11.11	0.012
6 June 2024	A (μmol m ^{−2} s ^{−1})		9.68 ± 0.35		9.38 ± 0.36	−3.1	0.396
	C _i (μmol mol ^{−1})		274.61 ± 3.53		264.03 ± 3.32	−3.85	0.01
	ETR (μmol s ^{−1})		158.57 ± 4.35		165.88 ± 4.99	4.6	0.281
	g _{tc} (mol m ^{−2} s ^{−1})	72	0.1 ± 0	75	0.09 ± 0	−10	0.019
	PSII		0.25 ± 0.01		0.26 ± 0.01	4	0.283
	T _{air} −T _{leaf} (°C)		−0.51 ± 0.06		−0.31 ± 0.07	−39.22	0.02
	T _r (mmol m ^{−2} s ^{−1})		3.36 ± 0.09		3.65 ± 0.09	8.63	0.009
	WUE (kg m ^{−3})		7.13 ± 0.22		6.32 ± 0.18	−11.36	0.03

¹ SPAD: SPAD value, NDVI: normalized difference vegetation index, A: photosynthetic assimilation rate, C_i: intercellular CO₂ concentration, ETR: electron transport rate, g_{tc}: stomatal conductance to CO₂, PSII: efficiency of photosystem II, T_{air}−T_{leaf}: leaf–air temperature difference, T_r: transpiration rate, WUE: water-use efficiency. ² N shows the number of measurements. ³ Stronger red colors indicate a higher positive relative difference to control than the lighter ones, stronger blue colors indicate a bigger negative difference to control than the lighter ones.

⁴ The *p*-values of Mann–Whitney U tests are bolded in case of significant differences.

The physiological and spectral responses of plants treated with *Trichoderma* were assessed in comparison to the control group across several parameters, as presented in Table 2. On 6 June, a significant decrease in internal CO₂ concentration (C_i) was observed in the *Trichoderma*-treated plants compared to controls (264.03 ± 3.32 vs. 274.61 ± 3.53 μmol mol^{−1}, *p* = 0.01), alongside a small but statistically significant reduction in stomatal conductance (g_{tc}) (*p* = 0.019). The leaf–air temperature difference (T_{air}−T_{leaf}) was notably smaller in the treated group (−0.31 ± 0.07 °C vs. −0.51 ± 0.06 °C, *p* = 0.02), indicating reduced transpiration-driven cooling. Accordingly, transpiration rate (T_r) was significantly higher in the *Trichoderma*-treated group (3.65 ± 0.09 vs. 3.36 ± 0.09 mmol m^{−2} s^{−1}, *p* = 0.009). Photosynthetic parameters such as the efficiency of photosystem II (PSII) and electron transport rate (ETR) did not differ significantly between treatments (*p* > 0.05). Similarly, photosynthetic assimilation rate (A) showed a non-significant trend toward a decrease in the *Trichoderma*-treated group (Table 2).

3.4. Outcomes of Endophytic *Trichoderma* Isolation and Molecular Identification

No *Trichoderma* was detected in the internal woody tissues of any of the saplings tested before treatment. However, two out of five individuals, sampled in June 2025 (19 months post-treatment) and collected from distinct plots, showed re-isolation of fungi with *Trichoderma*-specific colony and micromorphology. These re-isolations were from the underground woody tissues, respectively. Notably, no *Trichoderma* was isolated from any of the five plant samples collected from the control plots.

Amplified rDNA ITS regions of the isolates (GenBank accession numbers: PZ039495 and PZ039496) were identical to the *T. simmonsii* TR04 sequence (OK560828). Analysis using the ThSSR4, ThSSR6, TvSSR1, and TvSSR5 microsatellite primers revealed that both *Trichoderma* isolates produced identical amplicon profiles (Table 3). These profiles correspond to the established size for *T. afroharzianum* TR04 and *T. simmonsii* TR05 strains present in the applied product.

Table 3. Size (bp) of microsatellite markers of *Trichoderma afroharzianum* P. Chaverri, F.B. Rocha, Degenkolb & Druzhinina strain TR04 (TR04) and *Trichoderma simmonsii* P. Chaverri, F.B. Rocha, Degenkolb & Druzhinina strain TR05 (TR05) and re-isolated *Trichoderma* 1 and 2.

