

Article

Site-Specific Efficacy of Microbial Inoculants on Sunflower Development and Soil Metabolic Activity in Open-Field Trials

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Abstract

Modern agriculture faces critical challenges, including climate change, soil degradation, and reliance on synthetic inputs, necessitating sustainable alternatives. A promising approach is applying microbial solutions. This study investigated the effects of a commercially available powder-formulated seed coating product containing *Rhizoglossum irregularis* and *Azospirillum* spp. on high-linoleic sunflower (*Helianthus annuus* L.) under field conditions at three Hungarian sites with contrasting soil properties. Plant growth parameters, yield, and the rhizosphere's microbial carbon-source utilisation potential were assessed to evaluate treatment effects. Seed-applied inoculation increased plant height, stem diameter, and biomass, although responses varied with site conditions and sampling time. The agronomic response was highly site-dependent: the strongest effect was observed at a low-pH, phosphorus-limited site, where seed inoculation achieved a significant yield increase of 18–21%, while effects at sites with higher pH and sufficient phosphorus resulted only in minor, statistically non-significant numerical trends. Distinct shifts in culture-dependent carbon-source utilisation potential were detected by Biolog EcoPlate™ analysis, showing temporal and site-specific variability. These results indicate that seed-applied formulations offer a logistically feasible, targeted delivery method for plant-beneficial microbes, with their agronomic efficacy fundamentally governed by site-specific soil chemical profiles and baseline nutrient availability. Overall, the agronomic benefits of inoculation appear to be context-dependent and more pronounced under nutrient-limited conditions, highlighting the critical importance of site-specific application strategies and the need for further long-term studies.

Keywords: *Helianthus annuus* L.; arbuscular mycorrhiza; nitrogen-fixing bacteria; soil microbial activity; yield enhancement



Academic Editor: Luke Moe

Received: 28 May 2026

Revised: 9 July 2026

Accepted: 17 July 2026

Published: 20 July 2026

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1. Introduction

Global agricultural production relies heavily on key commodities such as cereals and oilseed crops, including sunflower (*Helianthus annuus* L.), which plays a significant role in European and Hungarian agriculture [1–4]. However, increasing climate variability, particularly drought, poses a serious threat to yield stability [5,6]. At the same time, regulatory and environmental pressures demand the enhancement of crop resilience while reducing dependence on synthetic inputs [7–9].

Microbial-based solutions, including arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR), offer promising alternatives. AMF enhance plant phosphorus uptake, water-use efficiency, and stress tolerance, while PGPR contribute to nutrient acquisition, phytohormone production, and stress mitigation through complex metabolic regulation [10–14]. Their combined application has been shown to produce synergistic effects on plant growth, yield, and stress resilience across various crops [15–17].

In sunflower, both AMF and PGPR have demonstrated positive effects on nutrient uptake, root colonisation, and yield [18–21]. Species such as *Rhizoglyphus irregularis* or *Azospirillum* spp. are widely recognised for their effectiveness and broad applicability [22,23]. However, plant–microbe interactions are strongly influenced by soil properties, microbial community structure, and environmental conditions, resulting in highly site-specific outcomes [24–26].

Despite extensive research on AMF and PGPR, most studies have focused on soil or root inoculation methods, while their application as seed coating agents under open-field conditions remains poorly documented, particularly in sunflower. Furthermore, field-scale evidence on the combined use of *R. irregularis* and *Azospirillum* spp. in oilseed crops is still limited. To address this gap, the present study evaluates a commercially available, powder-formulated AMF-PGPR consortium applied directly to the seed surface under open-field conditions across multiple sites with contrasting soil properties in Hungary. By integrating agronomic and functional soil microbiological analyses, this work aims to provide new insights into the site-specific performance and ecological functioning of microbial inoculants, contributing to their more effective and predictable use in sustainable crop production.

2. Materials and Methods

2.1. Experimental Design

The field experiments were conducted over two growing seasons (2023 and 2025). In 2023, the trial was established solely at the Beled (area: GPS: 47°27′53.6″ N 17°04′21.4″ E; alluvial meadow soil) site. In 2025, the study was expanded to three experimental locations: Beled, Zagyvarékas (area: GPS: 47°14′39.9″ N 20°06′24.0″ E; compacted meadow soil) and Gyomaendrőd (area: GPS: 46°54′05.6″ N 20°44′53.3″ E; brown clay forest soil). At all sites, cereals served as the preceding crop, and the trials were conducted under rainfed conditions. The trials were arranged in a randomised complete block design under open-field conditions with ten replicates. The total experimental area comprised 2 ha, with the inoculated and non-inoculated treatments each occupying a cumulative area of 1 ha. Each individual treatment plot consisted of 32 rows with 189 plants per row, resulting in plot dimensions of 24.0 m × 41.7 m (1000 m²). In 2023 sunflower was sown on the 24th of April 2023. In 2025, sowing was carried out on 17 April at Beled, 18 April at Gyomaendrőd, and April 22nd at Zagyvarékas. Sunflower seeds were sown at a density of 60,000 seeds ha^{−1} using a Väderstad Tempo F8 seeder (Väderstad, Sweden). The sowing configuration was maintained at a 75 cm row spacing, 22 cm plant spacing, and a 4 cm seeding depth, with an operating speed of 15 km/h.

Organic fertilisation was provided via poultry manure (25 t ha^{−1}) applied in the autumn of 2024 prior to the 2025 growing season. In contrast, the 2023 trial at the Beled site was established on an adjacent block characterised by an identical crop rotation history but without the application of organic amendments, representing a distinct baseline nutrient management background. All sites were managed under a uniform baseline agronomic protocol. The only treatment difference was the application of the product to the treated plots, whereas control plots received no such input. During the sowing process, a starter mineral fertiliser (Plantacote®; 30-7-7 + 2 MgO; Plantacote N.V., Antwerpen, Belgium) was

applied to the seedbed at a rate of 120 kg ha⁻¹. The sunflower seeds were pre-treated with the active ingredients fludioxonil (Maxim 025 FS[®]; Syngenta A.G., Basel, Switzerland), oxatiapiprolin (Plenaris[®]; Syngenta A.G., Basel, Switzerland). Weed control was primarily managed through the application of Gardo Sun[®] (600 g L⁻¹ aklonifen + 30 g L⁻¹ diflufenikan; Syngenta A.G., Basel, Switzerland) at a dosage of 1.9 L ha⁻¹.

During the experiment, the following sampling times were defined: T0 (pre-sowing), T1 (4–6 leaf stage), T2 (6–8 leaf stage), T3 (full flowering) and T4 (pre-harvest).

2.2. Plant Material and Microbial Inoculation

Seeds of the high-linoleic sunflower variety SY Fergus (CLP) Clearfield[®]PLUS (Syngenta A.G., Basel, Switzerland) (the variety carries M9 resistance, providing resistance against all *Plasmopara halstedii* pathotypes identified in Hungary, and has *Orobanche cumana* A-E race tolerance) were used in both years.

Seeds were inoculated with a commercial biofertiliser product (Kiplant Awaken[®], Asfertglobal, Várzea, Portugal; EU-registered organic fertiliser) according to the manufacturer's protocol or left untreated, as control plants. The sunflower-specific formulation contains arbuscular mycorrhizal fungus (*Rhizoglyphus irregularis*, 825 spores g⁻¹), plant growth-promoting rhizobacteria (*Azospirillum brasilense* and *A. lipoferum*, each 3 × 10⁴ CFU g⁻¹), seaweed extract (*Ascophyllum nodosum* extract, 11%), and micronutrients (Fe 0.2%, Mn 2%), stabilised in talc and kaolin powders.

The commercial powder-formulated biofertiliser was applied on the day of sowing at the manufacturer's recommended rate of 200 g per 150,000 seeds (approximately 1.33 mg per seed). To match the 1 ha cumulative area designated for the inoculated treatment at the seeding rate of 60,000 seeds ha⁻¹, the precise proportional amount of powder was thoroughly mixed with the required quantity of dressed seeds. Gentle tumbling was employed to ensure uniform adherence to the seed surfaces. The treated seeds were immediately poured into the seed drill tank and sown, thus completing the inoculation and sowing in a combined, single operation. Control seeds received identical handling without the addition of the powder-formulation product.

2.3. Assessment of Soil Properties and Characteristics of Its Microbial Community

To establish baseline soil properties, topsoil samples (0–20 cm) in ten replicates per plot were collected at T0 (pre-sowing) in both experimental years from all experimental sites. Soil organic matter content was determined using the modified Walkley–Black method [27] with spectrophotometric detection using a Genesys 150 UV–Vis, Thermo Scientific spectrophotometer (Waltham, MA, USA). The carbonated lime content of soil samples was determined using a FOG-L pressure laboratory calcimeter (UGT, Müncheberg, Germany). The soil pH was measured at a depth of 15 cm using a GroLine—HI981030 device (HANNA Instruments, Szeged, Hungary) at T0.

