Effect of *Bacillus subtilis* and *Aspergillus niger* inoculation on soil physicochemical parameters in agricultural soils from Béni Mellal (Morocco)

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Abstract. Soil quality plays a key role in sustainable agricultural productivity, particularly in semi-arid regions where fertility constraints limit crop performance. Microbial biofertilizers represent environmentally friendly alternatives to chemical fertilizers by enhancing nutrient availability and soil health. This study evaluated the effects of *Bacillus subtilis* and *Aspergillus niger* inoculations on the physicochemical properties of agricultural soils from the Béni Mellal region of Morocco. A total of 84 soil samples were analyzed, divided into four groups: untreated control, inoculated with *B. subtilis*, inoculated with *A. niger*, and combined inoculated. The results showed distinct treatment-dependent responses. *B. subtilis* significantly enhanced total nitrogen (N), while *A. niger* increased available phosphorus (P_2O_5) and influenced soil pH. Combined inoculation produced the most pronounced improvements, particularly for total nitrogen, available phosphorus, ammonium, and boron. Manganese (Mn) exhibited moderate but consistent variation among treatments, while organic matter (OM), carbon dioxide (CO_2), iron (Fe), copper (Cu), and zinc (Zn) showed no significant short-term changes. Correlation analysis indicated strong positive associations among key nutrients, suggesting synergistic effects of microbial inoculation. Overall, the findings highlight the potential of *Bacillus subtilis* and *Aspergillus niger*, especially when combined, to selectively improve soil fertility and support more sustainable agricultural practices in the Beni Mellal region.

1. Introduction

In agriculture, soil fertility refers to a soil's ability to supply plants with essential nutrients, in conditions that are favorable to their growth and productivity [1]. The increasing use of chemicals has deteriorated this fertility, posing a major threat to global food security and the sustainability of agroecosystems [2]. This situation leads to progressive depletion of nutrients, disruption of biogeochemical cycles, and deterioration of soil quality, particularly in vulnerable regions [3]. Soil physicochemical properties, including nutrient availability, pH, and organic matter content, play a central role in regulating crop productivity and plant

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adaptability [4]. Additionally, soil contamination by trace metals, particularly in areas irrigated with treated or untreated wastewater, can negatively affect both soil function and agricultural performance [5]. Globally, microbial biofertilizers, such as plant growth-promoting rhizobacteria (PGPR) and beneficial fungi, represent a sustainable alternative to chemical fertilizers [6, 7]. These microorganisms improve the availability of essential nutrients (nitrogen, phosphorus, potassium), solubilize poorly accessible forms of phosphorus, stimulate root development, and promote carbon sequestration in soils [8, 9]. In Morocco, agriculture is a strategic sector, contributing around 15% of gross domestic product, providing a source of income for nearly 40–45% of the population, and covering more than 80% of the land dedicated to crop production [10, 11]. However, this sector remains heavily dependent on soil quality and farming practices [12]. Recent studies have demonstrated that the use of microbial biofertilizers can enhance the sustainability of agroecosystems and mitigate the environmental impact of chemical inputs [6, 7]. Research, particularly in citrus orchards, has also highlighted the central role of microbial communities in the health and productivity of agricultural soils [13].

Among the most studied beneficial microorganisms in various agroecosystems, *Bacillus subtilis* and *Aspergillus niger* stand out for their positive effects on soil fertility and health [14]. *B. subtilis*, as a plant growth-promoting rhizobacterium (PGPR), contributes to plant growth stimulation, biological nitrogen fixation, phosphorus solubilization, and the degradation of certain pollutants, thereby enhancing agricultural productivity and soil resilience [6, 7]. *A. niger*, recognized as a phosphorus-solubilizing fungus (PSF), mobilizes nutrients through the production of organic acids, enhancing the availability of essential nutrients for crops [15, 16]. The combined action of these microorganisms promotes sustainable soil enrichment and supports biogeochemical processes indispensable to the health of agroecosystems [17]. However, few studies have quantified the direct, controlled, and comparative effect of microbial inoculation (PGPR and/or fungal biofertilizers) on the physicochemical parameters of soils in this specific context. To this end, the present study aims to evaluate the effects of inoculating soil samples with *Bacillus subtilis*, *Aspergillus niger*, or a combination of both, on the physicochemical properties of soils in the Beni Mellal region. The analysis focuses on major nutrients, pH, micronutrients, and multivariate interactions between these parameters.