Microsatellite Marker	Applied <i>Trichoderma</i> Strains		Re-Isolated <i>Trichoderma</i> Strains	
	TR04	TR05	1	2
ThSSR4	160	160	160	160
ThSSR6	390	390	390	390
TvSSR1	430	430	430	430
TvSSR5	200	200	200	200

4. Discussion

Black locust demonstrates notable drought tolerance [5], although its native range is associated with humid climatic zones, characterized by annual precipitation levels between 1020 and 1830 mm [36]. Nevertheless, it can survive in regions with as low as 400 mm of annual precipitation and is well known for its remarkable ecological plasticity. Therefore, this tree is widely cultivated in different regions. In Hungary, it is primarily planted for the stabilization and utilization of loose sandy soils, particularly in the Great Hungarian Plain region [37], where our study was conducted.

Targeted, assisted generative strategies can effectively strengthen the adaptive capacity and resilience of black locust against extreme climatic conditions. These include the transfer of seed lots from preadapted populations originating from warmer and drier regions (e.g., southeastern Europe, such as Bulgaria and Turkey), or the establishment of seed orchards using grafted or rooted cuttings derived from genotypes adapted to such climates [38]. Additionally, vegetative propagation techniques—such as tissue culture, micropropagation, the development of stool beds, or the production of rooted cuttings—offer viable means for the large-scale multiplication of drought-tolerant genotypes [36]. Stress tolerance may also be improved by plant growth-promoting microorganisms, such as *Trichoderma* fungi [15]. In forest sapling production, the inoculation with *Trichoderma* spp. can enhance sapling vigor, root architecture, nutrient uptake, and drought resilience [14]. In silvicultural systems, *Trichoderma longibrachiatum* inoculation improved *Pinus massoniana* drought tolerance and altered root-associated microbiomes, leading to better physiological performance under water stress [39]. Similarly, growth, rhizosphere enzymatic activity, and nutrient uptake were enhanced by the application of dual *Trichoderma* strains on *Pinus sylvestris* var. *mongolica* seedlings [40].

Jin et al. [41] reported that extreme drought leads to stunted growth and reduced overall productivity in black locust. Therefore, the 2024 growing season—characterized by exceptional heat and a precipitation deficit of over 100 mm compared to the 30-year average (1994–2023)—provided a uniquely challenging environment to evaluate the stress-buffering capacity of *Trichoderma* in saplings within a newly established plantation.

Following the six-month *Trichoderma* sapling priming, both RCD (21.96%, $p < 0.001$) and height (25.75%, $p = 0.001$) increased, indicating improved mechanical stability and water transport in the treated plants [42,43]. This, in turn, enhanced sapling performance during the critical early establishment phase. This early growth stimulation aligns with the previously described biostimulant effects of *Trichoderma* spp., which are known to facilitate nutrient uptake, stimulate phytohormone production, and improve abiotic stress tolerance [17,44]. Taller plants with thicker stems generally possess greater biomass and chlorophyll content, leading to higher NDVI values. In agreement with our growth data, we observed significantly higher NDVI values in the *Trichoderma*-treated plants.

Following the extreme summer drought, the difference in plant height was no longer observed in September. Black locust, being a fast-growing and stress-tolerant pioneer species [45], may have compensated for early growth disparities through its high intrinsic growth rate, thereby masking *Trichoderma* treatment effects by the final assessment. Another possibility is that the growth-promoting effects of *Trichoderma* are transient. Environmental conditions, particularly temperature, are critical determinants of *Trichoderma* activity [28,46]. Temperature directly influences spore germination and hyphal growth, which in turn affect the extent and success of root colonization [47], which may explain the observed results under the exceptionally high temperatures of the 2024 growing season.