Metabolic activity of microbial communities was assessed using EcoPlate[™] (Biolog Inc., Hayward, CA, USA) containing 31 different carbon sources, each with three replicates, where the manufacturer's protocol was followed. During the 2025 growing season, the topsoil samples were collected in three replicates per plot at T1 and T4 from all experimental sites. Absorbance was measured at 600 nm using BMR-100 Microplate Reader (BOECO, Hamburg, Germany). To account for baseline interference, measurements recorded at 24 h were used for data normalisation; these values were subtracted from each consecutive measurement at 120 h to minimise background noise, as established by

Urakawa et al. [28]. Average Well Colour Development (AWCD) quantified total metabolic activity was calculated according to Huang et al. [29],

$$AWCD = \frac{\sum(C_i - R)}{31} \quad (1)$$

where R is the average of the three water-containing control well, and C_i is the value of any substrate-containing well; negative values were set to zero [30]. The functional evaluation (Shannon–Wiener Index, H') was based on the substrate classification of Biolog® EcoPlate™, referring to Dimitrova's study [31]:

$$H' = -\sum \frac{C}{SumC} \times \ln \frac{C}{SumC} \quad (2)$$

where C is the value of R subtracted from a substrate-containing well (C_i) and $SumC$ is the sum of all the C values of a plate.

2.4. Plant Analysis

At Beled (2023 and 2025), Zagyvarékas (2025) and Gyomaendrőd (2025) plant samples were collected at two distinct phenological stages—T1 (4–6 leaf stage) and T2 (6–8 leaf stage)—to determine shoot biomass, as well as shoot moisture content (SMC). At each sampling interval, ten plants were randomly selected per plot, and the entire above-ground biomass of these plants was collected. To ensure a representative assessment of each plot and to avoid pseudoreplication, the measurements of these ten within-plot subsamples were averaged to yield a single plot-level mean value prior to statistical analysis. All samples were weighed immediately upon harvest to determine fresh weight (FW). Fresh and dry weights were measured using a precision scale (TN10001, TN Lab Supply, Louisville, TN, USA; 10 g capacity, 0.001 g precision). To obtain the dry weight (DW), the plant material was oven-dried at 65 °C for 48 h until a constant weight was achieved. The SMC was calculated relative to the fresh weight according to the methodology of Zhang [32] using the following equation:

$$SMC(\%) = \frac{(FW - DW)}{FW} \times 100 \quad (3)$$

At all experimental sites (at Beled both in 2023 and 2025), plant height and stem diameter were assessed by measuring ten randomly selected plants per plot at T1, T3 (full flowering) and T4 (pre-harvest). The head diameter of ten randomly selected plants per plot was measured at T3 and T4. For all of these biometric parameters, the measurements of the ten within-plot subsamples were averaged to yield a single, representative plot-level mean value per plot at each sampling time prior to statistical analysis.

2.5. Estimation of Mycorrhizal Colonisation Rate

In 2025, root samples were collected from 10 randomly selected plants per plot at all experimental sites at T1, T3 and T4. The ten root subsamples were averaged to yield a single, representative plot-level mean value per plot at each sampling time prior to statistical analysis, and the samples were stored at −20 °C until analysis.

The mycorrhizal colonisation rate was determined using the ink–vinegar root staining method. Small pieces (1 cm long) of the thoroughly washed fine roots were cut with a pair of sterilised scissors. Each sample was stained with Pelikan blue according to Vierheilig et al. [33]. Internal fungal structures—arbuscules, vesicles and hyphae—were examined under a stereomicroscope at ×100 magnification and the percentage of colonised root length was calculated using the gridline intersect method [34].

2.6. Statistical Analysis

Statistical analyses were performed using RStudio (version 2026.06.0-242) software. For all statistical models, the individual field plot ($n = 10$ per treatment) was defined as the primary experimental unit. Data entering into the models represented the plot-level composite values. To prevent rank deficiency from the asymmetric experimental design, data were partitioned into two independent subsets: a temporal model (Beled: 2023 vs. 2025) and a spatial model (2025 only: Beled, Zagyvarékas, and Gyomaendrőd). Both models assessed the main and interactive effects of the respective spatiotemporal variables, inoculation treatment, and sampling time. The interpretive scope of these models was strictly constrained: the temporal model evaluates seasonal shifts isolated exclusively to the Beled location (represented by uppercase letters in the figures and tables), while the spatial model describes site-specific variations restricted to the 2025 growing season (represented by lower case letters in the figures and tables), avoiding broader generalisations. Prior to hypothesis testing, normality and homoscedasticity were evaluated using visual diagnostics, the Shapiro–Wilk test, and Levene’s test (via the car package [version 3.1-5]). Variables meeting parametric assumptions were analysed using standard linear models and Type II Analysis of Variance (ANOVA). Variables violating these assumptions were analysed using Generalised Linear Models fitted with a Gamma distribution and log link function, evaluated via Type II Analysis of Deviance. For significant effects ($p < 0.05$), full-factorial post hoc pairwise comparisons were conducted using estimated marginal means (via the emmeans package [version 2.0.1]). To control the family-wise error rate, p -values were adjusted using Tukey’s HSD for standard linear models, and the exact probability Šidák method for Generalised Linear Models.

To visualise the overall shifts in microbial functional diversity across locations, treatments, and sampling times, a principal component analysis (PCA) was performed using the stats and ggfortify packages (version 4.3.2 and 0.4.19, respectively). The PCA was based on a correlation matrix of the 31 carbon sources of the EcoPlate, with data centred and scaled to unit variance to ensure equal weighting of all metabolic substrates. To statistically evaluate the main and interactive effects of location, inoculation treatment, and sampling time on these single-year carbon-source utilisation profiles, a three-way Permutational Multivariate Analysis of Variance (PERMANOVA) was performed using the vegan package (version 2.6-10) with 999 permutations based on a Euclidean distance matrix. To visualise the specific metabolic fingerprints of the microbial communities, with the application of pheatmap package (version 1.0.13), one heatmap was generated using the 31 carbon-source utilisation profiles and another, which grouped the 31 carbon sources. The metabolic data were Z-score normalised. Samples were organised via hierarchical clustering based on Euclidean distances using the Ward.D2 linkage method via pheatmap package.

3. Results

3.1. Soil Characteristics of the Experimental Sites

Pre-treatment soil analysis (Table 1) revealed distinct chemical profiles across the three experimental sites. The Beled site consisted of acidic alluvial meadow clay with moderate organic matter and nitrogen, alongside optimal potassium levels, while its total P_2O_5 concentrations represent a moderate supply. In contrast, the Zagyvarékas site was characterised by near-neutral, slightly calcareous soil with higher organic matter and substantial phosphorus and potassium enrichment, representing excessive baseline supply levels. While magnesium levels were optimal, manganese was present in excess. The Gyomaendrőd site exhibited a similar pH, phosphorus and organic matter profile to Zagyvarékas but was distinguished by exceptionally high magnesium concentration. Micronutrient analysis at this site indicated a deficiency in zinc alongside excessive manganese levels. Salinity and

carbonate content remained within low to adequate ranges across all locations, providing a stable baseline for the subsequent experimental treatments.

Table 1. Soil characteristics of the experimental locations determined from pre-treatment baseline sampling (T0: baseline, pre-sowing; autumn 2022 and 2024).

Location	Beled		Zagyvarékas	Gyomaendrőd
Sampling Year	2023	2025	2025	2025
pH (KCl)	4.94 ± 0.05	5.3 ± 0.3	6.8 ± 0.6	6.6 ± 0.3
Total water-soluble salt (% m/m)	<0.043 ± 0	<0.043 ± 0	0.09 ± 0	0.14 ± 0
Organic Matter (% m/m)	1.56 ± 0.08	2.5 ± 0.2	2.99 ± 0.01	2.98 ± 0.03
NO ₃ [−] + NO ₂ [−] + N (mg/kg)	23.9 ± 1.0	16.2 ± 0.4	15.5 ± 0.5	18.6 ± 0.2
P ₂ O ₅ (mg/kg)	78 ± 1	90.3 ± 0.2	278 ± 3	264 ± 3
K ₂ O (mg/kg)	305 ± 2	172.7 ± 0.2	482 ± 1	413.0 ± 0.6
Mg (mg/kg)	615 ± 8	377 ± 7	358 ± 5	544 ± 6
Carbonated lime (% m/m)	<0.1 ± 0	<0.1 ± 0	1.3 ± 0	2.8 ± 0

Mean values ± standard error, n = 10.

