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Seasonal variation in free-living nitrogen-fixing bacteria across soil types and depths in Eastern Georgia: Implications for sustainable horticulture crop production

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Abstracts

Free-living nitrogen-fixing bacteria contribute to soil N cycling, but their abundance may vary substantially with soil type, season, and position within the soil profile. Information on these interacting effects in horticultural soils of Eastern Georgia is limited. The study evaluated seasonal and depth-wise variation in culturable putative free-living nitrogen-fixing bacteria in Anthropogenic, Cinnamonic, Chernozem, and Alluvial soils. Samples were collected in spring, summer, autumn, and winter at depths of 5, 10, and 25 cm, with three independent field replicates per treatment combination. Bacterial abundance was expressed as CFU g^{-1} and analyzed after $\log_{10}$ transformation using three-way ANOVA followed by Tukey's HSD test. Soil type, season, and sampling depth significantly affected bacterial abundance, and all two-way interactions and the soil × season × depth interaction were highly significant ($P<0.001$). This trend shows that the effect of depth depended strongly on both soil type and season. The highest abundance occurred in Chernozem soil at 25 cm during spring (approximately 68.33×10468.33 10^4 CFU g^{-1}). Other major maxima occurred in Chernozem soil at 10 cm during summer and winter and in Anthropogenic soil at 25 cm during summer. Cinnamonic soil frequently showed higher abundance at 25 cm, whereas Alluvial soil showed its strongest populations during spring. No uniform increase or decrease with depth occurred across all soils and seasons. These results demonstrate pronounced soil-specific seasonal and vertical heterogeneity in culturable diazotrophic populations. Assessment of the biological component of horticultural soil fertility may therefore require sampling across both seasons and soil depths rather than relying exclusively on surface soil or a single sampling period.

Key words: Biological nitrogen fixation, diazotrophs, soil depth, seasonal dynamics, culturable bacteria, chernozem, soil microbial ecology, sustainable horticulture

Introduction

Nitrogen (N) is an essential macronutrient controlling plant growth, crop productivity, and soil fertility. Human activities, particularly the manufacture and intensive application of synthetic N fertilizers, have substantially altered the global nitrogen cycle, increasing the amount of reactive nitrogen entering terrestrial ecosystems (Canfield *et al.*, 2010; Fowler *et al.*, 2013). Although fertilizer N has contributed greatly to agricultural productivity, excessive or inefficient application may result in nitrate leaching, gaseous N losses, soil acidification, and other environmental impacts. Improving the contribution of biologically mediated N inputs is therefore increasingly important for developing productive and environmentally sustainable agricultural and horticultural systems.

Although molecular nitrogen (N_2) constitutes approximately 78% of the atmosphere, it cannot be directly assimilated by most plants. Biological nitrogen fixation converts atmospheric N_2 into biologically available forms through the activity of diazotrophic microorganisms possessing the nitrogenase enzyme complex. Studies have shown that beneficial soil microorganisms (*Azospirillum* and *Azotobacter*) may enhance nutrient availability. Soil microorganism activity minimized the mineral nitrogenous fertilizer requirement and improved crop performance or improved soil health for sustainable horticultural production (James and Umesh, 2025; Hamed *et al.*, 2025; Hashim *et al.*, 2026; Al-Khafaji *et al.*, 2026).

Free-living and associative N-fixing bacteria are an important component of agricultural soils, contributing to soil N inputs in the absence of the highly specialized symbioses of legume-rhizobium associations. Certain diazotrophic bacteria, including *Azotobacter* and *Azospirillum* spp., have been studied as plant-growth-promoting microorganisms and biofertilizer strategies to reduce mineral fertilizer use (Smercina *et al.*, 2019; Aloo *et al.*, 2022; Abdalla *et al.*, 2026). However, the abundance of culturable diazotrophs should not be interpreted directly as the rate of biological N fixation because microbial abundance and functional activity represent related but distinct ecological properties.