2. Materials and methods

2.1 Study area

The study was conducted in the Beni Mellal region (32°20′22″N 6°21′39″W), located in central Morocco [18], between the High Atlas Mountains and the Tadla Plain [5]. This area is one of the country's primary agricultural regions, benefiting from fertile soils, a favorable climate, and abundant groundwater and surface water resources [5]. It has a Mediterranean climate characterized by hot summers, with an average annual temperature of 24.5°C. Annual rainfall is relatively low, with an average of 53.36 mm [19].

According to the Moroccan soil classification, the soils in this region are mainly based on limestone substrates and are classified as Isohumic. The soil profiles encountered are of the A[B]C or ABC type, with an organic matter content exceeding 15% in the A horizon, indicating high potential for agricultural production and microbiological experimentation [20].

2.2 Soil sampling

A total of eighty-four agricultural soil samples were collected between November and December 2024 using a random sampling strategy to ensure representative coverage of the study area. Samples were taken from the surface layer (0–20 cm), placed in plastic bags, and transported to the laboratory for preparation and analysis. Upon arrival at the laboratory, visible plant residues and coarse particles exceeding 2 mm were removed by sieving, after which each sample was carefully homogenized to obtain a uniform matrix. The soils were then air-dried at 20 °C for 48 hours, ground with an agate mortar, and passed through a <63 µm sieve [21]. The processed samples were stored under appropriate conditions in labeled plastic containers until further analysis.

The microbial strains used were *Bacillus subtilis ATCC 6633* and *Aspergillus niger ATCC 16404*, obtained from certified culture collections. Pure cultures were prepared under sterile conditions and adjusted for soil inoculation following standard protocols. Soil samples were then distributed into four treatment groups, with twenty-one samples included in each group (Table 1).

Table 1. Distribution of samples according to soil microbiological treatment groups

Treatment group	**Description**	**Number of samples**
Control	Non-inoculated soil	21
Bacillus subtilis	Soil inoculated with *B. subtilis* only	21
Aspergillus niger	Soil inoculated with *A. niger* only	21
Combination (*B. subtilis* + *A. niger*)	Soil inoculated with both microorganisms	21

2.3 Soil analysis

Microbial inocula were prepared as mother suspensions for each microorganism. In sterile test tubes, 10 mL of peptone water were supplemented with a microbial load of 10^8 CFU/mL of *Bacillus subtilis* (ATCC 6633) in the first tube and 10^8 CFU/mL of *Aspergillus niger* (ATCC 16404) in the second. The optical density (OD) of each suspension was adjusted to 1.0 at 600 nm to standardize viable cells and spores concentrations. These suspensions were then diluted in 250 mL of sterile peptone water to obtain enough inoculum volume. Each soil sample, weighing 50 g, was inoculated with 5 mL of the diluted microbial suspension, and incubated at 25 °C under controlled laboratory conditions for 4 to 6 weeks, allowing microbial establishment and interactions between the soil and microbes to occur.

After incubation, soil samples were analyzed in the soil and water physicochemical laboratory. Soil pH was measured in a 1:5 soil–water suspension following ISO 10390 [23]. Organic carbon was determined by wet oxidation using a sulfochromic solution, following ISO 14235 [24], and total nitrogen was quantified using the Kjeldahl method according to ISO 11261[25]. Available phosphorus was measured following ISO 11263 [26], while ammonium (NH_4^+) was extracted with potassium chloride, as described in ISO 14256-2 [27]. Micronutrients, including iron, copper, manganese, and zinc, were extracted using EDTA, and quantified by atomic absorption spectrophotometry [28]. Boron was analyzed according to the French standard NF X 31-122 [29]. These analyses provide a comprehensive assessment of soil chemical properties and nutrient status, enabling evaluation of the effects of microbial inoculation on soil fertility.

2.4 Statistical analysis

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Statistical analyses were conducted using R software (version 4.5.0, R Core Team, 2025) [30]. Data normality was evaluated using the Shapiro–Wilk test [31], and visually assessed with QQ-plots [32]. As all variables deviated from normality ($p < 0.05$), non-parametric tests were applied [33]. Differences among microbial treatments (*Bacillus subtilis*, *Aspergillus niger*, and their combination) were assessed using the Kruskal–Wallis test [34]. Relationships between soil variables were analyzed using Spearman's correlation [35], and a Principal Component Analysis was conducted to investigate the multivariate structure of the dataset and to identify the most discriminant physicochemical parameters associated with microbial inoculation [36].