3.2. Estimation of Mycorrhizal Colonisation

The arbuscular mycorrhizal fungal (Figure 1) colonisation rates across the three field locations—Beled, Zagyvarékas, and Gyomaendrőd—exhibited a consistent and significant upward trend throughout the growing season (T1–T4) in 2025, influenced by location, sampling time and inoculation (Table 2). The Inoculation × Sampling time ($p < 0.001$) and Sampling time × Location ($p < 0.001$) interactions were significant (Table S1). At the initial vegetative stage (T1; 4–6 leaves), colonisation levels were relatively low, ranging from a minimum of 13% in the Gyomaendrőd control to 29% in the Beled inoculated group. As the plants transitioned to the reproductive phase (T3; main flowering), a marked surge in root occupancy was observed across all sites. During this period, inoculated plants consistently outperformed their respective controls; for instance, in Zagyvarékas, inoculated plants reached 57% colonisation compared to 38% in the control. Peak colonisation was recorded at the final sampling point (T4; before harvest), where levels reached their maximum in Beled at 89% for inoculated plants and 77% for the control. Statistical analysis confirms that, while inoculation consistently enhanced fungal occupancy, the native populations in the control plots were also highly active, particularly in the later stages of development.

Table 2. Dynamics of arbuscular mycorrhizal colonisation in inoculated and control areas at three sampling dates in sunflower.

Location	Sampling Time	Seed Inoculation	Colonisation (%)
Zagyvarékas	T1	Inoculated	21 ± 2.58 hi
		Control	16 ± 6.32 i
	T3	Inoculated	57 ± 4.76 e
		Control	38 ± 6.32 fg
	T4	Inoculated	75.5 ± 2.52 bc
		Control	63 ± 4.16 de
Beled	T1	Inoculated	29 ± 5.29 gh
		Control	24 ± 3.65 hi
	T3	Inoculated	74.5 ± 3 bcd
		Control	67.5 ± 5 cde
	T4	Inoculated	88.5 ± 1.91 a
		Control	77 ± 5.77 ab
Gyomaendrőd	T1	Inoculated	16.5 ± 5.74 i
		Control	13 ± 3.46 i
	T3	Inoculated	67 ± 6.63 cde
		Control	43.5 ± 3.42 f
	T4	Inoculated	81.5 ± 5.26 ab
		Control	67.5 ± 3.42 cde

T1: 4–6 leaf stage, T3: full flowering stage, T4: pre-harvest. Different letters within each measured parameter indicate significant differences ($p < 0.05$) among all treatment combinations (location, sampling time, and inoculation) according to Tukey HSD post hoc test.

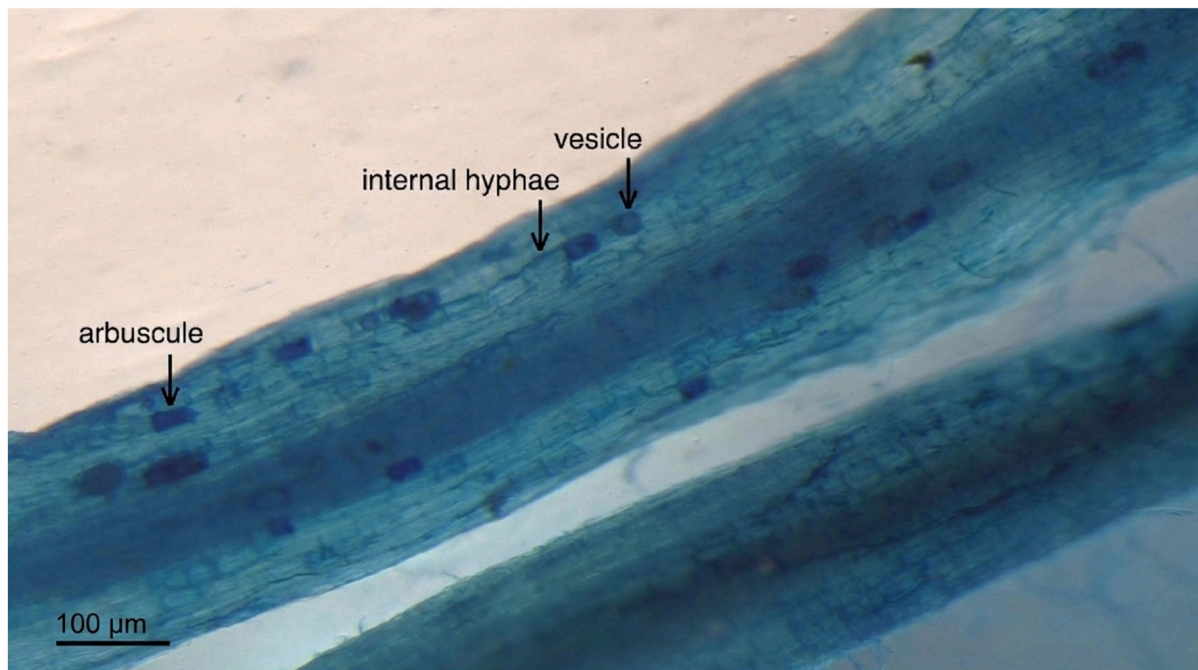


Figure 1. Arbuscular mycorrhizal colonisation in sunflower (*Helianthus annuus* L.) root showing vesicles and internal hyphae. Roots were stained with Pelikan blue and observed under a light microscope. Scale bar = 100 μ m.

3.3. Sunflower Morphological and Growth Characteristics

The morphological parameters of sunflower—plant height, stem diameter, and head diameter—were assessed to evaluate both temporal dynamics (2023 versus 2025 at Beled) and spatial variability across three experimental sites in 2025 (Beled, Zagyvarékas, and Gyomaendrőd), as summarised in Tables S2–S4. Across all assessments, inoculation consistently and independently exerted a highly significant ($p < 0.001$) influence on all measured traits (Table S1). Regarding temporal variation at Beled, the analysis revealed significant two-way interactions between inoculation and year for stem diameter ($p < 0.001$), head diameter ($p = 0.012$), and plant height ($p < 0.001$). Additionally, an inoculation by sampling time interaction was observed exclusively for head diameter ($p = 0.016$). No significant three-way temporal interactions (Inoculation $\times$ Sampling time $\times$ Year) were detected ($p > 0.05$). When spatial dynamics were evaluated across the three locations in 2025, the influence of inoculation on stem diameter and plant height fluctuated significantly depending on both location and sampling time ($p < 0.001$ for both two-way interactions). While head diameter was not significantly affected by any two-way spatial interactions, a complex three-way interaction (Inoculation $\times$ Sampling time $\times$ Location) was detected for this trait ($p = 0.021$), as well as for plant height ($p < 0.001$).

Stem diameter increased progressively from T1 to T4 at all experimental sites. At Beled and Gyomaendrőd, inoculated plants consistently exhibited significantly larger stem diameters compared to the control groups. In Beled (2023), inoculated plants reached a maximum stem diameter of 35.51 ± 2.09 mm at T4, while the control remained at 26.3 ± 1.3 mm. A similar trend was observed for head diameter, which was recorded starting at T3. Inoculated treatments typically resulted in broader plates. At Beled (2023), inoculated plants achieved a head diameter of 21.69 ± 1.74 cm by T4, significantly outperforming the control's 18.53 ± 2.08 cm. In 2025, a significant effect was observed in Beled during the early sampling stage, while in Zagyvarékas, it only appeared during the later sampling.

3.4. Fresh and Dry Biomass of Sunflower Shoots

Biomass accumulation, measured as shoot fresh and dry weight, was evaluated for both temporal and spatial variations (Figures 2 and 3). In the temporal analysis at the Beled site, inoculation and sampling time independently drove highly significant differences (Table S1) in both biomass parameters ($p < 0.001$), while the growing year had no significant main or interactive effects. The treatment's positive influence was consistent across the 2023 and 2025 seasons, primarily manifesting at the later sampling time (T2), where inoculated plants exhibited higher fresh and dry weights compared to the uninoculated controls. At the initial sampling stage (T1), treatment differences remained negligible. Conversely, the spatial analysis across the three experimental sites in 2025 revealed a highly context-dependent response. The efficacy of the inoculation varied significantly by location for both fresh ($p = 0.020$) and dry weight ($p < 0.001$). This geographic interaction is evident in the post hoc comparisons; while inoculation distinctly enhanced T2 biomass at Beled, this benefit was less pronounced at Zagyvarékas and virtually absent at Gyomaendrőd. Finally, the significant interaction between inoculation and sampling time for fresh weight across locations ($p < 0.001$) further confirms that the growth-promoting effects of the treatment are highly dependent on the developmental stage.