The occurrence and abundance of soil diazotrophs are influenced by interacting environmental and edaphic factors. Soil temperature, moisture, pH, organic carbon availability, mineral N status, vegetation and agricultural management can influence the size and composition of N-fixing microbial populations. Recent studies have shown responses of diazotrophic communities to soil physicochemical conditions, N availability, fertilization, and other environmental gradients (Li *et al.*, 2022; Wang *et al.*, 2024; Ren *et al.*, 2024). Thus, differences in the type of soil may produce different ecological conditions for the free-living nitrogen-fixing bacteria due to differences in aeration, availability of organic

matter, water-holding capacity, nutrient status and soil reaction.

Seasonality is another source of temporal variation in soil microbial populations. Changes in temperature, soil moisture, plant growth, rhizodeposition and nutrient availability with seasonality may affect microbial growth and survival. Large seasonal responses have been observed in diazotrophic community studies, although the magnitude and direction of these changes may be highly dependent on local soil and environmental conditions (Li *et al.*, 2022). Therefore, a uniform seasonal pattern, i.e. an increased abundance of diazotrophs in spring or summer and reduced abundance in winter, should not necessarily be expected across contrasting soils. Rather, soil type may modulate the response of nitrogen-fixing bacterial populations to seasonal environmental changes.

Vertical variation within the soil profile may further influence microbial distribution. Surface and subsurface horizons differ in organic-carbon inputs, root density, oxygen availability, moisture, temperature fluctuations, nutrient concentrations, and other characteristics that regulate microbial growth. The effects of sampling depth may consequently interact with both soil type and season. Such interactions are particularly relevant in horticultural fields, where cultivation, irrigation, fertilization, organic amendments, root activity, and other management practices can create pronounced spatial and temporal heterogeneity in the soil environment. Despite increasing interest in diazotrophic microorganisms for sustainable crop production, comparatively limited information is available on how soil type, season, and soil depth jointly influence culturable free-living nitrogen-fixing bacterial populations under field conditions.

Georgia provides a suitable setting in which to investigate these relationships because of its diverse climatic conditions, agricultural landscapes, and contrasting soil resources. Eastern Georgia contains horticultural production areas developed on several distinct soils, including Chernozem, Cinnamonic, alluvial, and anthropogenic soils. Differences in their physicochemical characteristics, together with pronounced seasonal climatic variation, may generate contrasting ecological conditions for free-living diazotrophic bacteria. Understanding these patterns can provide baseline information on the biological component of soil fertility. It may contribute to the development of soil-management strategies that make greater use of beneficial microorganisms in sustainable horticultural production.

Accordingly, the present study investigated the seasonal and depth-wise variation of culturable free-living nitrogen-fixing bacteria in four contrasting soil types used for vegetable crop production in Eastern Georgia.

Materials and methods

Study area and soil types: The study was conducted in horticultural production areas of Eastern Georgia representing four contrasting soil types. Chernozem soil was sampled in Dedoplistskaro Municipality, Cinnamonic soil in Bolnisi Municipality, Alluvial soil in the vicinity of Rustavi, and Anthropogenic soil in the vicinity of the Tbilisi Sea. These locations were selected to represent major soil environments associated with horticultural crop production in Eastern Georgia. Soil sampling, handling, and transportation to the laboratory were performed in accordance with standard microbiological procedures (Alef and Nannipieri, 1995).

Experimental design and soil sampling: We used a factorial sampling design to assess the effects of soil type, season and soil depth on the abundance of culturable free-living nitrogen-fixing bacteria. The four soil types were sampled during spring, summer, autumn, and winter at depths of 5, 10, and 25 cm.

For each soil type × season × depth combination, were collected and processed three independent field replicates separately. Thus, the three observations used for each treatment combination represented biological field replicates rather than repeated measurements from a single soil suspension.

Soil samples were collected aseptically and transferred into sterile sampling bags. Samples were transported to the laboratory and maintained at 4 °C until microbiological analysis. Serial dilution and culture procedures were performed within 24 h after sample collection.

Preparation of soil suspensions and serial dilutions: For each field replicate, 10 g of soil was aseptically transferred into 90 mL of sterile water to obtain the initial 10^{-1} soil suspension. The suspension was thoroughly homogenized, after which serial decimal dilutions were prepared under aseptic conditions.