3. Results

3.1 Assessment of Data Distributional Normality

The normality of the measured soil physicochemical variables was assessed using the Shapiro–Wilk test. All variables returned p-values below the 0.05 significance threshold, indicating rejection of the normality hypothesis. This departure from normality was confirmed visually by QQ-plots (Figure 1), where a clear deviation of the sample quantiles from the reference line is observed.

These results justified the use of non-parametric statistical methods for all subsequent analyses.

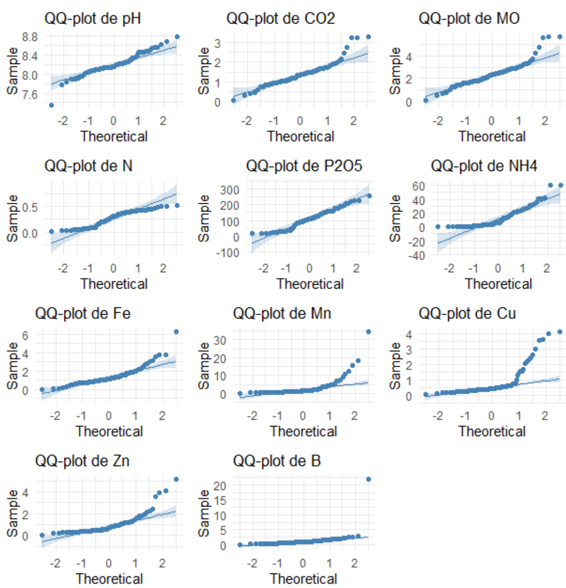


Fig. 1. QQ-plots of the measured soil physicochemical variables.

3.2 Effect of microbial treatments on soil parameters

The non-parametric Kruskal–Wallis test revealed significant differences among the four experimental groups (control, Bacillus subtilis, Aspergillus niger, and combined inoculation). Out of the eleven soil parameters analyzed, six showed clear treatment effects (Table 2). Total nitrogen (N) was markedly enhanced in soils inoculated with B. subtilis, consistent with its nitrogen-fixing activity, while the co-inoculated group displayed the highest overall N enrichment. Available phosphorus (P_2O_5) was most strongly increased under A. niger treatment, reflecting its ability to solubilize mineral phosphorus, whereas the combined treatment further amplified this effect. Ammonium (NH_4^+) concentrations followed a similar trend, with the co-inoculation producing the largest rise compared to single inoculations and the control. Boron (B) also responded significantly, particularly under the combined treatment, suggesting synergistic microbial mobilization mechanisms.

Soil pH shifted across treatments, with *A. niger* lowering pH through the release of organic acids, while *B. subtilis* had a milder effect. The co-inoculation resulted in an intermediate but significant alteration in acid–base balance. Manganese (Mn) exhibited moderate yet consistent variation, with higher levels observed in soils inoculated with *A.*

niger, likely due to fungal-mediated mobilization. In contrast, organic matter (OM), CO_2, iron (Fe), zinc (Zn), and copper (Cu) did not show significant treatment-related differences. This stability suggests that these parameters are less responsive within the short incubation period and may require longer timescales to detect measurable changes.

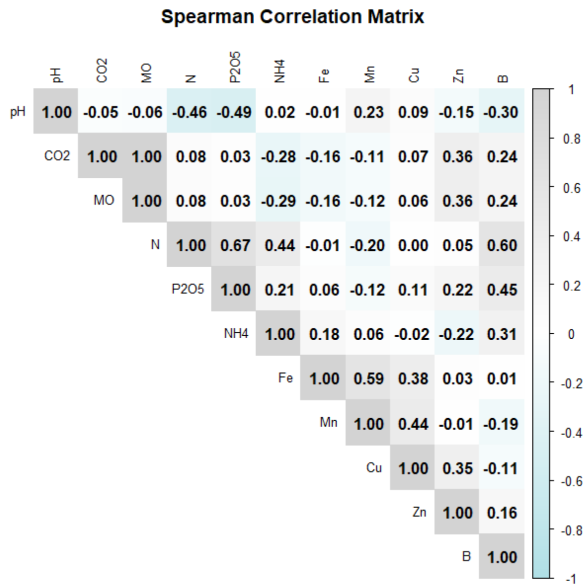


Table 2. Kruskal–Wallis test results for soil variables under microbial treatments