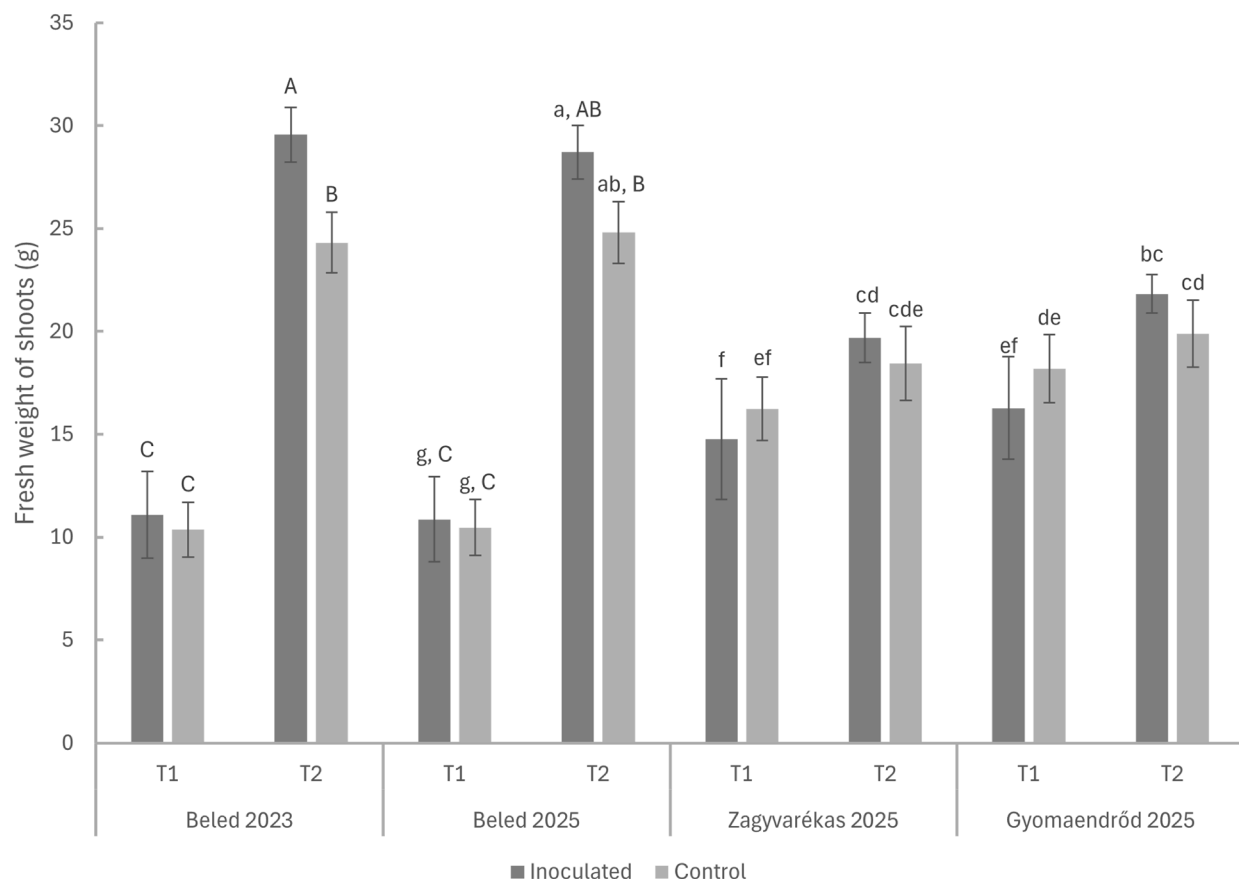


Figure 2. Analysis of fresh weight of shoots by treatment, location and sampling time at Beled (2023 and 2025), Zagyvarékas (2025) and Gyomaendrőd (2025). T1: 4–6 leaf stage, T2: 6–8 leaf stage. Uppercase letters denote significant differences within the temporal analysis (comparing the Beled location across 2023 and 2025). Lowercase letters denote significant differences within the spatial analysis (comparing Beled, Zagyvarékas, and Gyomaendrőd within the 2025 season). Different letters of the same case are significantly different ($p < 0.05$) according to the Šidák method post hoc test.

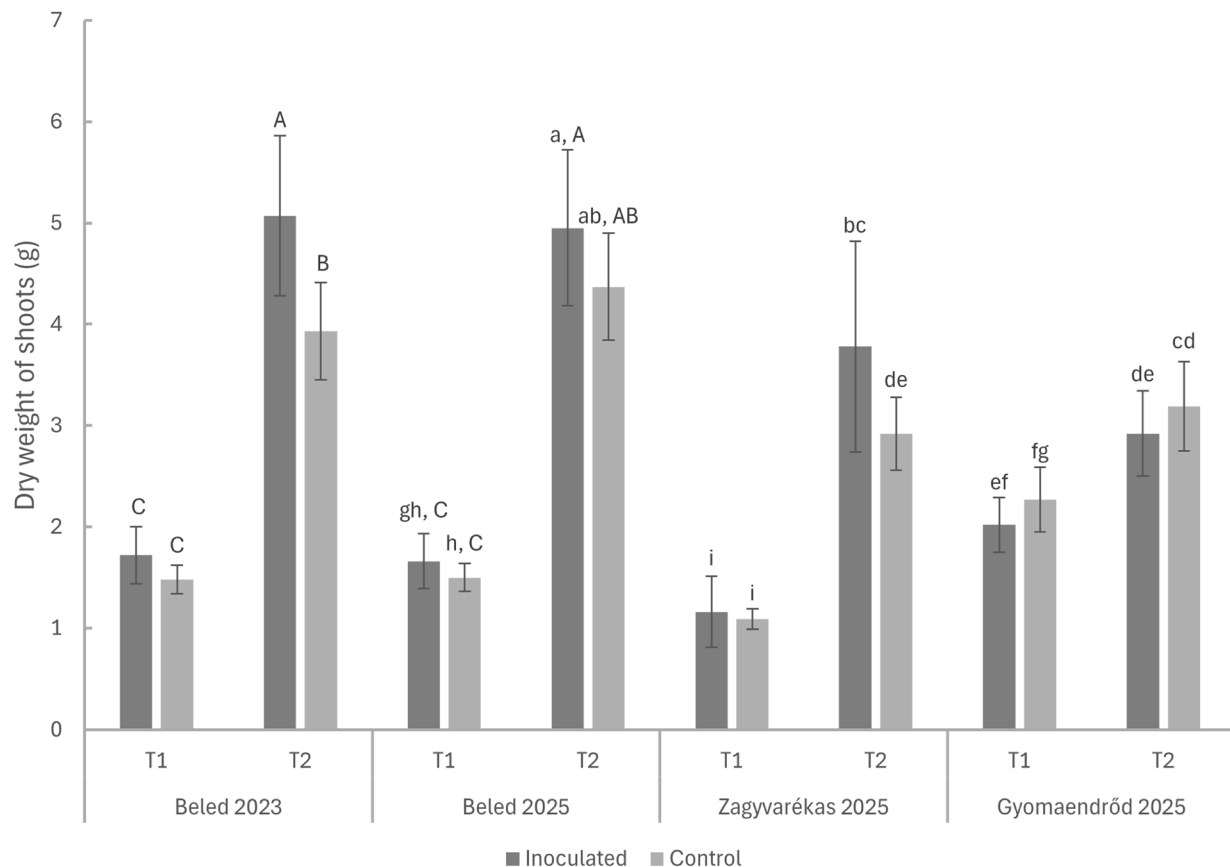


Figure 3. Analysis of dry weight of shoots by treatment, location and sampling time at Beled (2023 and 2025), Zagyvarékas (2025) and Gyomaendrőd (2025). T1: 4–6 leaf stage, T2: 6–8 leaf stage. Uppercase letters denote significant differences within the temporal analysis (comparing the Beled location across 2023 and 2025). Lowercase letters denote significant differences within the spatial analysis (comparing Beled, Zagyvarékas, and Gyomaendrőd within the 2025 season). Different letters of the same case are significantly different ($p < 0.05$) according to the Šidák method post hoc test.

3.5. Shoot Moisture Content of Sunflower Shoots

The shoots' *SMC* across the different sampling stages and locations remained relatively high (Figure 4), generally ranging between 80% and 94%. At T1, the highest *SMC* values were observed in Zagyvarékas, reaching approximately 92–93% for both inoculated and control plants, which was significantly higher than the values recorded at other locations in 2025. At Beled and Gyomaendrőd, *SMC* values at T1 were comparatively lower, fluctuating between 83% and 87%. By T2, a slight downward trend in moisture content was observed across most sites, though it remained statistically non-significant in many cases. Interestingly, in Zagyvarékas, there was a notable decrease from the elevated T1 levels to approximately 81–84% at T2. In the temporal analysis, only sampling time exerted a significant influence ($p < 0.001$), whereas inoculation, year, and all corresponding temporal interactions were non-significant (Table S1). Conversely, the spatial analysis revealed highly significant main effects for both sampling time ($p < 0.001$) and location ($p < 0.001$), while the overall main effect of inoculation remained non-significant ($p = 0.558$). However, within this spatial context, both the Inoculation $\times$ Location ($p = 0.014$) and the Sampling time $\times$ Location ($p < 0.001$) interactions were significant.