Appropriate dilutions were selected according to the expected bacterial abundance. Lower dilutions were used for samples with relatively low colony densities, whereas higher dilutions were used when necessary to obtain countable colonies.

Cultivation and enumeration of nitrogen-fixing bacteria: Free-living nitrogen-fixing bacteria were cultivated and enumerated in Jensen's nitrogen-free medium (Jensen, 1942). Jensen's medium was used because the lack of combined nitrogen promotes the growth of microorganisms that can obtain nitrogen through diazotrophic metabolism.

To each replicate prepared independently, 1 mL of the selected soil dilution was aseptically transferred to a sterile disposable Petri dish. Molten Jensen's medium was cooled to approximately 47 °C and poured on the plates. The contents of each plate were gently mixed to distribute the inoculum across the medium and allowed to solidify before incubation.

Aseptic techniques and sterile disposable Petri dishes were used to minimize contamination. Also, in pour plating, the addition of microorganisms to the agar creates an oxygen gradient in the medium, but this was not considered a direct measurement or controlled induction of microaerophilic conditions. Plates were incubated at 28° C for 72 Hours . Following incubation, colonies developing on Jensen's medium were counted and expressed as colony-forming units per gram of soil (CFU g^{-1}).

Because the method detects only organisms capable of growing under the specified culture conditions, the resulting values were interpreted as the abundance of culturable putative free-living nitrogen-fixing bacteria, rather than as the total diazotrophic community present in the soil.

Presumptive screening of nitrogen-fixing isolates: Representative colonies showing similar morphological characteristics were selected from Jensen's medium and subjected

to further screening. Individual colonies were transferred to liquid nitrogen-free medium and incubated for 24 h.

Following incubation, the cultures were tested using Nessler's reagent to detect ammonium accumulation. Development of the characteristic color reaction was considered evidence of ammonium production under nitrogen-free culture conditions and was used as a presumptive screening test for potential diazotrophic isolates.

Calculation of bacterial abundance: Bacterial abundance was calculated in accordance with the colony-counting principles described in ISO 7218 (ISO, 2013)

When colonies from two successive decimal dilutions were included in the calculation, the general expression was:

$N = \Sigma C / [V(n_1 + 0.1n_2)d]$

where: N = number of culturable bacteria, expressed as CFU g^{-1}; ΣC = sum of colonies counted on all plates retained for calculation; V = inoculum volume plated on each Petri dish (1 mL in the present study); n_1 = number of plates retained at the first selected dilution; n_2 = number of plates retained at the second successive dilution; and d = dilution corresponding to the first selected dilution.

Because the inoculum volume used in the present pour-plate procedure was 1 mL ($V = 1$), the equation simplifies to:

$N = \Sigma C / [(n_1 + 0.1n_2)d]$

When colony counts from only one selected dilution were used, bacterial abundance was calculated as:

$N = \Sigma C / (Vnd)$

where n represents the number of independent observations included at that dilution.

Results were expressed as CFU g^{-1} soil.

Statistical analysis: Microbial count data were examined for distributional characteristics before statistical analysis. Because CFU data covered a wide numerical range and showed positive skewness, bacterial abundance was transformed to $\log_{10}$(CFU g^{-1}) before inferential analysis.

The effects of soil type, season, sampling depth, and their interactions were evaluated using a three-way factorial analysis of variance (ANOVA). The statistical model included soil type, season, and depth as fixed factors together with all two-way and three-way interactions:

$Y = \mu + S + Se + D + (S \times Se) + (S \times D) + (Se \times D) + (S \times Se \times D) + \varepsilon$

where S represents soil type, Se represents season, and D represents sampling depth.

When significant treatment effects were detected, means were separated using Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. Because the soil type × season × depth interaction was of particular biological interest, Tukey comparisons were performed among season × depth combinations within each soil type.

Results were presented as mean ± standard deviation (SD), $n = 3$ in tables, while graphical error bars represented mean ± standard error (SE). Different lowercase letters indicate statistically significant differences according to Tukey's HSD test at $P \leq 0.05$, whereas means sharing at least one letter were not significantly different.