Parameter	**Statistic (χ^2)**	**p-value (exact)**	**Significance**
Total nitrogen (N)	63.14	1.25×10^{-13}	***
Available phosphorus (P_2O_5)	50.48	6.31×10^{-11}	***
Boron (B)	34.60	1.48×10^{-7}	***
pH	28.14	3.39×10^{-6}	***
Ammonium (NH_4^+)	22.80	4.44×10^{-5}	***
Manganese (Mn)	8.58	3.54×10^{-2}	*
Organic matter (OM)	7.71	5.24×10^{-2}	NS
Carbon dioxide (CO_2)	7.22	6.51×10^{-2}	NS
Iron (Fe)	3.56	0.312	NS
Zinc (Zn)	1.89	0.595	NS
Copper (Cu)	0.51	0.916	NS

Note: NS = not significant ($p \geq 0.05$). Significance codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3 Correlation analysis

Given the non-normal distribution of the data, Spearman's rank correlation was used to evaluate pairwise relationships between variables. The resulting correlation matrix (Figure 2) highlights several notable associations. In particular, strong positive correlations were observed between total nitrogen (N) and ammonium (NH_4^+), and between available phosphorus (P_2O_5) and boron (B). Moderate negative correlations were observed between pH and certain micronutrients, such as iron (Fe) and manganese (Mn), suggesting that soil acidity affects the availability of these elements.

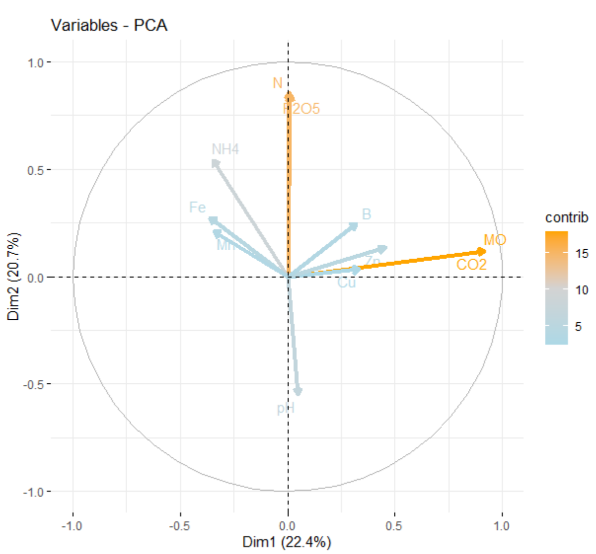
Fig. 2. Spearman correlation matrix of soil variables, with warm and cool tones indicating positive and negative correlations.

3.4 Multivariate Structure of Soil Data

A Principal Component Analysis (PCA) was used to explore the overall multivariate structure of the dataset and to identify the most discriminant variables. The first two principal components accounted for 43.1% of the total variance (Dim1 = 22.4%; Dim2 = 20.7%). The correlation circle (Figure 3) indicates that total nitrogen (N), available phosphorus (P_2O_5), organic matter (OM), and CO_2 are the variables that contribute most strongly to the construction of the principal axes (contribution > 15 %). In contrast, ammonium (NH_4^+) and pH are represented in light grey to denote their more moderate contributions; their specific orientations suggest distinct behavior compared with the other parameters. Trace elements (Fe, Mn, B, Cu, Zn) are located closer to the origin, reflecting relatively low contributions to the variability among treatments.

Fig. 3. PCA correlation circle (Dim1–Dim2) of soil variable; colors indicate variable contributions, with values ≥ 15 %.