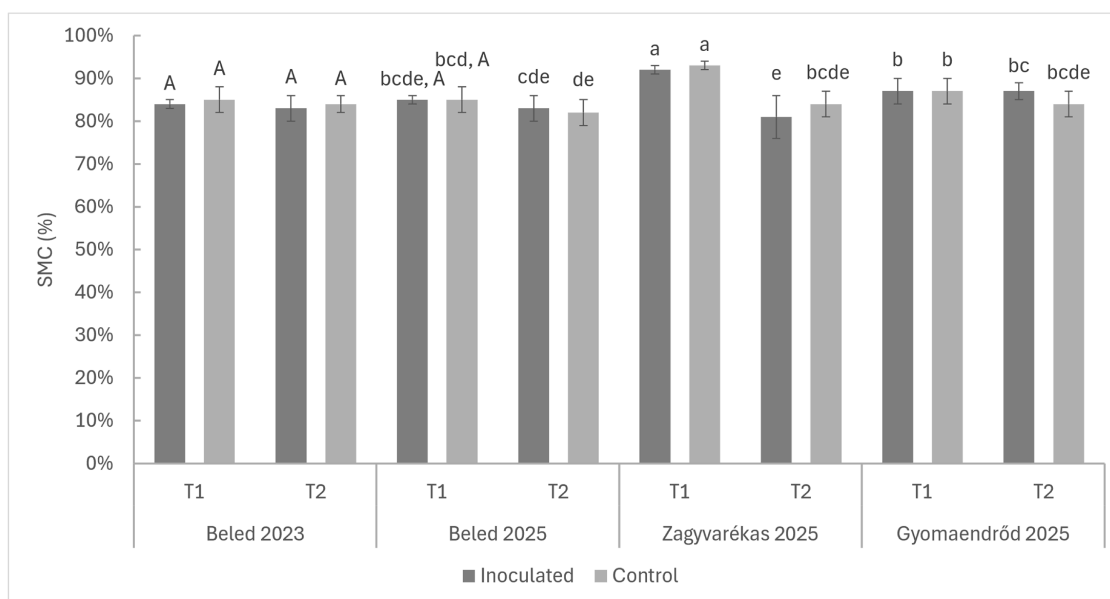


Figure 4. Results of shoots' shoot moisture content (SMC) by treatment, location and sampling time at Beled (2023 and 2025), Zagyvarékas (2025) and Gyomaendrőd (2025). T1: 4–6 leaf stage, T2: 6–8 leaf stage. Uppercase letters denote significant differences within the temporal analysis (comparing the Beled location across 2023 and 2025). Lowercase letters denote significant differences within the spatial analysis (comparing Beled, Zagyvarékas, and Gyomaendrőd within the 2025 season). Different letters of the same case are significantly different ($p < 0.05$) according to Tukey's HSD and the Šidák method post hoc tests, depending on the underlying statistical model (see Methods).

3.6. Evaluation of Yield

Seed yield (Table 3) was consistently enhanced by inoculation at the Beled site across both the 2023 and 2025 seasons (Table S1, $p < 0.001$), increasing productivity to as much as 4.0 tonnes per hectare in 2025. However, spatial analysis revealed a highly significant location-dependent response (Inoculation $\times$ Location, $p < 0.001$). While the treatment provided a clear advantage at Beled, it yielded no significant benefit at Zagyvarékas or Gyomaendrőd, where both inoculated and control plots produced statistically similar yields ranging from approximately 2.0 to 2.3 tonnes.

Table 3. Annual yield (q ha^{-1}) of inoculated and control sunflower plants across three sampling stages at Beled (2023 and 2025), Zagyvarékas (2025) and Gyomaendrőd (2025).

Location	Year	Seed Inoculation	Control
Beled	2023	36.7 ± 0.9 B	29 ± 2 D
	2025	40 ± 1 a,A	33 ± 1 b,C
Zagyvarékas	2025	23 ± 2 c	22 ± 2 c
Gyomaendrőd	2025	20 ± 1 c	19.6 ± 0.8 c

Values are presented as mean $\pm$ SD. Uppercase letters denote significant differences within the temporal analysis (comparing the Beled location across 2023 and 2025). Lowercase letters denote significant differences within the spatial analysis (comparing Beled, Zagyvarékas, and Gyomaendrőd within the 2025 season). Means with different letters of the same case are significantly different ($p < 0.05$) according to Tukey's post hoc test.

3.7. Assessment of the Metabolic Activity and Functional Diversity of the Soil Microbial Community

AWCD values (Figure 5), representing total microbial carbon-source utilisation potential, exhibited distinct temporal and spatial dynamics. In Gyomaendrőd, substrate utilisation potential remained relatively stable across the growing season for the inoculated plots, while the control group experienced a slight decline by the final sampling stage. A much sharper temporal effect was observed at the other sites: in Beled, the inoculated group showed a significant decline between T1 and T4, and in Zagyvarékas, both the inoculated and control

groups exhibited a severe, significant drop over time. Furthermore, significant treatment effects were recorded at T4 in two locations, but with contrasting outcomes. In Zagyvarékas, inoculated plots maintained significantly higher carbon-source utilisation potential compared to the controls at the final stage. Conversely, in Beled, it was the control plots that demonstrated a significantly higher utilisation potential than the inoculated group at T4. Functional diversity, assessed via the Shannon–Wiener index (Figure 6), demonstrated highly location- and stage-specific treatment responses. In Beled, a distinct temporal inversion occurred: inoculation significantly enhanced culture-dependent microbial diversity at the early vegetative stage (T1), but this effect reversed by the pre-harvest stage (T4), where control plots displayed significantly higher diversity. In Zagyvarékas, treatments did not differ at T1; however, despite an overall temporal decline, inoculated plots maintained a significantly higher functional diversity than the controls at T4. Conversely, in Gyomaendrőd, functional diversity remained relatively stable, with no significant differences observed between treatments at either sampling time. Functional diversity, assessed via the Shannon–Wiener Index (Figure 6), showed significant differences between inoculated and control groups were recorded in Beled and in Zagyvarékas at T4. Notably, Beled exhibited a distinct temporal trend change: while inoculation significantly increased culture-dependent microbial diversity during the early vegetative stage (T1), the effect inverted by the pre-harvest stage (T4), at which point inoculated plots displayed lower functional diversity compared to the control. The analysis of both *AWCD* and the Shannon–Wiener Index revealed that these culture-dependent metabolic potential metrics were significantly influenced by the main effects of inoculation ($p < 0.01$ for *AWCD*; $p < 0.001$ for Shannon–Wiener Index), sampling time ($p < 0.001$), and location ($p < 0.001$) (Table S1). While the two-way interaction between inoculation and sampling time was non-significant for both parameters, both the Inoculation $\times$ Location and Sampling time $\times$ Location interactions were highly significant ($p < 0.001$). Most notably, a highly significant three-way interaction (Inoculation $\times$ Sampling time $\times$ Location) was observed for both *AWCD* and the Shannon–Wiener Index ($p < 0.001$).

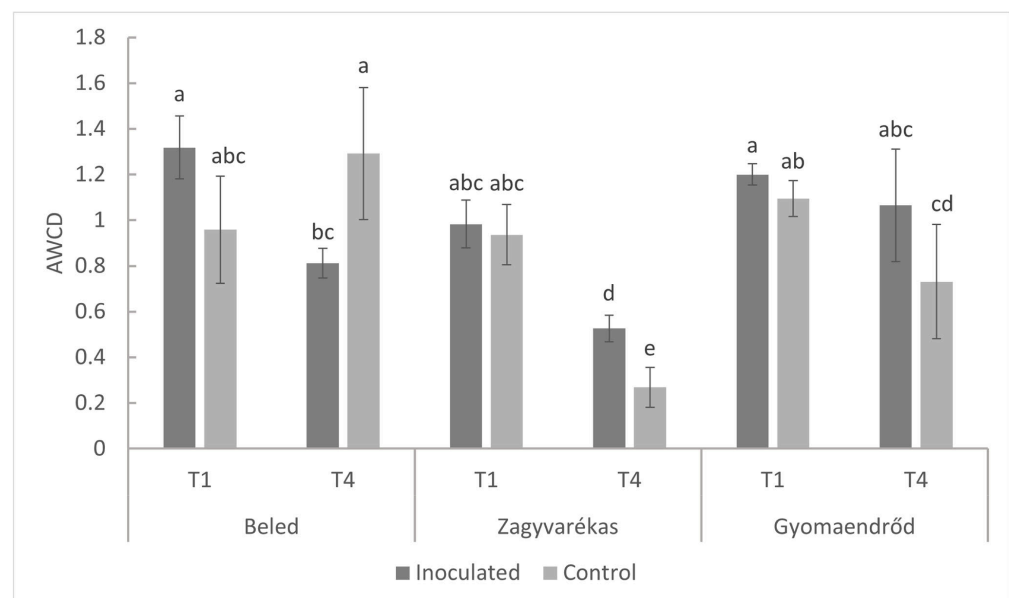


Figure 5. Total metabolic activity and oxidative capacity (*AWCD*) of the soil microbial communities by treatment across three experimental sites and two sampling times in 2025. T1: 4–6 leaf stage, T4: pre-harvest stage. Different letters within each measured parameter indicate significant differences ($p < 0.05$) among all treatment combinations (location, sampling time, and inoculation status) according to the Šidák method post hoc test.