Statistical analyses: Statistical analyses were performed in Python using the statsmodels package. Three-way ANOVA was applied to $\log_{10}$-transformed CFU g^{-1} data, followed by Tukey-adjusted multiple comparisons at P≤0.05 (Seabold and Perktold, 2010)

Results and discussion

Overall effects of soil type, season, and sampling depth
The abundance of culturable putative free-living nitrogen-fixing bacteria varied markedly among soil types, seasons, and sampling depths (Table 1; Fig. 1A–D). Three-way factorial ANOVA of $\log_{10}$-transformed CFU g^{-1} values showed highly significant effects of soil type ($F_{3,96} = 7113.61$, $P < 0.001$), season ($F_{3,96} = 3050.12$, $P < 0.001$), and sampling depth ($F_{2,96} = 3195.71$, $P < 0.001$).

All two-way interactions were also significant, including soil type × season ($F_{9,96} = 1185.99$, $P < 0.001$), soil type × depth ($F_{6,96} = 1012.81$, $P < 0.001$), and season × depth ($F_{6,96} = 780.89$, $P < 0.001$). Most importantly, the soil type × season × depth interaction was highly significant ($F_{18,96} = 1699.58$, $P < 0.001$). The significant three-way interaction shows that the effect of soil depth was not consistent across seasons or soil types, and that seasonal responses likewise depended on the soil environment. Therefore, the individual soil × season × depth combinations provide a more meaningful biological interpretation than the main effects considered independently.

Such context-dependent responses are consistent with current understanding of diazotrophic ecology. Free-living nitrogen-fixing microorganisms are susceptible to a variety of interacting factors such as carbon availability, mineral nitrogen, soil moisture, temperature, pH, oxygen status, vegetation and management practices (Smercina *et al.*, 2019; Zheng *et al.*, 2023). Thus, different soil types and depths can create new ecological niches for diazotrophs. As in studies on soil profiles, we observed significant vertical variation in diazotrophic abundance and community structure rather than a decreasing trend with depth (Wang *et al.*, 2021).

Table 1. Three-way factorial ANOVA

Effect	df	F	*P*
Soil	3	7113.61	<0.001
Season	3	3050.12	<0.001
Soil Depth	2	3195.71	<0.001
Soil x Season	9	1185.99	<0.001
Soil x Depth	6	1012.81	<0.001
Season x Depth	6	780.89	<0.001
Soil x Season x Depth	18	1699.58	<0.001
Residual	96		

Anthropogenic soil: In Anthropogenic soil, bacterial abundance ranged from 0.30 × 10^4 to 38.00 × 10^4 CFU g^{-1}. The greatest response was recorded in summer at 25 cm, with an abundance of 38.00 ± 0.00 × 10^4 CFU g^{-1}, which was significantly higher than all other season–depth combinations (Tukey's HSD test).

Spring abundance was highest at 5 cm (1.20 ± 0.10 × 10^4 CFU g^{-1}) and significantly lower at 10 and 25 cm. During autumn, abundance increased from 0.80 × 10^4 CFU g^{-1} at 5 cm to 1.20 ×