4. Discussion



The results demonstrate that microbial treatments with *Bacillus subtilis* and *Aspergillus niger* significantly influenced several soil physicochemical properties. Non-parametric statistical analyses revealed marked effects on key nutrients, while other parameters remained stable. The significant enrichment of total nitrogen, available phosphorus, and ammonium after inoculation is consistent with the functional traits of the introduced microorganisms. *B. subtilis* is well known for its ability to fix atmospheric nitrogen and solubilize insoluble phosphorus through the production of organic acids and phosphatase enzymes [6, 37], whereas *A. niger* releases citric acid and other metabolites that mobilize mineral phosphorus [38]. These microbial mechanisms explain the simultaneous increase in N, P_2O_5, and NH_4^+ observed in treated soils, confirming the efficiency of the inoculants in improving nutrient availability. Similar variations in soil nutrient contents have previously been reported in the Béni Mellal–Khénifra region in relation to agricultural practices [39].

Significant changes in soil pH observed after inoculation can be attributed to the metabolic activity of the introduced microorganisms. In particular, *A. niger* produces acidic exudates that modulate local soil acidity [15], a common mechanism among soil fungi that influences the rhizosphere environment [40]. This acidification has direct consequences for nutrient availability and metal mobility, further underscoring the regulatory role of microbial biofertilizers in maintaining soil chemical balance. At the regional scale, El Baghdadi et al. (2015) [39] reported that soil pH in the Béni Mellal area, especially along the Sabeq River, is highly sensitive to external inputs, such as contamination by solid waste [41], highlighting the vulnerability of this parameter to environmental disturbances. In contrast, organic matter, carbon dioxide, and trace elements, including iron, copper, and zinc, did not show significant variations during the experiment. This stability likely reflects the slow dynamics governing their biogeochemical cycling, which generally require longer incubation times to respond to microbial activity [42, 43]. In addition, these elements, especially metals, are partially immobilized within stable organo-mineral complexes, limiting their short-term bioavailability and mobility in soil systems [44].

Spearman's correlation analysis revealed strong positive associations between total nitrogen and ammonium [45], as well as between available phosphorus and boron [46], suggesting a coordinated mobilization of these nutrients driven by synergistic microbial activity. These interactions support the idea that microbial biofertilizers do not act on single elements but influence the entire nutrient cycling network. These results align with previous regional observations in agricultural soils of Béni Mellal, where similar nutrient correlations have been reported [5]. In addition, moderate negative correlations were observed between soil pH and certain micronutrients such as Fe and Mn, reflecting the strong dependence of their solubility on soil acidity [47].

Principal Component Analysis (PCA) provided a synthetic view of the studied variables and identified the most discriminant contributors [48]. The results showed that total nitrogen (N), available phosphorus (P_2O_5), organic matter (OM), and carbon dioxide (CO_2) were the strongest contributors to the first two principal components. This structuring highlights the predominant role of major nutrients in differentiating soils treated with microbial biofertilizers. In contrast, ammonium (NH_4^+) and pH, although less contributive, displayed specific orientations, indicating distinct behavior compared with other parameters. These findings are consistent with previous studies emphasizing the key role of major nutrients in the differentiation of microbiologically enriched ecosystems [49], also in line with the work of Fahde et al. (2025) [39], who demonstrated the importance of these nutrients in differentiating soils in the Béni Mellal region according to fertility status [4]. This research confirms that beneficial soil microorganisms, particularly *Bacillus subtilis* and *Aspergillus niger*, play a crucial role in regulating soil fertility and health by stimulating microbial processes associated with the nutrient cycle [50, 51].

5. Conclusion

The results of this study highlight the significant impact of microbial inoculations on the physicochemical properties of soils from the Béni Mellal region. Statistical tests showed that total nitrogen (N), available phosphorus (P_2O_5), ammonium (NH_4^+), boron (B), manganese (Mn), and pH, were strongly influenced by microbial treatments. At the same time, organic matter, carbon dioxide, iron, copper, and zinc remained stable during the experimental period. *Bacillus subtilis* enhanced nitrogen availability, *Aspergillus niger* primarily improved phosphorus solubilization and pH regulation, with the combined inoculation producing the most pronounced effects. Manganese was most affected under *Aspergillus niger* alone, while the stability of other parameters likely reflects their slower dynamics over short incubation times. Multivariate analyses confirmed the central role of N and P_2O_5 in structuring soil variability and differentiating microbial treatments. Overall, this study demonstrates that the co-inoculation of *Bacillus subtilis* and *Aspergillus niger* represents a promising biofertilization strategy to enhance soil fertility and resilience, offering a sustainable alternative to chemical fertilizers in semi-arid agroecosystems.

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