Despite severe drought and heat stress, *Trichoderma* was successfully re-isolated from the roots and belowground woody tissues of two of the five sampled plants 19 months following the treatment. This result is consistent with the reported colonization variability in woody hosts under field conditions [48,49]. The absence of the fungus in controls confirms treatment specificity. The potential for long-term colonization is suggested by the successful re-isolation of *Trichoderma* strains several months post-treatment, although this finding is limited by the small sample size. Furthermore, in contrast to grapevines, where systemic spread was detected [29], colonization in black locust was restricted to the root zone, particularly under drought conditions. *Trichoderma* treatment significantly modified several leaf physiological parameters. A modest decrease (3.85%) in intercellular CO₂ concentration (C_i) was observed together with reduced stomatal conductance (g_{tc}) in the *Trichoderma*-treated plants. This pattern is consistent with diffusional limitation of CO₂ supply due to partial stomatal closure, which is a common response under drought stress. Importantly, C_i is not determined solely by stomatal conductance but is also influenced by mesophyll conductance, which may decline under stress conditions and further restrict internal CO₂ diffusion. Since net assimilation (A) did not increase, the observed decrease in C_i likely reflects stomatal and internal diffusion constraints rather than enhanced carboxylation efficiency [50,51]. These results are consistent with previous studies that have shown that *Trichoderma* improves plant water balance regulation under drought conditions by partial stomatal closing and osmoregulation [44,52]. Consequently, rapid settlement appears to be a vital strategy for the long-term survival and recovery of the species, and surviving severe droughts outweighs the efficiency of gas exchange [53,54]. Despite the slightly lower stomatal conductance (g_{tc}), the leaf transpiration value (T_r) increased by 8.6%. However, higher transpiration generally requires wider stomatal openings; this opposite trend is consistent with the observations of Contreras-Cornejo et al. [15]. Improved root water uptake and modulated stomatal function, as well as leaf anatomy due to changes in surface or microstructural conductivity, may have led to this inconsistency [55]. Further research will be needed to gain a deeper understanding of the internal water transport mechanisms. *Trichoderma* colonization can promote such alterations [56,57], helping evaporative cooling even with partially closed stomata. The reduced $T_{leaf}-T_{air}$ value observed in treated plants supports the interpretation that cooling was better than in the control, despite slightly reduced stomatal conductance. This suggests that *Trichoderma* treatment may help to maintain more effective transpirational cooling under drought stress, which is critical for preventing thermal damage to the photosynthetic apparatus. In *Trichoderma*-treated plants, the electron flow was maintained to reduce heat stress, as suggested by sustained ETR. However, the quantum efficiency of photosystem II or assimilation rates (A) were not improved, suggesting that the extra energy was used to alleviate stress rather than to produce biomass. *Trichoderma* priming increased NDVI values significantly ($p = 0.05$), despite the similar net photosynthetic rates. These results show enhanced chlorophyll content and nitrogen use efficiency, so the treated sapling had better vigor or physiological state.

Root soaking with *Trichoderma* spore suspension can be easily incorporated into nursery practice; however, further research is needed to determine the duration of the effect. Additional studies are needed on single treatments or repeated applications required to provide long-term stress tolerance or resistance to pathogens.

5. Conclusions

This research highlights that endophytic *T. afroharzianum* TR04 and *T. simmonsii* TR05 have the potential to support black locust afforestation. Our findings demonstrate that this root inoculation strategy successfully enhances early growth and physiological stability, enabling saplings to better withstand the combined stress of poor soil and drought. Therefore, we propose that the targeted application of these strains could serve as a component of sustainable reforestation efforts. The high cost-effectiveness of this approach, stemming from improved plant survival rates and reduced replanting needs, makes it a viable and attractive option for large-scale ecological restoration projects.

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Abbreviations

The following abbreviations are used in this manuscript:

NDVI	normalized difference vegetation index
RCD	root collar diameter
SPAD	Soil–Plant Analysis Development
P_A	Arany plasticity index
SPM	settleable particulate matter
hy	Kuron’s hygroscopticity
RCBD	randomized complete block design
CFU	colony-forming unit
FAI	forestry aridity index
PPFD	photosynthetic photon flux density
ITS	Internal Transcribed Spacer
C_i	internal CO ₂ concentration
g_{tc}	stomatal conductance
$T_{air}-T_{leaf}$	leaf–air temperature difference
T_r	transpiration rate

PSII	efficiency of photosystem II
ETR	electron transport rate
A	photosynthetic assimilation rate
WUE	water-use efficiency
rDNA	ribosomal DNA

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