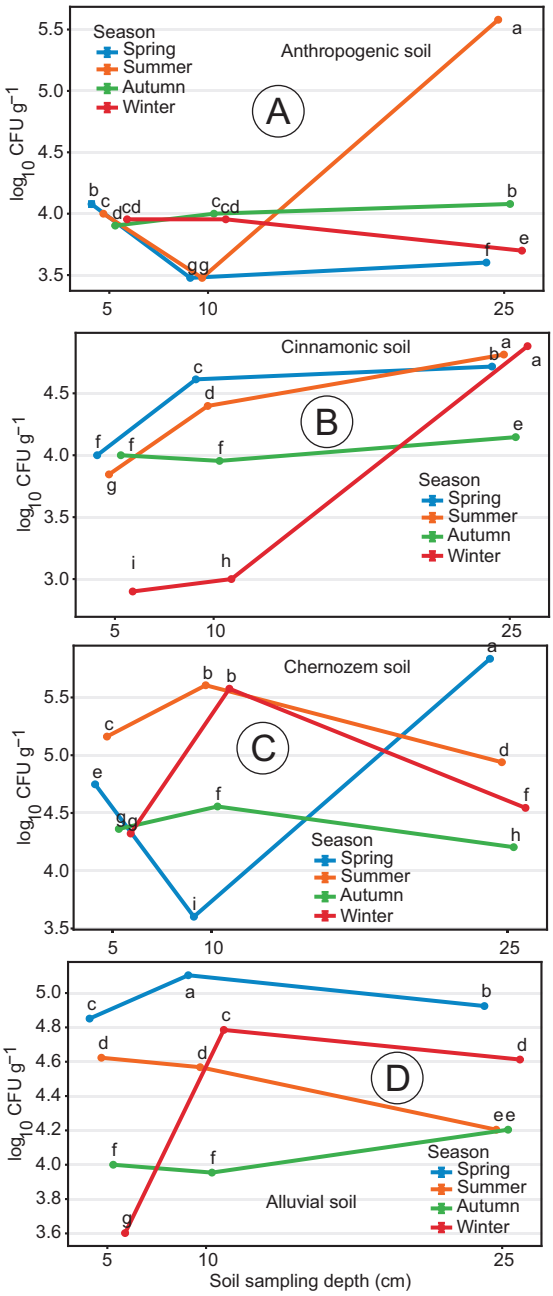


Fig. 1. Seasonal and depth-wise variation in culturable free-living nitrogen-fixing bacterial abundance in (A) Anthropogenic, (B) Cinnamonic, (C) Chernozem and (D) Alluvial soils. Values represent mean $\log_{10}$-transformed bacterial counts [$\log_{10}$(CFU g^{-1})] at soil depths of 5, 10, and 25 cm during spring, summer, autumn, and winter (n = 3 independent field replicates). Different lowercase letters indicate significant differences among season × depth combinations within each soil type according to Tukey's HSD test at $P \leq 0.05$; means sharing at least one letter are not significantly different.

10^4 CFU g^{-1} at 25 cm. Winter values were comparatively uniform at the two shallower depths but declined at 25 cm.

These results demonstrate the absence of a simple monotonic depth pattern in the Anthropogenic soil. The pronounced summer maximum at 25 cm suggests that the subsurface environment may at certain times provide more favorable conditions for culturable diazotrophs than the surface layer. Deeper soil horizons can be less variable in temperature and water availability than surface soil and provide refugia when surface soil dries or thermal stress occurs (Wang *et al.*, 2021). However, since the present study did not measure soil moisture, temperature, organic carbon, mineral N and pH concurrently, the mechanism responsible for the increase observed cannot be determined from the CFU data alone.

The particularly high summer value at 25 cm is also worthy of special attention because it was much higher than the corresponding spring, autumn and winter values. This temporal heterogeneity is consistent with the significant season × depth interaction but should be interpreted with caution due to the intrinsic spatial heterogeneity of anthropogenic soils, which can be affected by previous disturbance, irrigation, organic residues and horticultural management.

Cinnamonic soil: Cinnamonic soil showed a different vertical pattern, with bacterial abundance generally increasing toward the 25-cm depth. In spring, abundance increased from 1.00 ± 0.00 × 10^4 CFU g^{-1} at 5 cm to 4.10 ± 0.10 × 10^4 CFU g^{-1} at 10 cm and 5.20 ± 0.10 × 10^4 CFU g^{-1} at 25 cm. A similar pattern occurred during summer, when populations increased from 0.70 × 10^4 CFU g^{-1} at 5 cm to 2.50 × 10^4 CFU g^{-1} at 10 cm and 6.50 ± 0.10 × 10^4 CFU g^{-1} at 25 cm. Autumn values were lower and less variable.

The strongest depth contrast was detected in winter. Bacterial abundance was only approximately 0.07 ± 0.06 × 10^4 CFU g^{-1} at 5 cm and 0.10 ± 0.00 × 10^4 CFU g^{-1} at 10 cm but increased to 7.60 ± 0.10 × 10^4 CFU g^{-1} at 25 cm. The winter population at 25 cm was therefore more than an order of magnitude greater than the populations in the surface layers.