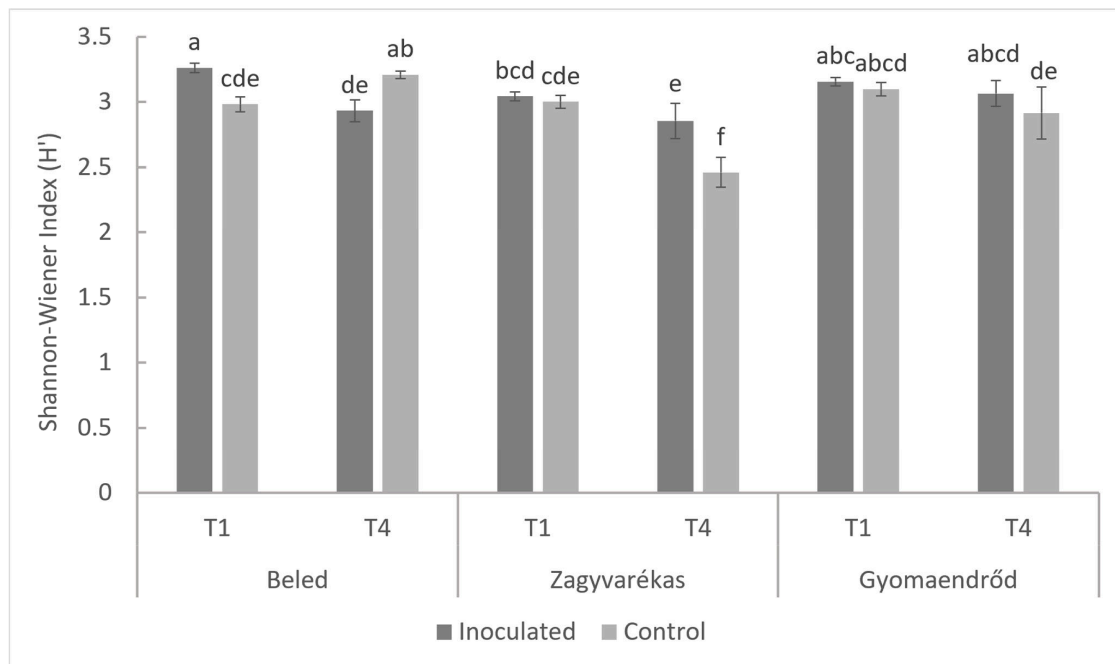


Figure 6. Functional diversity (Shannon–Wiener Index, H') of soil microbial communities based on carbon-source utilisation profiles by treatment across three sites and two sampling intervals. T1: 4–6 leaf stage, T4: pre-harvest stage. Different letters within each measured parameter indicate significant differences ($p < 0.05$) among all treatment combinations (location, sampling time, and inoculation status) according to the Šidák method post hoc test.

The temporal turnover was mirrored in the metabolic fingerprinting heatmap (Figure 7). In Beled and Gyomaendrőd, high diversity at T1 was supported by the more active potential utilisation of the substrates. Conversely, the distinctly lower substrate utilisation potential observed in Zagyvarékas (more visible without inoculation) at T4—characterised by reduced utilisation rates across most carbon catabolic pathways—corresponds with the significant decline in both AWCD and Shannon–Wiener Index at that site. The functional shifts in soil microbial communities were further elucidated by PCA of the EcoPlate carbon metabolic profiles (Figure 8). The first two principal components accounted for 47.22% of the total variance (PC1: 26.85%; PC2: 20.37%). Variance along PC1 is primarily driven by the utilisation of Tween.40, L-Serine, N-Acetyl-D-Glucosamine, β -HydroxyGlycyl-L-Glutamic Acid and L-Asparagine, while PC2 is predominantly influenced by i-Erythritol, 2-Hydroxybenzoic Acid, α -Keto-Butyric acid, Pyruvic Acid Methyl Ester and D-Galacturonic Acid. A three-way PERMANOVA formally substantiated that these culture-dependent metabolic profiles were significantly shaped by the main effects of location ($p < 0.001$) and sampling time ($p < 0.001$), and the three-way Location $\times$ Treatment $\times$ Sampling time interaction ($p < 0.001$). The PCA scores reveal a primary clustering pattern driven by location, which is particularly evident at the T1 sampling stage. At T1, samples from Zagyvarékas and Gyomaendrőd exhibited distinct metabolic profiles, largely separating along the PC2 axis. By T4, a significant temporal shift was observed, most notably in the Beled location. Specifically, the control samples from Beled at T4 shifted toward the positive end of the PC1 axis. The effect of the inoculation treatment varied across locations and time. At T1, inoculated samples and control samples within the same location often showed overlap. However, the separation between treatments became more pronounced in specific clusters at T4. Samples were situated at the positive extreme of PC1 (e.g., Beled T4 controls).

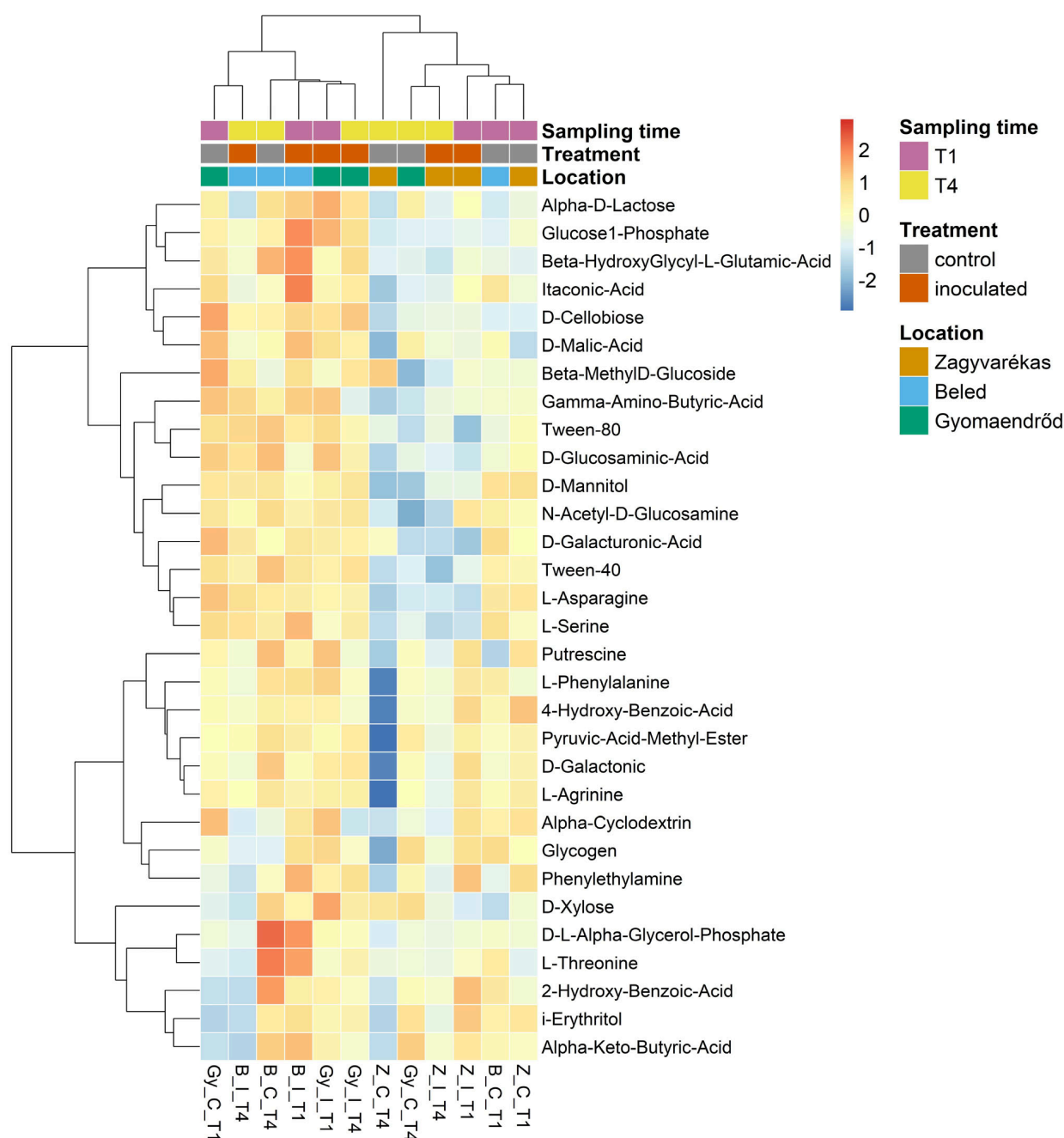


Figure 7. Heatmap and hierarchical clustering of microbial metabolic profiles across treatments and sampling times. The matrix displays the utilisation patterns of 31 carbon sources (rows) across soil samples (columns). Data are Z-score normalised by row. Abbreviations: Z—Zagyvarékas, B—Beled, Gy—Gyomaendrőd, C—control, I—inoculated, T1—4–6 leaf stage, T4—pre-harvest stage.