This pattern is compatible with the concept that deeper soil horizons may buffer microorganisms from seasonal environmental extremes. Studies of soil profiles have shown that diazotrophic communities can be abundant or differ in composition in deeper layers, as depth alters the availability of oxygen, the supply of substrates, the water status, the influence of roots and other environmental factors (Wang *et al.*, 2021). Depth-dependent biological nitrogen fixation has also been demonstrated in other soil and sediment systems, emphasizing that nitrogen-fixing communities need not be concentrated exclusively at the surface.

Nevertheless, the winter increase at 25 cm should not be interpreted as evidence of greater nitrogen-fixation activity at that depth. The present measurements represent culturable abundance, whereas actual nitrogen fixation depends on regulation and activity of the nitrogenase system. Diazotroph abundance and biological N2 fixation rate are differentially sensitive to environmental conditions (Smercina *et al.*, 2019; Chen *et al.*, 2022).

Chernozem soil: Chernozem soil exhibited the widest variation and produced the highest bacterial abundance recorded in the study. Values ranged from 0.40 × 10^4 to approximately 68.33 × 10^4 CFU g^{-1}. During spring, abundance was 5.60 ± 0.10 × 10^4 CFU g^{-1} at 5 cm and only 0.40 × 10^4 CFU g^{-1} at 10 cm, but increased dramatically to 68.33 ± 0.58 × 10^4 CFU g^{-1} at 25 cm. This interaction had the highest soil × season × depth value recorded across the entire experiment.

The pattern changed substantially during summer. Abundance reached 14.50 ± 0.10 × 10^4 CFU g^{-1} at 5 cm and peaked at 40.33 ± 0.58 × 10^4 CFU g^{-1} at 10 cm before declining to 8.70 × 10^4 CFU g^{-1} at 25 cm. Autumn populations were considerably lower, ranging from 1.60 to 3.60 × 10^4 CFU g^{-1}. In winter, abundance again peaked at 10 cm, reaching 37.67 ± 0.58 × 10^4 CFU g^{-1},

compared with 2.10 × 10^4 and 3.50 × 10^4 CFU g^{-1} at 5 and 25 cm, respectively. Chernozem therefore showed a pronounced shift in the depth of maximum bacterial abundance: the maximum occurred at 25 cm in spring, whereas it occurred at 10 cm during summer and winter. This finding provides particularly strong evidence for the soil × season × depth interaction.

The relatively large populations recorded in several Chernozem treatments may be associated with environmental conditions favorable to heterotrophic diazotrophs. In other agricultural soils, the abundance and community composition of diazotrophs have been related to soil organic carbon, pH, nutrient availability and management history (Wolińska *et al.*, 2017; Chen *et al.*, 2022; Zheng *et al.*, 2023). However, the present experiment did not quantify these physicochemical characteristics simultaneously; therefore, it cannot establish that the high Chernozem values were caused by greater organic matter or any other specific soil property. These factors should instead be considered hypotheses for subsequent investigation.

The results nevertheless suggest that Chernozem soils may provide particularly favorable niches for culturable free-living nitrogen-fixing bacteria during specific season–depth combinations. From a horticultural perspective, this is relevant because biologically active soil horizons can contribute to nutrient cycling and may influence the effectiveness of microbial-based fertility management. Further work linking CFU abundance with soil chemical properties and direct nitrogen-fixation measurements is needed to quantify this functional contribution.

Alluvial soil: Alluvial soil showed bacterial populations ranging from 0.40 × 10^4 to 12.70 × 10^4 CFU g^{-1}. The highest population was observed during spring and at 10 cm, which was 12.70 ± 0.10 × 10^4 CFU g^{-1}. Spring populations were also high at 5 cm (7.10 ± 0.10 × 10^4 CFU g^{-1}) and 25 cm (8.40 × 10^4 CFU g^{-1}). During summer, abundance decreased with depth from 4.20 × 10^4 CFU g^{-1} at 5cm to 1.60 × 10^4 CFU g^{-1} at 25cm. Autumn populations were relatively low, in the order of 0.90 to 1.60 × 10 4 CFU g^{-1}. The lowest population was observed at 5 cm (0.40 × 10^4 CFU g^{-1}) in the winter, while significantly higher populations were observed at 10 and 25 cm (6.10 × 10^4 and 4.10 × 10^4 CFU g^{-1}, respectively).

The clear spring peak indicates a strong effect of seasonal conditions on the culturable diazotrophs in Alluvial soil. Seasonal responses of nitrogen-fixing bacterial communities have been reported in other terrestrial systems and are often associated with concomitant changes in soil moisture, temperature, plant activity, and nutrient availability. Other studies have demonstrated that seasonal environmental changes can reorganize the community composition of nitrogen-fixing bacteria, altering their abundance (Li *et al.*, 2022; Zhao *et al.*, 2025).

The winter increase at 10 and 25 cm relative to the 5-cm layer again suggests that deeper soil layers may provide greater environmental buffering than the soil surface. However, the specific driver cannot be resolved without concurrent measurements of soil temperature and moisture.

Comparison among soils and implications of the three-way interaction: Across all treatments, the highest population occurred in Chernozem soil at 25 cm in spring (68.33 ± 0.58 × 10^4 CFU g^{-1}). Other particularly high populations occurred in Chernozem soil at 10 cm during summer (40.33 ± 0.58 × 10^4 CFU g^{-1}), Anthropogenic soil at 25 cm during summer (38.00 × 10^4 CFU g^{-1}), and Chernozem soil at 10 cm during winter (37.67 ± 0.58 × 10^4 CFU g^{-1}). In contrast, the lowest population was found in Cinnamonic soil at 5 cm during winter.

The results therefore do not support a universal assumption that culturable nitrogen-fixing bacteria are most abundant in surface soil or that their abundance consistently decreases with depth. Rather, the direction and magnitude of the depth response depended strongly on soil type and season. Molecular analyzes of agricultural diazotroph communities have also revealed soil-dependent patterns, with significant differences in diazotrophic composition and vertical distribution across soil types (Wang *et al.*, 2021). It is known that different agricultural management systems influence the diazotrophic networks and the effects are dependent on the soil (Zhang *et al.*, 2024).

Seasonal and depth-wise variation in free-living nitrogen-fixing bacterial abundance: Fig. 2 shows a significant seasonal, soil type and depth-wise variation in the abundance of culturable free-living nitrogen-fixing bacteria from 2.90 to 5.84 log10(CFU g-1). The highest abundance was in Chernozem soil in spring at 25 cm depth (5.84), Chernozem soil in summer at 10 cm (5.60), Anthropogenic soil in summer at 25cm (5.58) and Chernozem soil in winter at 10cm (5.58). Anthropogenic and Cinnamonic soils were generally lower in bacterial abundance than Chernozem and Alluvial soils. In spring, alluvial soil showed relatively high values at all depths (4.85–5.10), while Cinnamonic soil showed marked increase with depth in winter from 2.90 at 5 cm to 4.88 at 25 cm. The minimum abundance was recorded in Cinnamonic soil during winter at 5 cm (2.90) followed by 10 cm (2.95). The heatmap generally shows that seasonal effects differed between soil types and the depth of maximum bacterial abundance was not consistent across soils and seasons.

Season likewise did not produce a common response across all soils. For example, spring favored the largest populations in Alluvial soil and the deepest Chernozem layer, whereas summer

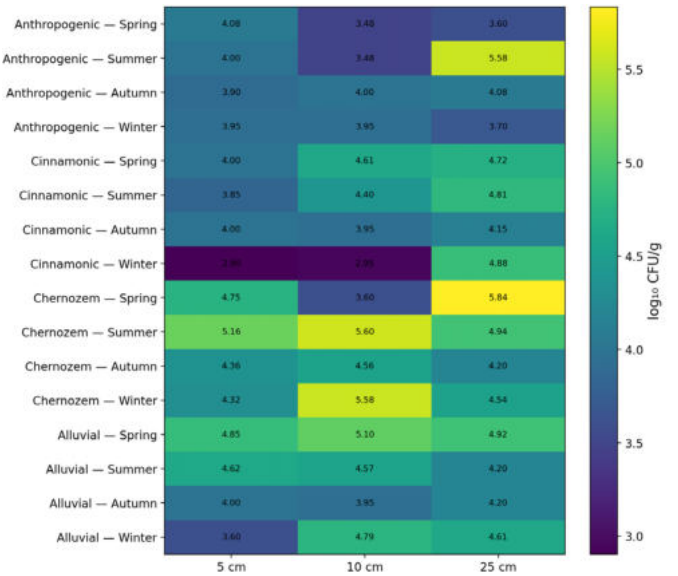


Fig. 2. Heatmap showing seasonal and depth-wise variation in culturable free-living nitrogen-fixing bacterial abundance [log_{10}(CFU g^{-1})] across Anthropogenic, Cinnamonic, Chernozem, and Alluvial soils. Cell values represent mean log_{10}-transformed bacterial abundance.