In Beled, the impact of inoculation was most distinct during the early vegetative stage (T1), where inoculated plots demonstrated significantly higher functional diversity compared to the control. Inoculated samples at T1 were characterised by the vigorous potential utilisation of carbohydrates (Glucose-1-Phosphate; D, L- α -Glycerol-Phosphate), amino acids (L-Threonine; β -Hydroxy-Glycyl-L-Glutamic Acid), and Carboxylic acids such as Itaconic acid. By T4, a functional inversion was observed; while the carbon-source utilisation potential of inoculated plots declined, the control plots exhibited significantly higher diversity. During this later period, control communities in Beled increased their consumption potential of specific Phenols (2-Hydroxybenzoic acid), carbohydrates (D, L- α -Glycerol-Phosphate) and amino acids (L-Threonine; β -Hydroxy-Glycyl-

L-Glutamic Acid), while decreasing their affinity for Polymers like Glycogen. At the Gyomaendrőd site, the differences between inoculated and control plots were less pronounced during the initial sampling. The inoculated community showed a balanced preference for carbohydrates such as D-Xylose and α -D-Lactose, while the control plot primarily consumed D-Cellobiose and β -Methyl-D-Glucoside. By T4, both treatments experienced a slight decline in overall substrate utilisation intensity, though the inoculated plots shifted toward exploiting Amines (Phenylethylamine) and amino acids (β -Hydroxy-Glycyl-L-Glutamic Acid). The control plot at T4 demonstrated a reduction in its primary carbohydrate utilisation (D-Cellobiose) but showed an increase in specific Carboxylic acids like α -Keto-Butyric acid. Zagyvarékas generally displayed the lowest overall culture-dependent metabolic response across the study, with both treatments showing moderate activity at T1. The most striking contrast between treatments appeared at T4, where the control plot reached a state of pronounced reduction in carbon utilisation potential across nearly all carbon-source categories, including amino acids (L-Phenylalanine; L-Arginine) and Phenols (4-Hydroxybenzoic acid). During this pre-harvest stage, the inoculated plots, while significantly less active than at T1, maintained significantly higher potential utilisation rates than their respective controls. This remaining activity was largely restricted to the moderate consumption of carbohydrates like D-Xylose and specific Carboxylic acids such as α -Keto-Butyric acid.

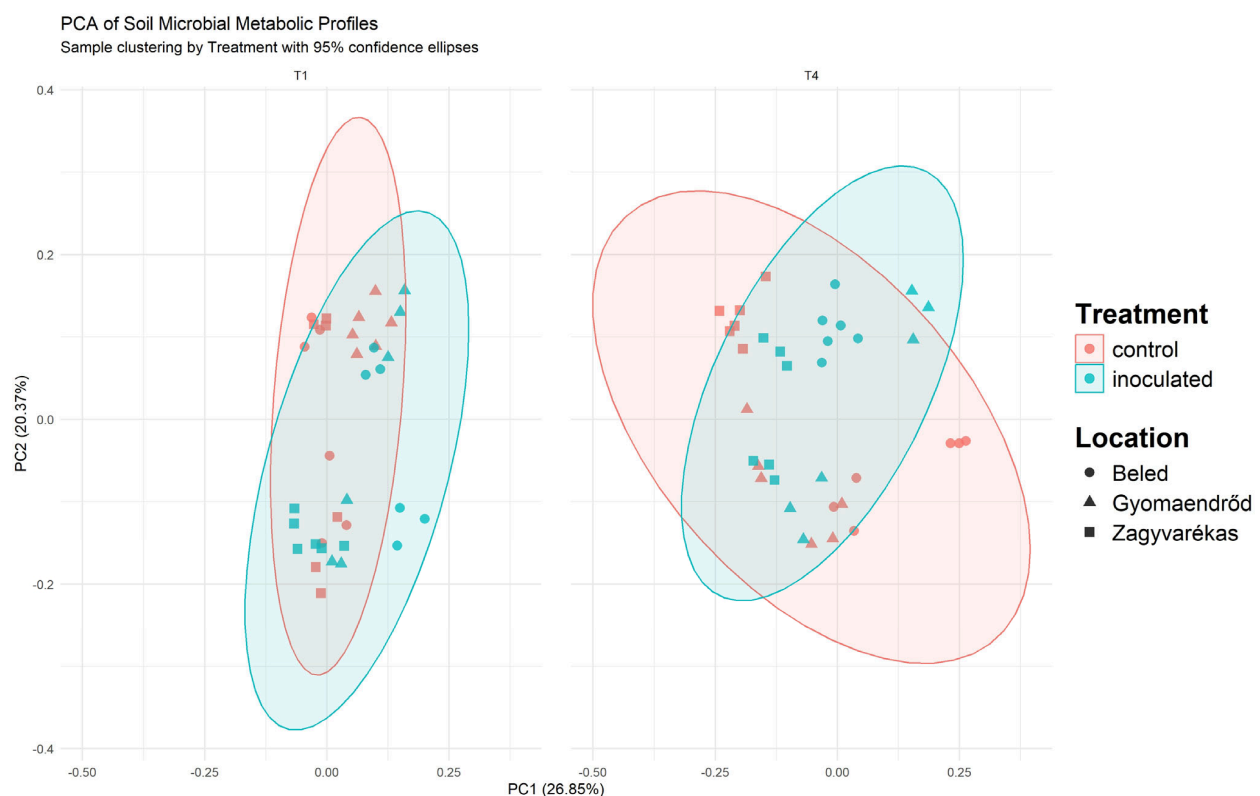


Figure 8. PCA of soil microbial functional profiles based on carbon-source utilisation. The ordination is based on the 31 individual substrate utilisation values. The analysis is partitioned by sampling interval, where the left side of the plot represents T1 (4–6 leaf stage) and the right side represents T4 (pre-harvest stage). Points represent individual soil samples, with colours indicating treatment levels and shapes representing location levels.

4. Discussion

While the general growth-promoting potential of AMF and PGPR is well-documented [18,24–26,35,36], this study provides a novel contribution by demonstrating the field efficacy

of a multi-component seed coating formulated with both AMF and beneficial bacteria offering a more simple and favourable application to traditional soil or root applications.

The site-specific responsiveness of treatment was most clearly demonstrated at the Beled site, where inoculation resulted in increased structural parameters, shoot biomass, and a yield increase of 18–21% across both years—which indicates clear agronomical benefits. Such pronounced effects are likely a direct response to the site's lower soil pH and limited phosphorus availability—conditions that typically increase plant reliance on symbiotic pathways for nutrient acquisition [18,37–41]. At the other sites, however, improved soil properties—particularly higher nutrient availability—likely reduced the plant's dependence on microbial symbiosis, and consequently, no additional phenological or yield-related benefits (e.g., shoot biomass or yield increase) were observed compared to the control plots. In this context, the commercial seed coating appears to bridge the gap between plant nutrient demand and soil supply. These effects are potentially mediated by the synergistic action of the formulation's components, including enhanced phosphorus acquisition via mycorrhizal hyphal networks and phosphatase activity, alongside nitrogen inputs from *Azospirillum*-mediated fixation, potentially supported by mycorrhizal contributions to nitrogen uptake. However, as the present study relied on indirect growth indicators rather than direct measurements of nutrient mobilisation or uptake, these mechanisms remain inferred rather than conclusively demonstrated, although they are well-supported by a substantial body of literature [11,14,36,42–44].