produced the maximum value in Anthropogenic soil. Cinnamonic soil retained a comparatively high population at 25 cm even during winter. This indicates that seasonal climatic conditions operate through soil-specific environments rather than acting independently.

Ecologically, these results make sense as free-living nitrogen fixation is energetically costly and highly sensitive to resource availability. Heterotrophic diazotrophs use organic carbon as an energy source, but the fixation of atmospheric N_2 may be less energetically favorable at high concentrations of readily available nitrogen (Smercina *et al.*, 2019; Zheng *et al.*, 2023). Therefore, both diazotroph abundance and community structure can be altered by agricultural management and soil amendments (Chen *et al.*, 2022). The contrasting responses observed among the four Eastern Georgian soils may therefore represent differences in the interaction between inherent soil properties, horticultural management, climatic conditions, and vertical resource distribution.

Relevance to sustainable horticultural soil management: Free-living and associative diazotrophic bacteria have attracted considerable attention as components of biologically based nutrient-management strategies. Diazotrophs that promote plant growth can improve nutrient cycling and, under favorable conditions, may supplement traditional fertilizer management (Aloo *et al.*, 2022). The strong seasonal and depth-dependent patterns observed in the present study suggest that assessments of soil biological fertility based on a single sampling date or a single surface depth may fail to represent the temporal and vertical heterogeneity of diazotrophic populations.

For horticultural fields in Eastern Georgia, monitoring multiple depths and seasons may therefore provide a more representative assessment of culturable nitrogen-fixing bacterial populations. The results also suggest that subsurface layers should not automatically be excluded from biological soil-fertility assessments, particularly because some of the highest populations in the present study occurred at 10 or 25 cm.

At the same time, high CFU values should not be interpreted directly as a capacity to replace mineral N fertilizer. Culture-based abundance indicates the number of microorganisms capable of developing under the selected Jensen medium conditions. In contrast, the actual agronomic contribution of biological nitrogen fixation depends on nitrogenase activity, environmental conditions, plant–microbe interactions, and the amount of fixed N ultimately available to crops.

Study limitations and future research: The present culture-based approach provides useful information on the spatial and temporal distribution of culturable putative nitrogen-fixing bacteria, but it represents only a fraction of the total soil diazotrophic community. Many soil microorganisms are not readily culturable under laboratory conditions, and growth on nitrogen-free medium does not by itself quantify in situ biological nitrogen fixation. Similarly, the Nessler reaction provides evidence of ammonium accumulation but is not equivalent to a direct nitrogenase assay.

Future studies should therefore combine culture-based enumeration with molecular approaches, particularly quantitative analysis of the *nifH* gene and sequencing of diazotrophic communities. Direct functional measurements, such as the acetylene-reduction assay or ^{15}N-based methods, would allow bacterial abundance to be related to actual N_2-fixation rates.

Concurrent determination of soil pH, moisture, temperature, organic carbon, total N, mineral N, available P and K, electrical conductivity, and root distribution would also help explain the mechanisms underlying the strong soil × season × depth interaction observed here. Such information would allow the present descriptive patterns to be converted into predictive relationships between soil environmental conditions and diazotrophic abundance.

Collectively, the results demonstrate that culturable putative free-living nitrogen-fixing bacterial populations in horticultural soils of Eastern Georgia exhibit pronounced soil-specific, seasonal, and depth-dependent variation. The strong three-way interaction indicates that soil type, season and depth alone are insufficient to characterize microbial abundance and emphasizes the need to consider both temporal and vertical variability when assessing the biological fertility of horticultural soils (Liu *et al.*, 2024).

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