The efficacy of the commercial formulation was reflected in enhanced overall total root colonisation across all study sites, occurring alongside robust indigenous mycorrhizal communities. This is consistent with the previous study of Arcidiacono et al. [18]. The temporal progression of this cumulative colonisation closely mirrored the plants' phenological development, particularly the rapid increase in plant height and stem diameter observed around the main flowering stage (T3). This synchronicity implies a functional alignment between the plant's increasing nutrient requirements and the expansion of mycorrhizal networks within the rhizosphere. Nevertheless, high total colonisation rates should not be viewed as a definitive proxy for enhanced performance. The net benefit of the symbiosis reflects a delicate balance between carbon costs and nutrient gains; under conditions of higher nutrient availability—particularly elevated phosphorus levels—the plant's reliance on arbuscular mycorrhizal fungi decreases, as the metabolic cost of maintaining the symbiosis may outweigh its benefits [40,41]. Consequently, in well-supplied soils, the functional importance of AMF is reduced and their contribution to plant performance becomes less pronounced. However, the relatively high total AMF colonisation observed at sites (Zagyvarékas, Gyomaendrőd), despite their more favourable nutrient status, was likely influenced by the extremely dry, precipitation-free periods recorded during the growing season (Table S5). Under such conditions, the stress-mitigating role of mycorrhizal associations becomes increasingly important, as fungal hyphal networks can enhance water uptake and improve plant tolerance to drought stress [18,19,21,45].

The capacity of the inoculation to provide such physiological support is likely intrinsically linked, besides AMF, to the broader microbial shifts occurring within the rhizosphere, particularly in resource-limited settings. Our analysis of microbial carbon-source utilisation potential at Beled (Figure 5) revealed an initial surge at T1 attributable to inoculation, followed by a gradual decline toward T4 as the growing season progressed. This temporal pattern is consistently reflected in the culture-dependent metabolic profiles (Figure 7). At T1, the inoculated treatment was characterised by a dominance of microorganisms capable of utilising amino acids and simple carbohydrates; however, this dominance diminished by T4, near harvest. A concurrent decrease in the Shannon–Wiener Index (Figure 6) at T4 suggests a shift toward functional specialisation. Nevertheless, alternative explanations—

such as substrate depletion or methodological bias—cannot be excluded [14,25,38,43,44]. These shifts in substrate utilisation patterns are further supported by principal component analysis (Figure 8), in which sample separation becomes more pronounced with plant development. Samples positioned at the positive extreme of PC1, particularly the T4 control at Beled, likely represent microbial communities with a narrower functional utilisation spectrum, specialising in specific substrate groups such as carbohydrates or amino acids. However, because PCA axes integrate multiple underlying variables, their biological interpretation remains inherently limited.

In contrast to the patterns observed at Beled, the responses at Zagyvarékas and Gyomaendrőd—where soil phosphorus availability was within an optimal range according to regional standards and pH values were comparatively higher—were limited, and the effects of inoculation on plant growth were generally not statistically significant at later phenological stages (T3–T4). This is consistent with the absence of significant increases in yield or shoot biomass following inoculation. These findings align with previous studies indicating that high phosphorus availability can reduce plant dependence on, and responsiveness to, arbuscular mycorrhizal symbiosis [40,46]. At Zagyvarékas, a pronounced decline in carbon-source utilisation potential was observed toward the end of the growing season, particularly in the control plots (Figure 5). Although inoculation appeared to partially mitigate this decrease in culture-dependent substrate consumption, the present study does not provide direct mechanistic evidence to substantiate regulatory interactions within the microbial community. Similarly, at Gyomaendrőd, inoculated plots exhibited relatively stable substrate utilisation patterns over time; however, these differences were not statistically significant and should therefore be interpreted as indicative trends rather than confirmed physiological responses. The modest, trend-level increases in yield (approximately 4–5%) at these sites further suggest that the marginal benefits of inoculation are reduced under nutrient-sufficient conditions, highlighting the importance of site-specific soil characteristics in shaping treatment outcomes. At the same time, the observed temporal variations at the Beled site must be interpreted with caution. Because the temporal model is restricted to a single location across two non-consecutive years, these dynamics cannot be extrapolated as generalised annual climate trends. Furthermore, because the 2025 trial was preceded by an autumn application of poultry manure which was absent in the 2023 baseline background, these temporal differences likely reflect a compounded effect of annual environmental fluctuations and the legacy of localised organic fertilisation management. Ultimately, the interpretation of these results requires caution. Data derived from EcoPlate™ assays reflect only the functional potential of the culturable fraction of the microbial community under optimised laboratory conditions and therefore may not fully represent in situ microbial dynamics.

Overall, the results demonstrate that the effectiveness of the applied inoculation is strongly context-dependent and cannot be generalised across contrasting environments. The most pronounced response—including a yield increase of 18–21%—was observed under low-pH and phosphorus-limited conditions (Beled), where inoculation enhanced plant growth and modulated microbial activity. These findings suggest that inoculants primarily act by alleviating nutrient constraints, thereby facilitating plant adaptation to suboptimal soil conditions rather than fundamentally restructuring the soil environment. In contrast, under nutrient-sufficient conditions, the effects were limited, mostly not statistically significant, and accompanied only by modest, trend-level yield increases. Although root colonisation increased consistently across all sites, the link between culture-dependent functional utilisation potential and plant performance remained indirect and should be interpreted with caution. The observed variability further highlights the critical role of site-specific soil properties—particularly phosphorus availability—in shaping inoculation

outcomes, underscoring the need for targeted application strategies in sustainable agricultural systems. At the same time, some limitations must be acknowledged. Open-field experiments inherently involve environmental heterogeneity, including microclimatic variation and soil spatial variability. While this study addressed such factors through intensive sampling and a high number of replicates, the two-season duration at one site constrains conclusions regarding long-term effects. Furthermore, because the evaluated commercial product is a complex composite formulation containing seaweed extract, micronutrients (Fe, Mn), and inert carriers alongside the microbial strains (*R. irregularis*, *A. brasilense*, and *A. lipoferum*), the individual mechanisms cannot be fully uncoupled in our experimental design. While the observed benefits demonstrate the practical efficacy of the product, these effects cannot be attributed exclusively to AMF-PGPR symbiosis. Additionally, our reliance on microscopic observation of stained roots means we evaluated total mycorrhizal colonisation rates rather than strain-specific tracking. Because native AMF communities were actively colonising the control plants at later growth stages, the microscopic data cannot definitively distinguish between the introduced *R. irregularis* and indigenous AMF. Moreover, methodological limitations—particularly the reliance on culture-based functional assays—restrict the extent to which in situ microbial dynamics can be inferred. Consequently, further research employing long-term experimental designs and high-resolution approaches, such as metagenomic analyses, is required to elucidate the persistence of introduced microorganisms, clarify their functional roles, and assess their cumulative impact on soil health.

5. Conclusions

Seed-applied inoculation with a commercial *Rhizoglossus irregularis* and *Azospirillum* spp. formulation improved sunflower performance under field conditions, but its effectiveness was strongly site-dependent. The greatest agronomic benefit, including an 18–21% yield increase, was achieved under low-pH, phosphorus-limited conditions, whereas responses in nutrient-sufficient soils were limited. Although inoculation consistently enhanced mycorrhizal root colonisation and influenced rhizosphere microbial functional activity, these changes did not always translate into improved plant performance. Overall, the results highlight seed coating as a practical delivery method for beneficial microorganisms and emphasise that successful application depends on site-specific soil conditions, particularly phosphorus availability. Further long-term molecular studies are needed to clarify the persistence and functional contribution of introduced microorganisms.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16141380/s1>, Table S1: Results of the ANOVA statistical analysis; Table S2. Stem diameter (mm) of inoculated and control sunflower plants across three sampling stages at Beled (2023 and 2025), Zagyvarékas (2025) and Gyomaendrőd (2025); Table S3. Head diameter (cm) of inoculated and control sunflower plants across two sampling stages at Beled (2023 and 2025), Zagyvarékas (2025) and Gyomaendrőd (2025); Table S4. Plant height (cm) of inoculated and control sunflower plants across three sampling stages at Beled (2023 and 2025), Zagyvarékas (2025) and Gyomaendrőd (2025); Table S5: Weather conditions of Beled, Gyomaendrőd and Zagyvarékas during the experiments.

Author Contributions: Conceptualisation, resources and supervision, K.P.; data curation, formal analysis and visualisation, L.L.; Investigation and writing—original draft, A.T.G.T.; Methodology and project administration, K.P. and A.T.G.T.; Writing—review and editing, K.P., L.L. and A.T.G.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research, Development and Innovation Office of the Hungarian Government (grant no: RRF 2.3.1 21 2022 00007 for Agribiotechnology and precision

breeding for food security National Laboratory), by the Flagship Research Groups Programme of the Hungarian University of Agriculture and Life Sciences, and by the National Research, Development and Innovation Office (grant no: OTKA142974).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Acknowledgments: During the preparation of this manuscript the authors used Gemini (Google LLC) for the purposes of language editing and refining. The authors reviewed and edited the output as needed and take full responsibility for the content of the published article.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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